BIOCONVERSION OF ISOFLAVONES IN SYNBIOTIC-SOYMILK USING PHYSICAL TREATMENTS (ULTRASONICATION, ELECTROPORATION AND ULTRAVIOLET) ON LACTOBACILLI AND BIFIDOBACTERIA

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LIST OF ABBREVIATIONS

ACE = Angiotensin I-Converting Enzyme
ANOVA = Analysis of Variance
ANS = 8-anilino-1-napthalenesulfonic acid
ATCC = American type culture collection
BT = Bioprocess Technology
CHD = Coronory heart disease
DBP = Diastolic blood pressure
DPH = 1,6-diphenyl-1,3,5-hexatriene
FAn = Fluorescence Anisotropy
FOS = Fructooligosaccharides
FTCC = Food Technology Culture Collection Centre
FTDC = Food Technology Division Culture Collection
HDL = High density lipoprotein
Hip-His-Leu = Hippuryl-L-Histidyl-L-Leucine Hydrate
HPLC = High performance liquid chromatography
HRT = Hormone Replacement Therapy
IPP = Ile–Pro–Pro
IQ = 2-amino-3-methylimidazo[4,5-f]quinoline
LAB = Lactic acid bacteria
LDL = Low density lipoprotein
MDA = Malonylaldehyde
MRS = de Mann, Rogosa, Sharpe
MRSA = Methicillin-resistant *Staphylococcus aureus*
ND = Not Detected
OPA = o-phtaldialdehyde
PBS = Phosphate buffered saline
pNPG = p-nitrophenyl-a-D-galactopyranoside
pNPGl = p-nitrophenyl-a-D-glucopyranoside
ROS = Reactive oxygen species
sIgA = Secretory immunoglobulin A
SBP = Systolic blood pressure

SCFA = Short chain fatty acids

SDS = Sodium dodecyl sulphate

SEM = Scanning electron microscope

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

UV = Ultraviolet

VPP = Val–Pro–Pro

VRE = Vancomycin-resistant *Enterococcus faecalis*

Biopenukaran Isoflavon di dalam Susu Soya-Sinbiotik dengan Menggunakan Rawatan Fizikal (Ultrasonikasi, Elektroporasi dan Ultralembayung) pada Laktobasili dan Bifidobakteria

ABSTRAK

Lima belas laktobasili dan bifidobakteria disaring dengan pertumbuhan di dalam susu soya. L. *casei* FTDC 2113, *L. acidophilus* FTDC 8033, *L. acidophilus* BT 4356, *L. casei* BT 1268, *Bifidobacterium* sp. FTDC 8943 dan *B. longum* FTDC 8643 menunjukkan kebolehhidupan yang lebih tinggi di dalam susu soya dan justeru itu dipilih untuk analisis-analisis berikutnya yang melibatkan penambahan dengan prebiotik seperti fruktooligosakarida (FOS), inulin, manitol, maltodekstrin dan pectin. Laktobasili dan bifidobakteria yang dipilih menunjukkan kebolehhidupan melebihi 7 Log_{10} CFU/mL selepas fermentasi selama 24 j pada suhu 37 ^{o}C dan meningkat apabila ditambah dengan maltodekstrin, manitol dan FOS.

Penambahan prebiotik juga meningkatkan aktiviti rencatan-ACE. Di samping itu, penambahan prebiotik seperti pektin juga meningkatkan aktiviti β-glukosidase ekstrasel. Aktiviti β-glukosidase intrasel juga dipertingkatkan apabila susu soya ditambah dengan pektin dan manitol. Hal ini kemudiannya disertai dengan peningkatan biopenukaran glukosida kepada aglikon di dalam susu soya prebiotik. Antara prebiotik tersebut, manitol menunjukkan kesan yang lebih ketara dalam menggalakkan penghasilan aglikon bioaktif di dalam susu soya.

Maka, susu soya-manitol digunakan dalam kajian-kajian seterusnya yang melibatkan ultrasonik (20-100 W; 1-3 min), sinaran ultralembayung (UVA-UVC, 30-90 J/m^2) dan elektroporasi (2.5-7.5 kV/cm; 3-4 ms). Rawatan-rawatan fizikal ini mendorong kebolehhidupan laktobasili dan bifidobakteria di dalam susu soya-manitol. Hal ini disebabkan oleh peningkatan kebolehtelapan membran yang berpunca daripada pengoksidaan lipid dan perubahan pada membran dwilapisan fosfolipid selepas rawatan-rawatan fizikal. Rawatan-rawatan tersebut juga menggalakkan aktiviti-aktiviti β-glukosidase intrasel dan ekstrasel laktobasili dan bifidobakteria, dan seterusnya meningkatkan biopenukaran glukosida kepada aglikon di dalam susu soya-manitol. Elektroporasi pada 7.5 kV/cm untuk 3.5 ms menunjukkan kesan yang lebih ketara di mana kepekatan aglikon meningkat sebanyak 78.2% berbanding dengan kawalan dan hal ini jelas diperhatikan di dalam susu soya-manitol yang difermentasikan oleh *B. longum* FTDC 8643. Ultrasonik (60 W; 3 min) dan sinaran ultralembayung (UVB; 90 J/m^2) juga berkesan dalam meningkatkan kepekatan aglikon dan paling jelas ditunjukkan di dalam susu soya-manitol yang difermentasikan oleh *L. casei* FTDC 2113 (43.1-46.7% lebih tinggi berbanding dengan kawalan). Rawatan-rawatan fizikal dan *Lactobacillus* and *Bifidobacterium* tersebut kemudian dipilih untuk analisis melibatkan warisan oleh subkultur berikutnya. Peningkatan kebolehhidupan dan biopenukaran glukosida kepada aglikon dan peningkatan ciri-ciri probiotik semasa rawatan-rawatan fizikal hanya terdapat dalam sel-sel induk tanpa diwarisi oleh subkultur berikutnya (subkultur pertama, kedua dan ketiga). Walaupun hanya untuk sementara, kajian ini jelas menunjukkan bahawa rawatan-rawatan fizikal tersebut sememangnya bermanfaat untuk menggalakkan potensi probiotik dan bioaktif *Lactobacillus* dan *Bifidobacterium* di dalam susu soya-manitol, untuk penghasilan susu soya sinbiotik berfungsi yang mempunyai bioaktiviti yang tinggi.

Bioconversion of Isoflavones in Synbiotic-Soymilk using Physical Treatments (Ultrasonication, Electroporation and Ultraviolet) on Lactobacilli and Bifidobacteria

ABSTRACT

Fifteen strains of lactobacilli and bifidobacteria were screened for growth in soymilk. *L. casei* FTDC 2113, *L. acidophilus* FTDC 8033, *L. acidophilus* BT 4356, *L. casei* BT 1268, *Bifidobacterium* sp. FTDC 8943 and *B. longum* FTDC 8643 exhibited higher ($P<0.05$) viability and were thus selected for subsequent analyses involving prebiotics such as fructooligosaccharides (FOS), inulin, mannitol, maltodextrin and pectin. All selected strains showed viability exceeding 7 Log_{10} CFU/mL upon fermentation in soymilk at 37 ^{o}C for 24 h and was higher upon supplementation with maltodextrin, mannitol and FOS.

Supplementation of prebiotics also increased angiotensin I-converting enzyme (ACE)-inhibitory activity. In addition, supplementation with prebiotics such as pectin also enhanced the extracellular β-glucosidase. The intracellular β-glucosidase activity was enhanced upon supplementation with pectin and mannitol. This led to a higher bioconversion of glucosides to aglycones in soymilk supplemented with these prebiotics. Among the prebiotics, mannitol showed a more prominent effect on promoting the production of bioactive aglycones in soymilk.

Therefore, mannitol-soymilk was used for subsequent evaluations upon application of ultrasound (20-100 W; 1-3 min), UV radiation (UVA-UVC, 30-90 J/m^2) and electroporation (2.5-7.5 kV/cm; 3-4 ms). These physical treatments significantly promoted the viability of lactobacilli and bifidobacteria in mannitol-soymilk mainly due to enhanced membrane permeability of cells upon treatments. Such changes were attributed to lipid peroxidation and alteration on membrane phospholipids bilayer. Such physical treatments also significantly promoted the intracellular and extracellular β-glucosidase activities of lactobacilli and bifidobacteria, and subsequently enhanced the bioconversion of glucosides to aglycones in mannitol-soymilk ($P<0.05$). Electroporation at 7.5 kV/cm for 3.5 ms showed a more prominent effect where concentrations of aglycones was increased by 78.2% compared to the control and this was clearly observed in mannitol-soymilk fermented by *B. longum* FTDC 8643. Ultrasound (60 W; 3 min) and UV radiation (UVB; 90 J/m^2) also effectively promoted the concentrations of aglycones and was most prevalent in mannitol-soymilk fermented by *L. casei* FTDC 2113 (43.1-46.7% higher compared to that of the control). These treatments and strains were then selected for analyses involving inheritance potential by subsequent subcultures. The increase in viability, bioconversion of isoflavones and enhancement of probiotic properties upon physical treatments were only prevalent in the parent cells ($P<0.05$), without inheritance by subsequent subcultures (first, second and third subculture) of treated cells. Although temporary, the results strongly illustrated that physical treatments may be beneficial for promoting the probiotic and bioactive potentials of *Lactobacillus* and *Bifidobacterium* in mannitol-soymilk, for the development of functional synbiotic-soymilk with enhanced bioactivity.

1.0 INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1 Background

Lactobacilli and bifidobacteria are known as the most common type of bacteria exhibiting probiotic properties. Probiotics are defined as ‘live microorganisms that when administered in adequate amounts confer health benefits on the host’ (Guarner *et al.*, 2005). These bacteria are proclaimed to impart health beneficial effects such as alleviation of immune system, modulation of gastrointestinal microbial balance and prevention of gastrointestinal infection (Liong, 2007). Due to their potential health benefits, there are growing interest to incorporate it into food preparation to produce healthy functional products.

Soybean (*Glycine max*) is well known as an inexpensive source of protein and carbohydrate for human consumption. It does not contain cholesterol and lactose and thus suitable for lactose intolerance consumer (Wang *et al.*, 2003). Past epidemiological studies and clinical trials have shown promising evidence on the importance of soy in prevention of postmenopausal symptoms, cardiovascular disease, bone health problems, and breast, prostate and colon cancers (Setchell and Cassidy, 1999). Despite the attractive nutritional attribute of soy, consumption of soy has been limited due to the undesirable beany flavour and the presence of oligosaccharides (stachyose and raffinose) that often lead to flatulence. Oligosaccharides could be hydrolyzed by α-galactosidase, an enzyme which is usually deficient in the human intestinal tract. Lactobacilli and bifidobacteria have been reported to possess varying levels of α-galactosidase. Fung *et al.* (2008a) demonstrated that *L. acidophillus* could hydrolyze the oligosaccharides in soy whey, while Liong *et al.* (2009) reported that the incorporation of *Lactobacillus* with α-galactosidse activity significantly increased the hydrolysis of soybean oligosaccharides and subsequently reduced such antinutritive property in soy cream cheese.

In addition, soy contains reasonable amounts of proteins which when metabolized by *Lactobacillus* and *Bifidobacterium*, yield bioactive peptides known to confer health benefits (Liong *et al.*, 2009). These bacteria were found to possess proteolytic activity which could cleave the soy protein into various amino acid and peptides. Some of the peptides produced by *Lactobacillus* and *Bifidobacterium* are found to be bioactive and could impart antihypertensive effect (Ng *et al.*, 2008).

Soybeans also contain appreciable amounts of isoflavones which are responsible for the vast beneficial effects of soy. Isoflavones are nonsteroidal phytoestrogenic and antioxidative polyphenolic molecules with the potential to protect against hormone-dependent diseases due to its partial agonist and antagonist estrogens effects (Kano *et al.*, 2006). Izumi *et al.* (2000) demonstrated that aglycones are absorbed faster and in higher amount than glucosides in human. However, approximately 80% - 95% of isoflavones in unfermented soybean exist as glucosides which are less bioactive and nonbioavailable. β-Glucosidase producing lactobacilli and bifidobacteria has been reported to biotransform isoflavones glucosides to biologically potent aglycones (Tsangalis *et al.*, 2002).

Prebiotics are food ingredients that are neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract, and is selectively used as a substrate for beneficial bacteria in the colon (Liong and Shah, 2006). Oligosaccharides are the most common and widely researched prebiotics, especially fructooligosaccharides (FOS) and inulin. The viability and enzyme activity of *Bifidobacterium* was reportedly induced by prebiotics (Rastall and Maitin, 2002). Therefore, interest has been raised to incorporate prebiotics into probiotic preparations which is term as synbiotics. Synbiotics are defined as 'a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract and thus improving host welfare' (Tuohy *et al.*, 2003).

Supplementation of skim milk powder and lactulose in *Lactobacillus*-fermented soymilk has been demonstrated to increase the production of β-glucosidase enzyme and subsequently enhanced the bioconversion of isoflavones in soy (Pham and Shah, 2007; Pham and Shah, 2008a). However, up to date, little information is available on the effect of prebiotics on the bioconversion of isoflavones by lactobacilli and bifidobacteria in soy medium. To our knowledge, there have been no studies evaluating the effects of prebiotics such as pectin, mannitol and maltodextrin on the growth characteristic, antihypertensive properties and bioconversion of isoflavones in soy-products fermented by lactobacilli and bifidobacteria.

It is important to note that, the efficient bioconversion of isoflavones in soy products by lactobacilli and bifidobacteria is strongly influenced by permeability of cellular membrane. Generally, cellular membrane acts as a semipermeable barrier for transport of substance into and out of cells (Tryfona and Bustard, 2008). Selective permeability of the cellular membrane may prevent an efficient transport of isoflavones and β-glucosidase enzyme across the membrane. This subsequently impedes the bioconversion of isoflavones in soy products by cells. Furthermore, the cellular membrane could also limit the release of transformed bioactive products extracellularly. Therefore, permeabilization of cell membrane is essential to overcome the limitation of material transfer across the cellular membrane.

Physical treatments such as electroporation, ultrasound and ultraviolet radiation could efficiently permeabilize cellular membrane and promote the transport of substances across the cellular membrane without causing cell death. Considering the enhanced permeabilization, these physical treatments have demonstrated to impart various biotechnological applications. Loghavi *et al.* (2007) previously reported that electroporation (1V/cm, 60Hz, for 40h) promoted the production of bacteriocins (lacidin A) by *L. acidophilus* without causing cell death. In another study, Ohshima *et al.* (1995) demonstrated that application of electroporation on brewers' yeast, *Saccharomyces cerevisiae* increased the extracellular activities of invertase and alcohol dehydrogenase without destruction of cells. All these were attributed to the temporary pores created by the external electric field which increased the diffusive permeability of bioactive components and enzymes across the cellular membrane.

Ultrasound treatment has also been demonstrated to enhance biotechnological potential where Wu *et al.* (2000) reported that ultrasound treatment stimulated the acid production by starter culture and reduced the fermentation time in yogurt without inactivating the cultures. In addition, Wang *et al.* (1995) demonstrated that ultrasound increased the viable cell counts and β-galactosidase activity of *Lactobacillus* and *Bifidobacterium* in milk which led to subsequent increased of lactose hydrolysis in milk. In another study, ultrasound followed by static incubation was reported to increase the viable cell counts of *Brevibacterium* sp. and production of cholesterol oxidase (Yang *et al.*, 2010). These were due to the reversible alteration of membrane properties upon ultrasound treatments on the cells.

In addition, UV radiation has also shown promising evidences in promoting permeabilization and bioprocesses. Despite conventionally used for sterilization purpose, UV radiation, under appropriate dose would only caused sublethal injuries where the cells exhibited a longer lag phase prior to resuming viability (Berney *et al.*, 2007). This was in agreement with the study of Kramer and Ames (1987) who reported that *S. typhimurium* recovered and resumed viability after exposure to a low intensity of UV radiation. UV radiation (253.7 nm; 4.7 W/m^2) has also been reported to increase permeability of ions across the membrane of *Chara corallina* (Doughty and Hope, 1973), attributed to the reversible alteration of membrane properties. In another study, Shimizu and Sekiguchi (1979) reported that UV radiation effectively permeabilized *E. coli* cells and thus enabled the transport of macromolecules such as enzyme across the membrane while retaining the colony forming ability.

Thus, we hypothesized that the transient elimination of permeability barrier by physical treatments could enhance growth properties and yield of bioactive compound by whole cells. However, up to date, no attempt has been made to utilize such treatments to improve the production of bioactive isoflavones aglycones by lactobacilli and bifidobacteria. In addition, the effects of physical treatments on bioactivities of the offspring cells are also not well understood with the currently available limited information. Therefore, more studies are needed to better understand the effects of physical treatments on the bioactive potentials in subsequent offspring cells generated after few growth cycles (first, second and third subculture of cells).

1.2 Aim and Objectives for Research

The aim of this study is to evaluate the effects of prebiotics on the growth characteristics of lactobacilli and bifidobacteria and bioconversion of isoflavones in soymilk, and the effects of subsequent physical treatments on the bioactivity of synbiotic-soymilk.

Specific and measurable objectives

1. To screen and select strains of lactobacilli and bifidobacteria based on viability in soymilk.
2. To screen and evaluate the effects of prebiotics on the growth properties and bioactive potential of lactobacilli and bifidobacteria in soymilk.
3. To evaluate the effects of physical treatments on viability and membrane properties of lactobacilli and bifidobacteria in synbiotic-soymilk.
4. To assess the effects of physical treatments on bioconversion of isoflavones by lactobacilli and bifidobacteria in the synbiotic-soymilk.
5. To determine possible carry-over effects of physical treatments on viability and bioactivity in subsequent subcultures of cell.

2.0 LITERATURE REVIEW

CHAPTER 2

LITERATURE REVIEW

2.1 Probiotics

Probiotics are defined as ‘live microorganism which when added to foods help restore gut microflora of the host and subsequently confer health beneficial properties’ (Desai *et al.*, 2004). The beneficial health promoting effects of probiotics include immune modulation, antihypertensive, anticarcinogenic, reduction of serum cholesterol and prevention of gastrointestinal infection.

2.1.1 *Lactobacillus*

Lactobacilli are straight Gram-positive, non-motile and non-spore forming organisms that commonly form chains, with optimum pH of 4.5 - 6.0. Owing to their microaerophillic nature, they can tolerate oxygen or live anaerobically. Lactobacilli have complex nutritional requirements for carbohydrates, amino acids, peptides, fatty acids, nucleic acid derivatives, vitamins and minerals. *Lactobacillus* could be either homofermentative or heterofermentative where carbohydrates are used as carbon and energy source. These sugars or oligosaccharides are transported into the cell by the phosphotransferase system or the permease system (Konig and Frohlich, 2008). *Lactobacillus* can be characterised based on their physiological properties to three groups which include obligate homofermentation, facultative heterofermentation and obligate heterofermentation. *L. acidophilus* and *L. bulgaricus* are mainly obligate homofermentaters, *L. casei* are characterised as facultative heterofermentative while *L. fermentum* are obligate heterofermentative. Homofermentation ferment glucose via the Embden-Meyerhof-Parnas pathway converting 1 mol glucose to 2 mol lactic acid while heterofermentation proceed via the hexosemonophosphate pathway resulting in 1 mol each of lactic acid, ethanol/acetate and CO_2.

2.1.2 *Bifidobacterium*

Bifidobacteria are Gram-positive, saccharolytic anaerobes which occur ubiquitously in the human gut, with optimum pH of 6.0 and 7.0 and optimum temperature of 37 ^{o}C-41 ^{o}C. Carbohydrates are degraded exclusively and characteristically by the fructose-6-phosphate shunt. In pure glucose medium, bifidobacteria produce acetic and lactic acid in a molar ratio of 3:2. They are also capable of utilizing a variety of carbohydrate as carbon sources because of their ability to produce several intracellular and extracellular depolymerizing enzymes (Amaretti *et al.*, 2006). Complex carbohydrate such as oligosaccharides and polysaccharides were initially depolymerized to their respective monomeric constituents prior to incorporation into the fructose-6-phosphate shunt and fermented to lactic and acetic acids.

2.1.3 Health Promoting Benefits of Probiotics

Conventionally, probiotics have been demonstrated to promote gastrointestinal health by modulating gut microbial balance. In recent years, application of probiotics has been extended beyond gastrointestinal health to include prevention of killer disease such as cancer. *In vivo* studies have shown promising evidence that there is a strong correlation between probiotics and reduced risks of colon cancer induced by mutagens such as heterocyclic amines. Tavan *et al.* (2002) conducted a randomised study involving 60 weanling male rats which were induced with 250 mg of mutagens/carcinogens. These mutagens induced rats were randomly assigned to four groups each fed with water, non-fermented milk, *B. animalis* (5.4 ±

1×10^8 CFU/day) fermented milk or *Streptococcus thermophilus* ($5.4 \pm 1 \times 10^8$ CFU/day) fermented milk for 7 weeks. The authors found that rats fed with both probiotics-fermented milk significantly decreased the incidence of aberrant crypts foci compared to rats fed with water and unfermented milk. Ingestion of *B. animalis* and *St. thermophilus* fermented milk inhibited the incidence of colon aberrant crypts foci by 96% and 93%, respectively. In another study, Reddy and Rivenson (1993) demonstrated that a diet containing *B. longum* (2×10^{10} live bacterial cells/g) inhibited colon carcinogenesis induced by 2-amino-3-methylimidazo[4,5-f]quinoline (IQ). A total of 156 rats (78 female and 78 male) were fed with a control diet (high fat diet without containing *B. longum*) or experimental diet containing 0.5% lyophilized *B. longum* (2×10^{10} live bacterial cell/g) with or without IQ (125 ppm) for 58 weeks. This randomized, placebo-controlled study found that IQ induced gut carcinogenesis while dietary supplementation of *B. longum* significantly inhibited the incidence of colon and small intestinal tumour in the rats.

Several mechanisms have been proposed to explain the efficiency of probiotics in suppressing and preventing colon cancer. One of the potential mechanisms is removal of mutagens/ carcinogens via binding ability of probiotics to those compounds. Previous *in vitro* studies have reported that probiotics could permanently bind to dietary mutagens/ carcinogens thus inhibited the activity of the compounds (Bolognani *et al.*, 1997; Lankaputhra and Shah, 1998). Another possible anticancer mechanism by probiotics involved the production of bioactive compounds (Rhee and Park, 2001) and metabolites such as short chain fatty acid (SCFA; Lankaputhra and Shah, 1998) which inhibited the activity of mutagens/carcinogens.

In addition to prevention of colon cancer, probiotics are seen as an alternative therapy to antibiotic treatment for various infections due to their ability to improve immune functions. Probiotics have been found to influence the immune functions by affecting components related to immunologic responses (Erickson and Hubbard, 2000). The consumption of probiotics is capable of stimulating 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 prevention of binding of foreign bacteria to epithelial cells and penetration of harmful microorganisms (Erickson and Hubbard, 2000). Thus, probiotics could protect the gastrointestinal tract from the invasion of pathogens and opportunistic bacteria, which would subsequently reduce the risk of infection. In such cases, the use of antibiotics to treat illnesses would be reduced. 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 also led to the suggestion that they could be used as an alternative to antibiotic treatment.

In addition, probiotics could exert antimicrobial activity against various pathogenic and antibiotic-resistant strains. This antagonistic action is due to the production of antimicrobial substances such as bacteriocins and hydrogen peroxide. The use of bacteriocins is often preferred compared to 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 involved a lipid II binding step by the LtnA1 peptide, followed by insertion of LtnA2 peptide into the membrane. This led to formation of pores and ultimately cell death (Morgan *et al.*, 2005). Therefore, bacteriocins and other antimicrobial peptides produced by probiotics could act as promising therapeutic agents to treat various infections.

Probiotics could also be applied for prevention of antibiotic resistance by disrupting the transfer of antibiotic resistance genes. Moubareck *et al.* (2007) reported that probiotics could limit the emergence of antibiotic resistance. The authors evaluated the inhibitory effects of different bifidobacteria strains on the transfer of resistance genes among enterobacteriacea in a gnotobiotic mouse. Three of the five selected bifidobacteria strains successfully inhibited the transfer of antibiotic resistance genes and subsequently decreased the development of antibiotic-resistant enterobacteriacea in digestive tract. Similarly, Zoppi *et al.* (2001) reported that *Bifidobacterium* and *Lactobacillus* effectively prevented antibiotic resistance. These probiotics were able to decrease the production of beta-lactamase in fecal flora after treatment with a β-lactam antibiotic. This finding suggested that probiotics could prevent the establishment of antibiotic resistance among intestinal microflora because β-lactamase is an enzyme that breaks the β-lactam ring structure subsequently leading to the deactivation of the β-lactam antibiotic. Production of this enzyme often leads to increased bacterial resistance to β-lactam-based antibiotics.

Probiotics have also been investigated for their roles in reducing the risk of coronary heart disease (CHD). The risk of CHD generally increases with increasing levels of serum cholesterol. Past studies have demonstrated that probiotics exerted hypocholesterolemic effects. Liong and Shah (2005) reported that *Lactobacillus* removed cholesterol via three possible mechanisms including assimilation of cholesterol, incorporation of cholesterol into cell membrane and binding of cholesterol to cell surface. In another study, Nguyen *et al.* (2007) reported that ingestion of 10^7 CFU/day of *L. plantarum* by hypercholesterolemic mice reduced serum cholesterol and triglycerides levels by 7% and 10% respectively, compared to that of the control. Thus, considering the reduction of cholesterol level by probiotics in *in vivo* models, these beneficial bacteria could possibly reduce the risk of CHD.

In addition, previous studies have reported promising evidences that probiotic fermented food exerted angiotensin I-converting enzyme (ACE)-inhibitory activity and antihypertensive effects due to the production of bioactive peptides (Seppo *et al.*, 2003). ACE is an enzyme that plays an important role in the regulation of blood pressure and inhibition of ACE will lead to lowering of blood pressure. *Lactobacillus* and *Bifidobacterium* strains have been demonstrated to possess proteolytic activity that could hydrolyze long oligopeptides to produce ACE-inhibitory peptides (Donkor *et al.*, 2005) with antihypertensive properties. *L. acidophilus*, *L. casei* and *B. lactis* fermented yogurt have been found to contain ACE inhibitor peptides such as Val–Pro–Pro (VPP) and Ile–Pro–Pro (IPP). *L. delbrueckii* subsp. *bulgaricus* and *L. lactis* subsp. *cremoris* was also reported to liberate ACE-inhibitory peptides with IC_{50} ranging from 8.0 to 11.2 mg/L in milk (Gobbetti *et al.*, 2000). This indicates that probiotic fermented food could be used as an alternative treatment for hypertension.

2.2 Prebiotics and Synbiotics

Prebiotics are defined as “a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon” (Gibson and Roberfroid, 1995). Prebiotics have a long history of safe use and occur naturally in food. Food components such as oligosaccharides and polysaccharides are of great importance to exert prebiotic effects. In addition, food additives and sugar alcohols such as sorbitol, xylitol and mannitol are also potential prebiotics which are not absorbed by the small intestines (Gibson and Roberfroid, 1995). Prebiotics are widely utilized in the food and beverage industries to improve organoleptic qualities.

Consumption of prebiotics has been associated with various health promoting effects such as protection against colon cancer, enhancement of calcium absorption and reduction of cholesterol. The principle action of prebiotics is to modify the composition of intestinal microflora and thus improve the bowel health (Bruzzese *et al.*, 2006). Prebiotics act as substrates to the beneficial bacteria and fermentation of prebiotics could produce compound with protective effects such as short chain fatty acids (SCFA) in the gastrointestinal tract, such as acetate, propionate and lactate which subsequently reduce the pH of colon (Wong and Jenkins, 2007). The acidic environment exerts an antibacterial effect against other pathogens in the gastrointestinal tract, leading to improve gastrointestinal microbial balance and bowel health (Gibson and Roberfroid, 1995).

Prebiotics ingestion and the production of SCFA have also been associated with improved calcium absorptions. A randomized, double-blind, crossover study has reported that consumption of 15 g/day of oligofructose for 9 days enhanced the calcium absorption of twelve male adolescent (van den Heulen *et al.*, 1999). Prebiotics could increase the absorption of calcium via binding or sequestering calcium in the upper gastrointestinal tract. Bound or sequestered calcium on prebiotics would then reach the colon where it was being released from the prebiotics matrix and absorbed in the colon (Roberfroid, 2000). Therefore, the ingestion of prebiotics would increase the absorption of calcium. The absorption of calcium has also been found to increase in the presence of acids in the intestines (Scholz-Ahrenz *et al.*, 2001). Prebiotics are indigestible where they escape digestion of upper intestine and serve as the substrate for microflora population in lower intestines to produce SCFA. SCFA and H^+ have been found to exchange for Ca^{2+} in the distal colon regions (Trinidad *et al.*, 1996). This would then increase the concentration of Ca^{2+} which favour passive diffusion and consequently absorbed by the human colon (Roberfroid, 2000).

In addition, insoluble prebiotics have also been demonstrated to exert hypocholesterolemic effects. The levels of cholesterol have been reported to reduce via the binding effect of prebiotics. These insoluble prebiotics could absorb cholesterol, fat and phospholipids in the lower intestines and subsequently excrete them in faeces. The insolubility of prebiotics could shorten the time of gastric transit and thus rapidly excrete the cholesterol and fats. Gallaher *et al.* (2002) proposed that fiber could bind with bile acids and reduce solubilisation of cholesterol leading to a cholesterol lowering effect. The reduction of total cholesterol regulated the receptors of LDL and thus increased the clearance of LDL cholesterol (Aller *et al.*, 2004). This overall cholesterol lowering effect could reduce the stiffness of large arteries and potentially reduce blood pressure (Ferrier *et al.*, 2002).

In addition to cholesterol lowering effects, fibrous prebiotics has also been associated with the well-being of blood glucose profile and attenuation of insulin resistance, attributed to the insoluble fractions of prebiotics. Insoluble prebiotics are often extracted from native plant fibres such as cellulose, hemicelluloses, lignin and wheat bran. They have been found to improve postprandial glucose response and decrease secretion of insulin via a lowered glycemic index. The lower circulation of insulin up-regulated the insulin receptors and secondary signalling molecules resulting in increased tissue insulin sensitivity (Robertson *et al.*, 2003). In addition, other insoluble prebiotics such as high cereal fiber has also been associated with the reduced risk of diabetic, mainly attributed to their metabolism in the colon by indigenous microflora producing SCFA and their effects on hepatic insulin sensitivity (Schulze *et al.*, 2007). SCFA has been suggested to improve hepatic insulin sensitivity (Weickert *et al.*, 2006). Cereal fiber has a low glycemic index and has been studied for their roles in managing diabetes via lowering of early postprandial hyperglycemia and decreasing risks of post-absorptive hypoglycemia (Ludwig, 2002). The beneficial effects of fibrous prebiotics on blood glucose and lipid profile was showed in Table 2.1.

Prebiotics such as fructans has been demonstrated to decrease the incidence of obesity. Past studies involving animal models mainly rats has shown promising evidences that ingestion of inulin-type fructans could regulate body weight via the promotion of endogenous glucagon-like peptide-1 (GLP-1) in the gut (Cani *et al.*, 2005). GLP-1 is a key hormone released from enteroendocrine-L cells in response to nutrient ingestion and is the key modulator of food intake by promoting satiety (Delzenne *et al.*, 2007). This consequently reduces the intake of food which leads to a decreased in body weight and body mass index. Most of the studies involving the promotion of satiety by fructans via increased production of GLP-1 were performed in animal models and little information is available on human subjects. However, this remains a possible mechanism of fructans in promoting satiety. Piche *et al.* (2003) had previously demonstrated that ingestion of 6.6 g of oligofructose three times a day for 7 days increased the released of GLP-1 in nine subjects. Although the GLP-1 level was not directly associated with satiety in this study, the promotion of GLP-1 in human was a plausible finding of the effects of fructans in human. Additionally, it is interesting to note that ingestion of 16 g/day of oligofructose for 2 weeks decreased the total energy intake by 5% and increased satiety following breakfast and dinner in human subjects (Cani *et al.*, 2006).

Table 2.1: Effects of fibrous prebiotics on blood glucose and lipid profiles

Intervention	Prebiotics	Dose; duration of the study	Experimental design	Animals/ Subjects	Effects	Ref
Blood Glucose	Alginate fibre	5.0 g sodium alginate supplement (algae isolate, 75% soluble fibre); for two days	Randomized, placebo-controlled	Seven men with type 2 diabetes; mean age of 53 years	31% reduced in postprandial rise of blood glucose (P< 0.05) and 42% reduced in serum insulin (P< 0.02)	Torsdottir *et al.* (1991)
	Soy hulls	26 g of soy hulls which incorporated into 7 slices of bread daily; for 4 weeks	Randomized, double-blind, placebo-controlled	Ten subjects (5 male and 5 female) with type 2 diabetes; mean age of 65 ± 5.9 years	Significantly improved the glucose score (P< 0.05) and the total area under the glucose curve (P< 0.05) by 6.7% and 7.1%, respectively	Mahalko *et al.* (1984)
Lipid Profile	Pectin	75 g citrus pectin daily; for four weeks	Randomized, placebo-controlled	Six male adult hypercholserolemic minipigs	67.1% decrease in VLDL- cholesterol (P< 0.05); 41.1% decrease in LDL-cholesterol (P< 0.05); 49.4% decrease in total serum cholesterol (P < 0.05)	Ahrens *et al.* (1986)
	Fiber (*Plantago ovata* husk)	10.5 g *Plantago ovata* husk daily; for eight weeks	Randomized, crossover, placebo-controlled, single-blind	Twenty-eight men with myocardial infarction or stable angina	6.7% decrease in plasma triacylglycerol (P < 0.02), 6.7% increase in HDL-cholesterol (P < 0.006); 10.6% decrease in the total cholesterol/HDL ratio (P < 0.002); 14.2% decrease in LDL/HDL ratio (P< 0.003)	Sola’ *et al.* (2007)

In addition to prebiotics, another possible approach to promote a healthier gastrointestinal balance is via consumption of synbiotics. Synbiotics are ingredients that contain a mixture of both probiotics and prebiotics to produce synergistic effects on the maintenance of a desirable microbial population in the intestinal microbiota (Smejkal *et al.*, 2003). The concept and beneficial effects of synbiotics has been widely reported in past studies. Liong and Shah (2006) has found that a synbiotic diet that contained *L. casei*, FOS, and maltodextrin significantly improved the bowel microbial balance and reduced serum total cholesterol. Synbiotics has also been suggested to reduce infection and inflammation in organ transplantation patients (Bengmark, 2004). In another study, Gallaher and Khil (1999) demonstrated that oral administration of oligofructose and bifidobacteria reduced the risk of colon cancer in carcinogen-treated rats. However, individual administration of bifidobacteria or oligofructose insignificantly reduced the aberrant crypt counts. These show that the use of probiotics and prebiotics in combination could provide additive health promoting effects.

2.3 Soy

The consumption of soy (*Glycine max*)-based food has increased tremendously due to epidemiological and clinical evidence which showed health promoting effects of soy-based food. Soy provides a good source of protein as it contains appreciable amount of essential amino acids, low quantity of saturated fat without containing cholesterol. In addition to the nutritional attraction, new soy-based products with better organoleptic properties have been produced to improve the acceptability of consumer towards soy-based food.

2.3.1 Nutritional Profile and Health Benefits

Consumption of soy has been associated with the prevention of hormone dependant diseases including osteoporosis, cardiovascular diseases and cancer.

Generally, these vast beneficial effects are attributed to isoflavones that occur abundantly in soy-based products. Isoflavones are a group of phytoestrogens which are structurally similar to human estradiol. Considering their structural similarity, isoflavones could bind to estrogen receptors and provide agonist or antagonist actions.

Previous studies have demonstrated that soy and soy isoflavones reduced the risk of prostate and breast cancer. Kurahashi *et al.* (2007) conducted a population-based prospective study with a 5 year follow up involving 43,509 Japanese men (45 - 74 years old) and found that isoflavones intake was associated with a decreased risk of localized prostate cancers. In another population based case-control study, Shu *et al.* (2001) reported that high soy intake during adolescence reduced the risk of breast cancer in later stage of life. The population-based study involved 1459 chinese women (25 - 64 years old) with newly diagnosed breast cancer and 1556 healthy women subjects as control.

Soy and soy isoflavones have also been found efficient in reducing the risk of osteoporosis. Arjmandi *et al.* (1996) demonstrated that soybean diets exhibited a protective effect on bone. A total of 32 female rats were ovariectomized or sham operated and were fed with either control diet (casein) or soy protein isolate. The bone density was significantly reduced after ovariectomized but the rat fed with soy diet showed greater vertebral and femoral bone densities compared to the ovariectomized rat fed with control diet. In another population-based studies, Ikeda *et al.* (2006) reported that intake of fermented soy products (natto) prevented postmenopausal bone loss. The study involved 944 women (20-79 years) and a follow up was conducted after 3 years. High consumption of fermented soy products increased bone mass density in postmenopausal women.

Soy-based food has also received considerable attention for their potential roles in preventing cardiovascular disease. Such protective roles of soy are attributed to soy protein and isoflavones that occurs naturally in soy (Sacks *et al.*, 2006). Sagara *et al.* (2004) reported that dietary intake of soy for 5 weeks effectively reduced the risk of cardiovascular disease among high-risk middle-aged men. Sixty-one men with relatively higher blood pressure and/or total cholesterol levels were randomly assigned to either soy-containing diet (soy powder containing at 20 g soy protein and 80 mg isoflavones) or placebo diet (olive oil). The authors found that there was a significant reduction from baseline in total cholesterol, systolic blood pressure and diastolic blood pressure in the soy-containing diet group, but not in the placebo group. In another study, Crouse III *et al.* (1999) conducted a double blind randomized parallel trial and found that naturally occurring isoflavones in soy protein could reduce plasma total and low density lipoprotein (LDL) cholesterol. A total of 156 healthy men and women were assigned to a control diet containing 25 g casein or 25 g soy protein diet containing 3, 27, 37 or 62 mg isoflavones. Administration of soy protein with 62 mg of isoflavones lowered total cholesterol by 4% and LDL by 6% compared to that of the control.

2.3.2 Antinutritive Factors

Soybeans like many other legumes may contain several undesirable antinutritive components. These components include trypsin inhibitors, phytate (inositol hexaphosphate), oligosaccharides and saponins. However, these antinutritive factors could be reduced and bioconverted into other compounds upon processing (Anderson and Wolf, 1995).

Trypsin inhibitors occur naturally in whole soybean in an amount of 17 - 27 mg/g of soybean. Trypsin inhibitors are considered antinutritive due to its interference with protein digestion. Therefore, elimination of trypsin inhibitors is beneficial and this could be achieved via heat processing. Being a protein, trypsin inhibitors are susceptible to heat and are readily inactivated and denaturated by heat (Anderson and Wolf, 1995). In addition, fermentation could also decrease the concentration of trypsin inhibitors. Hong *et al.* (2004) has previously reported that fermentation of soybean and soybean meal by *Aspergillus oryzae* eliminated most of the trypsin inhibitors in soy.

Saponins are glycosides composed of polar sugar residues attached to a lipid soluble aglycones with triterpenoid structure (Messina, 1999). These components have been postulated to be toxic (Anderson and Wolf, 1995). However, fermentation could reduce the content of these components. Fermented soy products such as natto and miso have been reported to exhibit lower saponins content compared to unfermented counterparts (Anderson and Wolf, 1995). Phytic acids have been recognized to reduce the absorption and bioavailability of mineral in beans (Messina, 1999). Phytic acids concentrations in soybean vary considerably and on average the concentration is approximately 1% - 2%. Fermentation of soybean could reduce the concentration of phytic acids. Egounlety and Aworh (2003) reported that fermentation of soybean with *Rhizopus oligosporus* reduced the concentration of phytic acids by 30%.

Soybean oligosaccharides such as stachyose and raffinose are also regarded as antinutritive, as these sugars are not digested in the small intestine due to deficient of α-galactosidase enzyme in human intestinal. These sugars are degraded by human intestinal bacteria to carbon dioxide, hydrogen and methane, leading to flatulence and abdominal pains. However, these sugars have also been reported to exert prebiotic effects (Roberfroid, 2007), and was found useful to improve the microbial balance in gastrointestinal tract. The antinutritive properties of soybean oligosacharides could be eliminated by hydrolysis with extracellular α-galactosidase enzyme or fermentation with microorganism that possess this enzyme such as lactobacilli and bifidobacteria.

2.3.3 Potential Risks of Soy Consumption

Naturally, soy contains a reasonable amount of isoflavones which has chemical structure similar to that of estrogen. In recent years, estrogen used in hormone replacement therapy (HRT) has raised several controversies. The estrogen therapy has been reported to stimulate the proliferation of endometrial and mammary gland tissue and subsequently increase the risk of breast cancer (Wuttke *et al.*, 2008). Considering the structural similarity of isoflavones and estrogens, it has been postulated that consumption of soy/soy isoflavones may also lead to the same side effects as estrogen.

Studies on the negative effects of soy isoflavones on the mammary gland tissue and risks of breast cancer are limited. One of the most detailed studies was a cross-sectional study (<1 year) by Maskarinec and Meng (2001) which involved 514 women (mean age: 53.9 years old) and has reported that soy intake were positively associated with percent mammographic densities. Mammographic densities refer to the distribution of fat, connective, and epithelial tissue in female breast and are associated with increased breast cancer risk. The study demonstrated that percent mammographic density of women with higher soy intake (>38.4 g/day) was 11% greater than that of women with lower soy intake (<6.7 g/day). However, controversial finding has been reported. In another randomised placebo-controlled study (220 premenopausal women), women consuming 2 serving of soy/day (50 mg isoflavones/day) for 2 years showed no significant differences in mammographic densities compared to the control group (Maskarinec *et al.*, 2008). It has been suggested that isoflavones do not exert an estrogenic effect similar to hormone replacement therapy on mammographic density and are safe for long term consumption.

The safe consumption of soy by men has also been debated lately. A population-based prospective study (follow up from 1994 through 2005) involving 43,509 Japanese men (ages: 45 - 74 years) has reported that consumption of soy isoflavones was associated with increase risk of advanced prostate cancer (Kurahashi *et al.*, 2007). However, in the same study, the authors demonstrated that soy food/soy isoflavones intake significantly decreased the risk of localized prostate cancer. In another study, consumption of soy protein (107 mg isoflavones/day) has been reported to exert preventive effects against advanced prostate cancer in 58 men. This randomised study demonstrated that consumption of soy protein for 6 months significantly suppressed androgen receptor expression, and thus may exhibit anti-prostate cancer potential (Hamilton-Reeves *et al.*, 2007). Up to date, studies on negative effect from the consumption of soy are scarce. On the other hand, numerous clinical and epidemiological studis has shown promising evidences on the potential application of soy for the prevention of prostate cancers in men.

2.4 Probiotics and Soy Products

In the past, dairy-based products have been widely used as carriers for probiotics both in the developing and industrialized country. However, the drawbacks of dairy-based carriers associated with cholesterol contents, lactose intolerance and desire for vegetarian alternative have prompted the development of alternative carriers for probiotics (Farnworth *et al.*, 2007). Considering the nutritional content and nature of soy, soy-based products could substitute dairy-based products as carriers for probiotics. Soybean, a vegetarian diet is well-known as an inexpensive source of protein and calories for human consumption. It also contains reasonable amounts of bioactive ingredients without containing cholesterol or lactose (Scalabrini *et al.*, 1998). Therefore, much attempt has been made to develop soy-based product as probiotic dietary adjuncts in recent years.

Despite having such attractive nutritional values, consumption of soy has been limited mainly due to its poor organoleptic properties. Soy products often exhibit unpleasant beany flavour due to the presence of aldehydes such as n-hexanal and pentanal, produced upon hydrolysis of unsaturated acids in soymilk. One possible solution to overcome this limitation is via fermentation by probiotics that possess reductase to reduce the content of n-hexanal and pentanal in soymilk (Scalabrini *et al.*, 1998). It was reported that n-hexanal was reduced from 16.5 ppb to 4 ppb, whereas pentanal was not detected in soymilk upon fermentation with bifidobacteria for 24 h. Tsangalis and Shah (2004) demonstrated that *Bifidobacterium* metabolized the entire hexanal and pentanal in soymilk after 12 h of fermentation. According to the authors, probiotics possess complex enzyme systems, which responsible for catalyzing the oxidation of aldehydes into carboxylic acids. This indicates that probiotics could improve the organoleptic properties of soy products, subsequently increase the acceptability and consumption of soy products.

Consumption of soy products fermented with probiotics has been found to be beneficial especially for modulation of gastrointestinal microbial balance, leading to improvement of human health. Shimakawa *et al.* (2003) demonstrated that administration of *B. breve* strain Yakult-fermented soymilk significantly increased the counts of beneficial bacteria in gastrointestinal tract of 15 healthy male subjects. The subjects were blindly and randomly assigned to two groups each receiving either 500 mL/day of unfermented soymilk or fermented soymilk containing 1.6 x 10^9 CFU/mL of *B. breve* for seven days. The study indicated that upon ingestion of fermented soymilk, total counts of *Bifidobacterium* in faeces were significantly higher than before ingestion. Thus, it appears that fermentation with probiotics is beneficial for development of soy products with health-promoting effects.

2.4.1 Soy as Carrier for Probiotics

In order to exert optimal probiotics effects, it is important that the numbers of live bacterial in food and fecal recovery of the administered bacteria are high. It has been suggested that probiotic organisms should be present in a food product at a minimum concentration of 10^6 CFU/g (Gomes and Malcata, 1999), or the daily intake of approximately 10^8 CFU to exhibit health-promoting effects. Past studies have reported that fermented soy-based products could fulfil both these requirements indicating that it serves as a potential vehicle for probiotics.

Up to date, various studies have been conducted to verify the efficiency of soy-based products as a potential carrier of probiotics. Donkor *et al.* (2007a) reported that probiotics could reach the desired therapeutic level (10^6 CFU/mL) in fermented soymilk due to its ability to metabolize soy oligosaccharides during fermentation. In addition to soymilk, other soy products were also evaluated for their efficiency to act as probiotic carriers. Probiotics was found to thrive well in soy yoghurt with viability above therapeutic levels (10^8 CFU/mL) and remained consistent even after 28 days