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THESIS

PRODUCTION OF FERMENTED SOYMILK POWDER CONTAINING LACTIC ACID BACTERIA BY SPRAY DRYING

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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The objective of this study was to develop a probiotic soymilk powder by spray drying. Three strains of lactic acid bacteria, *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49 was evaluated for their tolerance to gastrointestinal tract conditions. During exposure to simulated gastric juice the viable cell of all test strains decreased dramatically to 2 log CFU/ml after 30 min and an undetectable level after 60 min. The strains showed high tolerance to simulated bile juice with the viable cell number remained constant after 240 min incubation. During fermentation of soymilk by the strains, *L. plantarum* P49 was found to increase calcium solubility and exploit raffinose and stachyose more efficiently than other strains. Furthermore, effect of sugar (glucose, galactose and sucrose) on calcium solubility and oligosaccharide reduction in soymilk fermented by *L. plantarum* P49 was evaluated. Dramatic increase in soluble calcium was obtained in the fermented soymilk with sucrose addition, 2.5-fold compared to the control. Moreover, the addition of sugar was found to enhance the reduction of raffinose and stachyose. Survival of all test strains after spray drying and during a 6-month period of storage was examined. After spray drying, *L. casei* subsp. *tolerans* TISTR 1341 showed a highest survival percentage of 107.69 %. A significantly ($p<0.05$) higher percent of survival was also noted when the spray-dried fermented milk in sealed aluminum foil bag was stored at 4 °C than 30 °C. During storage at 4 °C for 6 months, the viability of all samples was quite stable with viable cell of 10^8-10^9 CFU/g. While, the viable cell of the strains after 4 months of storage at 30 °C was not detectable. Finally, spray-dried cell encapsulated with soymilk exhibited high tolerant to gastric and bile juices. This finding suggested that fermented soymilk powder containing lactic acid bacteria could be used as a dietary adjunct.

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INTRODUCTION

Soymilk provides a high quality of protein, iron, unsaturated fatty acids and niacin. Based on free of cholesterol, gluten and lactose, soymilk could serve as beverage for lactose-intolerance consumers, vegetarians and milk-allergy patients. They also contain isoflavone which have been reported to have the potential to reduce the total and LDL cholesterol, the risk of age-related and hormone-related diseases including cancer, menopausal symptoms, cardiovascular diseases and osteoporosis (Setchell and Cassidy, 1999). However, the consumption of soymilk is limited because of the beany flavor and the presence of non-digestible oligosaccharides such as stachyose and raffinose. These non-digestible oligosaccharides are not digested by human beings and may cause flatulence. Further, soybeans are rich with phytic acid which is negatively charged, it complexes with positively charged ions or proteins and subsequently decrease the bioavailability of these compounds (Lopez *et al*., 2000; Shirai *et al*., 1994). Lactic acid fermentation is the way to overcome these limitations.

In the last few decades, lactic acid bacteria have attracted enormous attention for food manufacturer due to their potential associated with health-promoting effects. They are classified as probiotics which do not pose any health risk to the man and some are designated as “GRAS” (Generally Recognised As Safe) organisms. Probiotic lactic acid bacteria are provides substantial health benefits to human health by means of maintenance of normal intestinal flora. Some of probiotic strains have been shown to be effective in reducing the severity and duration of diarrhea, lowering cholesterol, increasing of turnover of enterocytes and neutralizing of dietary carcinogens (Saito, 2004).

Through fermentation of lactic acid bacteria, fermented products are preserved and improved of nutritive properties and the texture. Lactic acid bacteria also found to

produce nutraceuticals (e.g., vitamins) and reduced toxic (e.g., biogenic amines) in fermented products. There has been reported that soymilk fermented with lactic acid bacteria overcome the problem of beany flavour and flatulence result in an increased the acceptability and the nutritional value (Liu and Lin, 2000; Fávaro Trindade *et al*., 2001; Wang *et al*., 2003). They possess an enzyme of α-galactosidase which hydrolyzes α-galactosides of stachyose and raffinose in soymilk (Donkor *et al*., 2007). Also, fermentation of soymilk by lactic acid bacteria can enhance the calcium bioavailability due to increased calcium solubility (Tang *et al*., 2007).

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Technological challenge associated with the application of probiotic microorganism in functional food is the maintenance of viability during processing. This is a particular concern, given that high levels (at least 10^7 CFU/g) of live microorganisms are recommended for probiotic products. Production of an instant fermented powder would provide benefit of shelf-life extension and convenience of preparation and storage. Spray drying may be a suitable method for production of soymilk powder enriched with high numbers of viable lactic acid bacteria. The successful spray drying of probiotic has been reported for a number of different strains (Conrad *et al.,* 2000; Desmond *et al*., 2002; Hamsupo, 2005; Hamsupo *et al*., 2005; Morgan *et al*., 2006, Nguyen *et al.,* 2007). However, cell injury occurs during spray drying, potentially destroying the properties and performance characteristics of the probiotic culture.

The aim of the present study is to produce fermented soymilk powder containing lactic acid bacteria by spray drying. The strain viability in fermented soymilk was evaluated after spray drying and storage at different temperature for 6 months. The growth of lactic acid bacteria, the consumption of oligosaccharides and the change in calcium solubility were evaluated during fermentation of soymilk with and without sugar addition by lactic acid bacteria. Additionally, acid and bile tolerances of the strain were evaluated for probiotic characteristic.

The objectives of this study were:

1. To investigate the viability of *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49 in fermented soymilk after spray drying and storage at different temperature for 6 months.

2. To evaluate the oligosaccharides content and the calcium solubility in soymilk with and without sugar addition during fermentation by lactic acid bacteria.

3. To investigate acid and bile tolerances of free cells and spray-dried cells of the test strains.

LITERATURE REVIEW

1. Lactic acid bacteria and their applications

1.1 Background information of lactic acid bacteria

The lactic acid bacteria (LAB) are a group of Gram-positive bacteria, non-respiring, non-spore forming, cocci or rods, which produce lactic acid as the major end product of the fermentation of carbohydrates. They can be classified into two groups including homofermentative and heterofermentative. The homofermentative LAB convert glucose almost exclusively into lactic acid, while the heterofermentative LAB catabolize glucose into ethanol and CO_2 as well as lactic acid (Figure 1) (Wee *et al*., 2006).

The term LAB is conventionally reserved for genera in the order *Lactobacillales*, which includes *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus* and *Streptococcus*, in addition to *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weisella*. LAB are normal flora of humans in the oral cavity, the intestinal tract and the vagina, where they play a beneficial role. However, there have been many publications describing the use of probiotics to prevent and treat a variety of gastrointestinal disorders, only a few have contributed convincingly of the health effects of probiotics in humans. A few LAB are pathogenic for human, most notably some members of the genus *Streptococcus* especially the hemolytic streptococci, others like the *enterococcus* may be pathogens if they penetrate to unusual places in the body causing for example endocarditis or urinary tract infections. LAB are also found in environment of human beings including milk and milk products and in decaying plant materials. They contribute to the taste and texture of fermented products and inhibit food spoilage bacteria by producing growth-inhibiting substances and large amounts of lactic acid. As agents of fermentation, LAB are involved in making yogurt, cheese, kefir, salami, sikhae, kimchi and sauerkraut (Table 1).

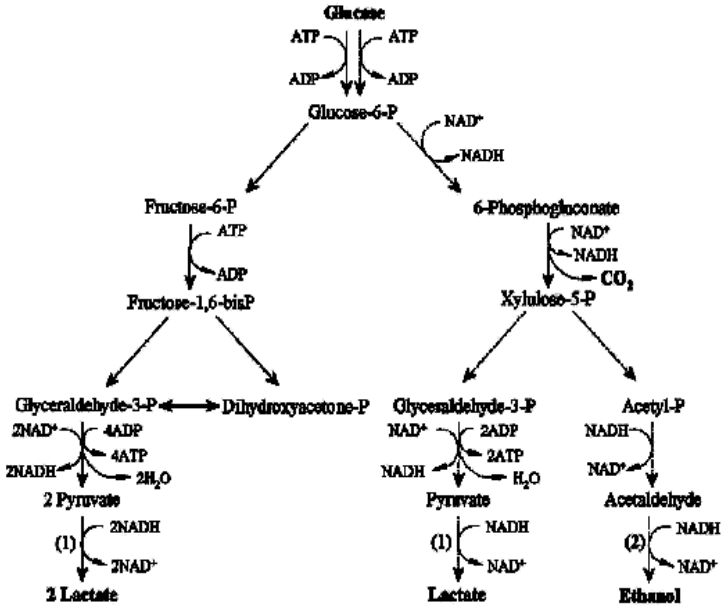


Figure 1 Metabolic pathways of homofermentative and heterofermentative LAB

Source: Wee *et al*. (2006)

1.2 Role of lactic acid bacteria in food preservation

LAB are thought to be safe bacteria that have been ingested from foods without any problems for many years and some are designated as "GRAS" (Generally Recognised As Safe) organisms (Saito, 2004). They have a long and safe history of use as preservatives in food fermentations, especially in dairy products where they are commonly employed as starter cultures. They are associated with the human environment and their beneficially interactions are both in food and in the human intestinal tract. Probiotics could maintain the healthy intestinal microbiota through competitive exclusion and antagonistic action against pathogenic bacteria in the animal intestine (Fuller, 1989). It has been proposed that probiotics could maintain the healthy intestinal microbiota through competitive exclusion and antagonistic action against pathogenic bacteria in the animal intestine. The ability of LAB to inhibit the growth of certain pathogens may be due to the production of organic acids such as lactic and acetic acids, hydrogen peroxide, bacteriocins and bacteriocin-like

substances. On the other hand, the adhesion of probiotic bacteria to intestinal mucosa could prevent the attachment of pathogens and stimulate their removal from the infected intestinal tract (Lee *et al*. 2000). These antagonistic properties could be very useful in probiotic products. Metabolic products of LAB with antimicrobial properties are presented in Table 2. Each of these properties or a combination of some of properties can be used to extend the shelf life and safety of food products.

Table 1 Example of fermented foods involving lactic acid fermentation

Product name	Major ingredients	Microorganisms
Sour bread	Wheat	Lactic acid bacteria, yeast
Gari	Cassava	*Leuconostoc*, *Alcaligenes*, *Corynebacterium*, *Lactobacillus*
Mungbean starch	Mungbean	*L. mesenteroides*, *L. casei*, *L. cellobiosus*, *L. fermenti*
Sauerkraut	Cabbage, salt	*L. mesenteroides*, *L. brevis*, *L. plantarum*
Kimchi	Korean cabbage, radish, various vegetables, salt	*L. mesenteroides*, *L. brevis*, *L. plantarum*
Sikhae	Sea-water fish, cooked millet, salt	*L. mesenteroides*, *L. plantarum*
Pla-ra	Fresh-water fish, salt, roasted rice	*Pediococcus* sp.
Nham	Pork, garlic, salt, rice	*P. cerevisiae*, *L. plantarum*, *L. brevis*
Nem-chua	Pork, salt, cooked rice	*Pediococcus* sp., *Lactobacillus* sp.
Salami	Pork	*Lactobacillus*, *Micrococcus*
Yogurt	Milk	*L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*
Kefir	Milk	*L. kefir*, *L. kefiranofacies*, *L. brevis*
Cheese	Milk	*E. faecium*
Rice wine	Rice	*L. sakei*
Natto	Soybean	*B. subtilis*

Source: Modified from Lee (1997)

There has been reported that LAB growth in meat can cause microbial interference to spoilage and pathogenic bacteria through several mechanisms like nutrient and oxygen competition, competition for attachment/adhesion sites and production of a wide range of inhibitory substances primarily lactic acid or lactic and

acetic acids, acetoin, diacetyl, hydrogen peroxide, reuterin and bacteriocins (Hugas, 1998).

During lactic acid fermentation, production of lactic acid contributes to a major safety factor in fermented food. The organic acids lower the pH and thereby indirectly affected growth of the pathogen. In addition, the lactic and acetic acids can be toxic to microbes. The antimicrobial action of these acids is related to the ability of the undissociated acid molecules to penetrate through the bacterial plasma membrane as a function of their diffusion constant. In the cytoplasm, the acid dissociates to release protons and conjugate bases with higher pH, this disrupts the membrane proton-motive force, thus disabling the energy-yielding and transport process dependent upon it (Savard *et al*., 2002).

Table 2 Metabolic products of lactic acid bacteria with antimicrobial properties

Product	Main target organisms
Organic acids	
Lactic acid	Putrefactive and gram-negative bacteria, some fungi
Acetic acid	Putrefactive bacteria, clostridia, some yeasts and fungi
Hydrogen peroxide	Pathogens and spoilage organisms, especially in protein-rich foods
Enzymes	
Lactoperoxidase system with H_2O_2	Pathogens and spoilage bacteria (milk and dairy products)
Lysozyme (by recombinant DNA technology)	Undesired gram-positive bacteria
Low-molecular-mass metabolites	
Reuterin (3-OH-propionaldehyde)	Wide spectrum of bacteria, moulds, and yeasts
Diacetyl	Gram-negative bacteria
Fatty acids	Different bacteria
Bacteriocins	
Nisin	Some lactic acid bacteria and gram-positive bacteria, notably endospore-formed
Other	Gram-positive bacteria, inhibitory spectrum according to producer strain and bacteriocin type

Source: Karovičová and Kohajdová (2005)

Hydrogen peroxide is generated by different mechanisms by certain lactobacilli. Accumulation of hydrogen peroxide can be occurred and be inhibitory to some microorganisms. Inhibition is mediated through the strong oxidizing effect on membrane lipids and cell proteins (Mayra-Makinen and Bigret, 2004; Karovičová and Kohajdová, 2005). Hydrogen peroxide may also activate the lactoperoxidase system of fresh milk with the formation of hypothiocyanate and other antimicrobials (Caplice and Fitzgerald, 1999).

Diacetyl (2, 3-butanedione, biacetyl) is perhaps best known as the compound responsible for the characteristic aroma and flavor of butter. It is produced by some species and strains of the genera *Streptococcus*, *Leuconostoc*, *Lactobacillus*, and *Pediococcus*, as well as by other organisms. Although diacetyl was identified as a flavor compound but its antibacterial action was noted by many researchers. Its mode of action is believed to be due to interference with the utilization of arginine (Caplice and Fitzgerald, 1999). Jay (1982) tested commercial diacetyl preparations at 172 and 344 μg/ml against the set of 40 cultures and found that the Gram-positive non-LAB (*Corynebacterium striatum*, *B. subtilis*, *S. epidermiditis*) were not inhibited by 344 μg/ml.

Bacteriocins from LAB are ribosomally produced peptides (usually 30–60 amino acids) that display potent antimicrobial activity against certain other Gram-positive organisms. They function by disruption of the membrane of their targets, mediated in at least some cases by interaction of the peptide with a chiral receptor molecule (e.g. lipid II or sugar PTS proteins) (Garneau *et al*., 2002). According to Schillinger and Lücke (1989), a total of 221 strains of *Lactobacillus* isolated from meat and meat products were screened for antagonistic activities under conditions that eliminated the effects of organic acids and hydrogen peroxide. Nineteen strains of *L. sake*, three strains of *L. plantarum*, and one strain of *L. curvatus* were shown to inhibit the growth of some other lactobacilli. The compound excreted by *L. sake* Lb 706 was active against various LAB and *Listeria monocytogenes*. Its proteinaceous nature, narrow inhibitory spectrum, and bactericidal mode of action indicated that this substance is a bacteriocin, which we designated sakacin A. Maisnier-Patin *et al*.

(1992) demonstrated the potential of using nisin-producing starters for the inhibition of *L. monocytogene*s in Camembert cheese. The nisin-producing starter was composed of a pair of isogenic protease-positive and protease-negative strains of *L. lactis*. In this case, use of the nisin-producing strain was associated with a reduction in *L. monocytogenes* levels by over three log cycles compared to the initial level in the cheese-milk.

2. Probiotics and their applications

Probiotics are defined as live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance (Fuller, 1989). The claims made for probiotics mainly include improvement of health via consumption of probiotic products. Such products are gaining more widespread popularity and acceptance throughout the developed world and are already well accepted in Japan and the USA. The dairy products, specifically yogurt-like products-form are the largest segment by far of the market for probiotic products. Furthermore, increased commercial interest in exploiting the proposed health attributes of probiotics has contributed in a significant way to the rapid growth and expansion of this sector of the market.

2.1 Mode of action of probiotics

Proposed mechanisms of pathogen inhibition by the intestinal microbiota include competition for nutrients, production of toxic conditions and compounds (volatile fatty acids, low pH, and bacteriocins), competition for binding sites on the intestinal epithelium, and stimulation of the immune system. The same probiotic may inhibit different pathogens by different mechanisms. In addition, some microorganisms may effect change with a single mechanism, whereas others may use several mechanisms. Such probiotic microorganisms appear to be promising candidates for the treatment of intestinal disorders produced by abnormal gut microflora and altered gut mucosal barrier functions.

(i) Production of inhibitory substances

LAB have been shown to inhibit the in vitro growth of many enteric pathogens. The ability of lactic acid bacteria to inhibit the growth of various Gram-positive or Gram-negative bacteria may be due to the production of organic acids such as lactic and acetic acids, hydrogen peroxide, bacteriocins, bacteriocin-like substances and possibly biosurfactants, which are active against certain pathogens. The types of interactions of antimicrobial mechanisms include competitive colonisation as well as adhesion and growth inhibition. *L. acidophilus* and *L. gasseri* have been shown in vitro to strongly inhibit same enteropathogenic bacteria without interfering with the normal bacterial residents of the gastrointestinal tract, lactic acid could account for this antimicrobial activity (Fernández *et al*., 2003).

(ii) Competition for binding sites

Adhesion of the probiotic microorganisms to the intestinal mucosa is a prerequisite for colonization and for antagonistic activity against enteropathogens (Owehand, 1998). The results of competitive binding assays clearly showed that *L. gasseri* effect the attachment of enteropathogen *E. coli* to Caco-2 cells under the condition of exclusion (Fernández *et al*., 2003).

(iii) Competition for nutrients

The evidence of probiotics competition for nutrients in vivo is lacking. However, competitive colonisation refers to the fact that the probiotic strain can successfully outcompete the pathogen for either nutrients or the site of colonization (Conway, 1996).

(iv) Alteration of microbial metabolites

Several studies in both animals and humans have shown the ability of LAB to reduce the toxicity of intestinal contents by suppressing the levels of bacterial

enzymes such as β-glucoronidase, nitroreductase, azo-reductase and urease, all of which activate procarcinogens (Soomro *et al*., 2002).

(v) Stimulation of immunity

There is sufficient evidence suggesting that probiotics specifically (e.g. antibody production, cytokinase production, lymphocyte proliferation, delayed-type hypersensitivity) and nonspecifically (e.g. phagocyte function, NK cell activity) modulate the host's immune system. Because phagocytic activity is associated with natural immunity and phagocytes are involved in antibody immune responses as antigen-presenting cells, it is possible that the stimulation of intestinal IgA antibody responses induced by LAB may be explained partly by an effect on phagocyte cell function (Salminen *et al*., 1998).

2.2 Microorganisms used as probiotics

The probiotic preparations may be presented in the form of powders, tablets, capsules, pastes or sprays depending on the animal or human receiving the supplement and the condition to be treated. They may be administered by direct insertion into the mouth or by inclusion in the food or water. Experiments have also been done with the administration to newly hatched chicks by spraying into the surrounding atmosphere (Fuller, 1992). For human, probiotics may be consumed either as a food component or as a non-food preparation. To date, the most popular food delivery systems for these cultures have been freshly fermented dairy foods such as fermented milk and unfermented milk with cultures added (Stanton *et al*., 1998; Sanders *et al*., 1996).

The composition of the probiotic preparations varies from those containing one or several strains of microorganisms. A variety of microbial species have been used as probiotics, including species of *Bacillus*, *Bifidobacterium*, *Enterococcus*, *E. coli*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, a variety of yeast species (Table 3). *Lactobacillus* and *Bifidobacterium* species have been used most extensively in

humans, whereas species of *Bacillus*, *Enterococcus*, and *Saccharomyces* yeast have been the most common organisms used in livestock (Simon *et al*., 2001).

Table 3 Microorganisms considered as probiotics

Lactobacillus	*Bifidobacterium*	Other lactic acid bacteria	Non-lactic acid bacteria
L. acidophilus	*B. adolescentis*	*E. faecalis*	*Bacillus cereus var. toyoi*
L. amylovorus	*B. animalis*	*E. faecium*	*Escherichia coli* Nissle 1917
L. casei	*B. bifidum*	*L. lactis*	*Propionibacterium freudenreichii*
L. crispatus	*B. breve*	*Leu. mesenteroides*	*Saccharomyces cerevisiae*
L. delbrueckii subsp. *bulgaricus*	*B. infantis*	*P. acidolactici*	*Saccharomyces boulardii*
L. gallinarum	*B. lactis*	*S. thermophilus*	
L. gasseri	*B. longum*	*Sporolactobacillus inulinus*	
L. johnsonii			
L. paracasei			
L. plantarum			
L. reuteri			
L. rhamnosus			

Source: Holzapfel *et al*. (2001)

2.3 Selection of a good probiotic strain

The features of a good probiotic were summarized in Table 4. For probiotic microorganisms to exert beneficial health benefits it must fulfill several criteria. It is important that the culture that is added at the time of manufacture remain viable at high concentrations during the relevant shelf-life or storage period. In general, the minimum concentration of probiotic microorganisms necessary to exert a beneficial effect remains unclear. However, the therapeutic minimum dose of 10^5

viable cell/g or ml of product has been proposed (Lee and Salminen, 1995). Yogurt and fermented milks have received most attention as carriers of probiotic cultures. However, in more recent times, other fermented products have also been studied as carriers of probiotic organisms. Gardiner *et al*. (1999) found that *E. faecium* could survive to high numbers in Cheddar cheese during ripening at 8 °C for 15 months ($4x10^8$ CFU/g) and in yogurt during storage at 4 °C for 21 days ($4x10^7$ CFU/g). According to Dinakar and Mistry (1994), Cheddar cheese into which bifidobacteria was incorporated was examined as the vehicle. Bifidobacteria remained viable at approximately $2x10^7$ CFU/g during 24 weeks study but did not affect the flavor, flavor intensity, texture, or appearance of the cheese. In addition, probiotic culture must survive passage through the harsh environment of the gastrointestinal tract (GIT) with large numbers, hence the need to select strains that are acid and bile tolerant and possess the capability to adhere to intestinal cells. The food product used to deliver the probiotic culture may influence the ability of the probiotic to survive in the GIT. Gardiner *et al*. (1999) evaluated the delivery of viable microorganisms of *E. faecium* to the gastrointestinal tract using Cheddar cheese as a food carrier. In an in vitro model system, the results demonstrated that no viable probiotic cells were detectable after only 8 min in gastric juice, pH 2. In a parallel experiment in which the probiotic strain was added in Cheddar cheese to gastric juice, pH 2, no loss of viability was observed. Similarly, when probiotic yogurt was added to gastric juice, only a 10-fold reduction in probiotic numbers was observed over the 2 h exposure period. These data showed that food carriers such as Cheddar cheese or yogurt greatly enhance the survival of this strain in gastric juice, which is most likely due to the buffering capacity of the food product. Subsequently, a feeding trial involving 8 pigs per group was performed in which a rifampicin-resistant variant of the probiotic strain was fed for 21 days at a mean daily intake of Cheddar cheese or yogurt. During the feeding period, Cheddar cheese yielded a significantly higher mean fecal probiotic count than did yogurt. These data indicate that mature Cheddar cheese compares very favorably with fresh yogurt as a delivery system for viable probiotic microorganisms to the gastrointestinal tract.

Bile salts are detergent and disorganize the membrane lipid structure, and it is difficult for microorganisms to live under high concentration of bile salts conditions.

One hundred twenty two strains LAB isolated from traditional fermented dairy products in Inner Mongolia, China, were supplied to bile tolerance trials as indicator tests of probiotics. Results showed that *L. plantarum* strain 301102 exhibited the growth rate of 60 % after 6 h in MRS broth containing 0.3 % oxgall, survival rate of 71 % after 3 h in artificial gastric juice adjusted pH 2.0 and could grow in artificial intestinal juice. This suggested that the strain might be grown in intestine after overcome against low pH condition in stomach and bile acid. As a characteristic of this strain, the tolerance to the bile was extremely high by growing in MRS broth containing 20 % oxgall (Tsuda *et al*., 2007). Recently, fermented cereal has been looked to as a way of delivering LAB. Michida *et al*. (2005) investigated the effect of cereal extracts and cereal fiber on the viability of *L. plantarum* under gastrointestinal tract conditions. Regarding gastric tolerance, the addition of cereal extracts significantly improved the viability of *L. plantarum* while immobilization within cereal fiber slightly improved its viability. Meanwhile, immobilization within cereal fiber played a major role in bile tolerance and the presence of cereal extracts further enhanced the tolerance of *L. plantarum* to bile juice. In both media, cell immobilization within cereal fiber and the presence of cereal extracts had a synergistic effect on the gastrointestinal tolerance.

Table 4 Properties of a good probiotic strain

(i) exert a beneficial effect on the host
(ii) withstand into a foodstuff at high cell counts, and remain viable throughout the shelf-life of the product
(iii) withstand transit through the GI tract
(iv) adhere to the intestinal epithelium cell lining and colonize the lumen of the tract
(v) produce antimicrobial substances towards pathogens
(vi) stabilize the intestinal microflora and be associated with health benefits

Source: Parvez *et al*. (2006)

2.4 Health benefit and therapeutic effect of probiotics

There are a variety of proposed beneficial health effects of probiotics. Several probiotics including *S. boulardii*, *E. faecium* and *Lactobacillus* have been shown to be clinically effective in preventing antibiotic-associated diarrhea (Rolfe, 2000). The modulation of the intestinal microbiota by probiotics can be a useful tool for both the dietary management and the prevention of some allergic diseases. Perinatal administration of *L. rhamnosus* GG decreased subsequent occurrence of eczema in at-risk infants by one-half (Isolauri *et al*., 2000). Probiotics may also be helpful in alleviating some of the symptoms of food allergies such as those associated with milk protein (Majamaa and Isolauri, 1997). Consumption of certain strains of lactobacilli has shown an improvement in symptoms of inflammatory bowel disease (IBD), pouchitis and ulcerative colitis (Parvez *et al*., 2006). There is evidence that supplementation of the diet with fermented milks (yoghurt) has a beneficial hypocholesterolemic effect (Khedkar *et al*., 1993). Isolates of *L. acidophilus* from human intestinal material are able to assimilate cholesterol and actively deconjugate bile salts into free acids that are excreted more rapidly from the intestinal tract than are conjugated bile acids (Buck and Gilliland, 1994). In addition, Ling *et al*. (1993) has shown in human that oral administration of yogurt containing viable *Lactobacillus* strain GG decreases the activities of fecal enzymes that may convert procarcinogens to carcinogens in the colon.

The action of probiotics during the preparation of cultured foods or in the digestive tract has been shown to improve the quantity, availability and digestibility of some dietary nutrients. Fermentation of food with LAB increases minerals in carrot juice, whole wheat flour and soymilk (Bergqvist *et al*., 2005; Lopez *et al*., 2000; Tang *et al*., 2007). Bergqvist *et al*. (2004) improved iron solubility in carrot juice fermented by homo- and heterofermentative LAB. Results showed that *L. pentosus* FSC1 (homofermentative) had better capacity to solubilize iron than *Ln. mesenteroides* FSC2 during carrot juice fermentation. Interestingly, the level of soluble iron in the combined-strain fermented juice was in between that when both strains were inoculated separately. Tang *et al*. (2007) reported that fermentation of calcium-

fortified soymilk with *L. acidophilus* ATCC 4962 and *L. casei* ASCC 290 significantly increased ($P<0.05$) soluble calcium with 89.3 % and 87.0 % after 24 h, respectively. The increase in calcium solubility observed was related to lowered pH associated with production of lactic and acetic acids.

Furthermore, fermentation of LAB in soymilk has been reported to decrease the level of carbohydrates as well as some non-digestible oligosaccharides. This reduces side effects such as abdominal distension and flatulence. Many evidence showed that the appropriate strain of LAB reduced the content of stachyose and raffinose in soymilk (Wang *et al*., 2003; Scalabrini *et al*., 1998). Several lines of evidence showed that the appropriate strain of LAB can alleviate symptoms of lactose intolerance. Alm (1982) reported that eight lactose intolerant individuals showed symptoms of abdominal distress and diarrhea following consumption of 500 ml of low fat milk whereas ingestion of the same quantity of yogurt or acidophilus milk did not result in any symptoms. This was suggested that fermented milk products should be considered in formulating diets for lactose-intolerant subjects. In addition, fermentation of LAB assists in digestion of protein. According to Sindhu and Khetarpaul (2001), indigenously developed BCGT food mixture which contained barley flour, milk coprecipitate, sprouted green gram paste and tomato pulp was fermented with single culture (*L. casei*, *L. plantarum*) and mixed cultures (*S. boulardii* + *L. casei*; *S. boulardii* + *L. plantarum*). Results showed that all the fermentations improved the *in vitro* digestibilities of protein. Mixed culture fermentations brought about higher change as compared to single culture fermentations. Protein digestibility increased by 49, 47, 50 and 50 %, over the unprocessed control after fermentations with *L. casei*, *L. plantarum*, *S. boulardii* + *L. casei* and *S. boulardii* + *L. plantarum*, respectively.

3. Fermented soymilk

Soymilk provides a plentiful and inexpensive supply of protein and calories. It is considered as a suitable economical substitute for cow's milk and an ideal nutritional supplement for lactose-intolerant population. Additionally, soybean is a

rich source of isoflavone, which are reported to have beneficial estrogenic effects with potential bioactive antioxidant properties. Asian populations, with their intake of soy-derived isoflavones, are known to have the lowest incidence of osteoporosis, menopausal symptoms, mortality from cardiovascular disease, and cancer (Rekha and Vijayalakshmi, 2008; Chun *et al*., 2008). Jacobsen *et al*. (1998) reported that frequent consumption (more than once a day) of soymilk was associated with 70 % reduction of the risk of prostate cancer. The association was upheld when extensive adjustments were performed. Furthermore, the possibility that phytoestrogens (e.g., daidzein, genistein) may offer a natural alternative to conventional hormone replacement therapy (HRT) for the prevention of bone loss due to estrogen deficiency associated with loss of ovarian function during the menopause (Setchell and Cassidy, 1999). Soy protein is correlated with significant decreases in serum cholesterol and the risk of heart disease. There has been suggested by Carroll *et al*. (1995) that the presence of factors in soy protein can counteract the effects of hypercholesterolemic amino acids. Substitution of soy protein for animal protein in the diet reduced the concentration of serum cholesterol in humans. The difference effects of dietary proteins on serum cholesterol concentrations in humans and in rabbits are primarily due to changes in LDL cholesterol, and the hypercholesterolemia produced by dietary casein which associated with down-regulation of hepatic LDL receptors. Furthermore, phytic acid content in soybean has also been associated with reduced risk of cancer, reducing inflammation and minimizing diabetes (Vucenik and Shamsuddin, 2003; Yoon *et al*., 1983; Sudheer *et al*., 2004). However, some evidences have been reported that taking soy products associated with health risks and phytic acid-containing soybeans is also criticized for reduction of mineral availability.

Fermented soymilk by LAB gained much interest in the past. However, fermented and non-fermented products derived from soymilk contain assumed probiotic bacteria have gained popularity in recent years. Soymilk was considered as a suitable economical substitute for cow's milk and an ideal nutritional supplement for lactose-intolerant population. Furthermore, consumption of probiotic bacteria *via* food products is also an ideal way to reestablish the intestinal microflora balance. Soymilk is suitable nutrient sources for the growth of LAB and it was used to prepare many

fermented products such as tofu, miso, tempeh, natto, Chinese cheese (sufu) and soy yogerts (Table 5) (Liu *et al*., 2006). Many published reports about fermented soymilk by LAB were concerned with the bacterial growth or the taste of the product, but not with evaluation as a probiotic food. According to Liu *et al*. (2006), the soy cheese was made from soymilk fermented with soy cheese bacterial starter cultures (DH1 and GH4) and *L. rhamnosus* 6013. After 6 h of fermentation, *L. rhamnosus* 6013 was capable of growing in soymilk as high as 10^8–10^9 CFU/ml. After being stored for 30 days at 10 °C, the viable count of the strain was noticed. The viable counts of *L. rhamnosus* 6013, DH1 and GH4 were 10^7, 10^6 and 10^6 CFU/g, respectively. Furthermore, *L. rhamnosus* 6013 could utilize the soybean oligosaccharides as carbon sources. In addition, NaCl (2–4 %) had little effect on the survivability of *L. rhamnosus* 6013. It indicated that *L. rhamnosus* 6013 could withstand the technological processing of soy cheese and had no negative effect on the fermentation and the sensory properties of the soy cheese. The unfavorable beany flavor and the high content of indigestible α-D-galactosyl oligosaccharides such as raffinose and stachyose, the flatulence factors, limited the consumption of soybean as a raw food material. LAB appeared promising starter cultures for the production of quality fermented soymilk products containing reduced quantities of antinutritional factors and beany flavor. Hou *et al*. (2000) showed that fermentation of soymilk with either *B. infantis* or *B. longum* resulted in reduced contents of stachyose and raffinose. The content of stachyose exhibited the largest magnitude of reduction during fermentation, reduced from 5.88 to 2.07-2.90 mmol/l after 48 h of fermentation. According to Wang *et al*. (2003), two strains of LAB, *L. acidophilus* CCRC 14079 and *S. thermophilus* CCRC 14085 were used in single culture and in combination with either *B. infantis* CCRC 14633 or *B. longum* B6 for the production of fermented soymilk. They found that *L. acidophilus* and *S. thermophilus* were capable of metabolizing stachyose and raffinose in soymilk and *S. thermophilus* exploited these substrates more efficiently than *L. acidophilus*. A further reduction in the content of stachyose and raffinose were found in soymilk fermented with mixed cultures of bifidobacteria and LAB than that fermented with single culture of the respective LAB.

n-Hexanal and pentanal, which are naturally present of in soymilk as products of the breakdown of unsaturated acids, are responsible for the unpleasant off-flavor (Matoba *et al*., 1985). Scalabrini *et al*. (1998) found that *B. breve* MB233 could metabolize pentanal and *n*-hexanal in soymilk. The natural contents in soymilk of *n*-hexanal and pentanal are 16.5 and 8.9 ppb, respectively. After 24 h of batch fermentation, *n*-hexanal was reduced to 4 ppb, whereas pentanal was no longer detectable; over the following 24 h, no further decrease in the *n*-hexanal content was observed. Lactic acid fermentation together with the addition of sweeteners is possible to obtain products with better acceptance by consumer. Fávaro Trindade *et al*. (2001) produced yogurts by fermentation of soymilk with a mixed starter culture containing *L. bulgaricus* and *S. thermophilus*. Soymilk at 9 Brix soluble solids was homogenized and fermented with and without addition of sucrose (2.0 and 2.5 g per 100 g) for 4, 5, 6 and 7 h. Result showed that a yogurt with the best sensory quality was obtained using the homogenized soymilk with sucrose (2 %) addition and fermented for 6 h.

Table 5 Proximate composition (g/100 g fresh weight basis) of some traditional soyfoods

Soy food	Moisture	Protein	Fat	Carbohydrate	Ash
Soybean	12.0	34.3	17.5	31.2	9.0
Soymilk	90.8	3.6	2.0	2.9	0.5
Soft tofu	90.3	5.3	0.9	2.6	0.9
Firm tofu	84.0	10.7	2.1	2.3	0.9
Deep-fried tofu	45.2	24.6	20.8	7.9	1.5
Okara	87.0	2.6	0.3	9.4	0.7
Yuba	7.1	50.5	23.7	15.6	3.1
Rice miso (light)	50.0	12.6	3.4	21.2	12.8
Rice miso (red)	50.0	14.0	5.0	16.2	14.8
Soybean miso	47.5	16.8	6.9	15.9	13.0
Jiang (Chunky)	48.6	11.6	5.2	29.3	7.4
Tempeh	64.0	18.3	4.0	12.7	1.0
Natto	58.5	16.5	10.0	12.4	2.6
Sufu (red)	55.5	14.6	5.7	6.4	17.8
Sufu (white)	56.5	14.4	11.2	5.5	12.4

Source: Liu (1999)

In general, isoflavones in soybeans exist mainly as glucoside forms and rarely as aglycone forms. Aglycone isoflavones are absorbed faster and in higher amounts than their glucosides by human. The growth of probiotic in soymilk has been reported to bioconvert the glucoside isoflavones into their respective aglycones. Chien *et al*. (2006) investigated the change in the content of various isoflavones in soymilk during fermentation with LAB. It was observed that fermented soymilk contains lower total isoflavone content than soymilk without fermentation. Regardless of starter organism employed, fermentation causes a major reduction in the contents of glucoside, malonylglucoside and acetylglucoside isoflavones along with a significant increase of aglycone isoflavones content. Among all the fermented soymilk tested, soymilk fermented with *S. thermophilus* showed the highest β-glucosidase activity and the greatest increase in the contents of aglycones. The percentage of aglycone isoflavones of daidzein, genistein and glycitein to total isoflavone content in *S. thermophilus*-fermented soymilk increases from an initial 14.24 %, 6.89 % and 2.45 %, respectively, to 36.20 %, 28.80 % and 12.44 % after 24 h of fermentation. There was a continuing need to improve existing cultures or to screen new organisms for development of new dairy products. Recently, Chun *et al*. (2008) studied the change in the contents of isoflavone glucosides and aglycones and other characteristics in soymilk fermented with *S. infantarius* 12 (*Si* 12), *Weissella* sp. 4 (Ws 4), or their mixed cultures with different mixing ratios (Si 12:Ws 4 = 1:1, 1:3, 1:5 and 1:10, v/v). The results showed that a sharply increase in β-glucosidase activity corresponded well with a rapid decrease in isoflavone glucosides and an increase in isoflavone aglycone contents. The rate of hydrolysis of isoflavone glucosides was the least with *Si* 12 while the highest with *Ws* 4, resulting in about 23 %-33 % and 98 %-99 % hydrolysis of the glucosides with *Si* 12 and *Ws* 4, respectively, after 12 h. Mixed cultures with 1:3, 1:5 and 1:10 ratios seemed to be more effective starters for bioactive fermented soymilk with more aglycones and appropriate acidity in a short time than single cultures. According to Otieno *et al*. (2007), soymilk fermented with 3 selected *L. acidophilus* strains were stored at various temperatures and the concentration of isoflavones was determined. Isoflavone aglycones as well as isoflavone glucosides largely appeared to be stable during storage. Interestingly, the aglycone forms showed much smaller degradation as compared to glucoside forms at all the storage temperature studied. Isoflavone

aglycones showed smaller degradation constants in fermented soymilk at lower storage temperatures (-80 °C and 4 °C) and higher degradation constants at higher storage temperatures (25 °C and 37 °C) with each strain. In contrast, isoflavone glucosides showed higher degradation at lower storage temperatures and lower degradation at higher storage temperature.

Recently, fermented soymilk as probiotic food has been reported by Shimakawa *et al*. (2003). The effects of *B. breve*-fermented soymilk on probiotic function were evaluated. An administered strain of *B. breve* strain Yakult was capable of growing in soymilk with no additives as high as 10^9 CFU/ml. During storage of the fermented soymilk at 10 ºC for 20 days, viable counts of the strain did not change. The growth inhibition of the strain in a bile containing medium was lessened by the addition of soy protein. In human feeding experiments, the administered *B. breve* was recovered at a level of over 10^9 CFU/g faeces, accompanied by an increase in the total number of bifidobacteria. These results indicate that fermented soymilk with *B. breve* strain Yakult could be a novel type of probiotic food.

A prebiotic substance has been defined as a non-digestible 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 (Cashman, 2003). Soybean oligosaccharides have been shown to have selectively stimulating the growth of bifidobacteria (Saito *et al*., 1992). When the two components (prebiotic and probiotic) are combined to achieve synergistic effects, they are called synbiotics (Shioiri *et al*., 2006). Shioiri *et al*. (2006) evaluated the effects of ingestion of a synbiotic fermented milk beverage containing *L. casei* strain Shirota and transgalactosylated oligosaccharides on the defecation frequency in student with constipation as well as the defecation frequency, intestinal microflora, and intestinal environment in elderly persons in whom the intestinal microflora and the levels of putrefactive metabolites were abnormal in a placebo-controlled double-blind study. In the female students, the defecation frequency after 1 week of synbiotic fermented milk beverage ingestion was significant higher than that after 1 week of placebo ingestion or before ingestion. In the elderly persons, the fecal *Bifidobacterium* and *Lactobacillus* bacterial counts after

1 and 2 weeks of synbiotic fermented milk beverage ingestion were significantly higher than those after placebo ingestion or before ingestion. The fecal lecithinase positive *Clostridium* bacterial count after 1 week of synbiotic fermented milk beverage ingestion and the fecal Enterobacteriaceae bacterial counts after 1 and 2 weeks of synbiotic fermented milk beverage ingestion were significant lower than those after placebo ingestion. The acetic acid levels after 1 and 2 weeks of synbiotic fermented milk beverage ingestion were significantly higher than those after placebo ingestion. The stool pH values, the ammonia and phenol levels after 2 weeks of synbiotic fermented milk beverage ingestion were significantly lower than those after placebo ingestion. These results suggest that ingestion of the synbiotic fermented milk beverage containing *L. casei* strain Shirota and transgalactosylated oligosaccharides improves the stool quality, intestinal microflora and intestinal environment.

3. Spray drying of lactic acid bacteria

Spray drying is one of the common methods used to prepare food adjuncts or preservation and concentration of microorganisms. It is an inexpensive process and high production rate which contributes to dry, stable and occupy small volume (Lian *et al*., 2002). In this process, the starting material is in liquid or paste form. The slurry from a nozzle is atomized at high velocity into a flow of hot air (up to 200 ºC) and it is then dried into granules before they hit the side of the chamber (Morgan *et al*., 2006). Consequently, this process results in exposure of the drying medium to high temperature for a short time which can be detrimental to the integrity of live bacterial cells (Ananta *et al*., 2005). During spray drying, lethal thermal injury is the main reason for reduced cell viability (To and Etzel, 1997). The other factors which had affected on the stresses of bacterial was also reported including oxidative, dehydration-related stresses (osmotic, accumulation of toxic compounds, etc.) acting either simultaneously or successively on bacteria, which potentially lead to cell death (Ananta, 2005). Daemen and van der Stege (1982) showed that the destruction of bacteria during heat stress and spray drying can not only be ascribed to a thermal effect but also to a non-thermal drying effect caused by the loss of bound water at the cell surface. Teixeira *et al*. (1995) studied the effect of spray drying on the cell

membrane of *L. bulgaricus*. The results showed that *L. bulgaricus* has increased sensitivity to lysozyme and NaCl and partial loss of some cytoplasmic material was observed, indicators of cell membrane damage. In addition, other possible sites where damage may occur as a result of heat stress and spray drying include cell wall, DNA and RNA (Teixeira *et al*., 1997). However, there are a number of attempted to improving culture viability during spray drying such as the addition of protective substances, pH adjustment and heat adaptation of LAB culture prior to spray drying (Johnson and Etzel, 1995; Desmond *et al*., 2002).

Addition of various protective agents such as carbohydrate (e.g. trehalose, maltodextrin, gum arabic, starch) and protein (reconstituted skim milk, gelatin, monosodium glutamate) to the media prior to spray drying has been reported to successfully improved probiotic viability during spray drying (To and Etzel, 1997; O'Riordan *et al*., 2001; Desmond *et al*., 2002; Lian *et al*., 2002; Sunny-Roberts and Knorr, 2009). Lian *et al*. (2002) investigated the survival of bifidobacteria after spray drying. *B. infantis* and *B. longum* were first spray-dried with different carrier media. It was found that survival of bifidobacteria after spray drying was highly dependent on carriers or protective agents and the strains. Regardless of the *Bifidobacterium* strains, skim milk exhibited the higher survival of bifidobacteria after spray drying than other carriers. Comparing the effect of gum arabic, gelatin and soluble starch on the survival of bifidobacteria after drying, it was noted that *B. infantis* strains survived better with gum arabic compared to gelatin and soluble starch. On the other hand, *B. longum* strains survived better with gelatin as a carrier compared to gum arabic and soluble starch. Increasing the concentration of gelatin, gum arabic or soluble starch from 10 % to 20 % or more caused reduced survival of *Bifidobacterium*. Furthermore, the inactivation caused by increased outlet air temperature varied with the carrier used, with the greatest reduction observed using soluble starch and the least with skim milk. Gardiner *et al*. (2002) investigated whether the probiotic *L. paracasei* NFBC 338 culture in spray-dried form is a suitable inoculant for probiotic Cheddar cheese manufacture. 20 % (w/v) Reconstituted skim milk containing probiotic *L. paracasei* NFBC 338 was produced at pilot scale inlet air and outlet air temperatures of 175 ºC and 68 ºC, respectively. After spray drying, survival of *L. paracasei* NFBC 338 was

84.5 %. The powder which contained *L. paracasei* NFBC 338 was used as an adjunct inoculum during probiotic Cheddar cheese manufacture. Probiotic numbers were $2x10^7$ CFU/g in the cheese after 1 day and grew to $7.7x10^7$ CFU/g after 3 months of ripening, without adversely affecting cheese quality. The data demonstrate that probiotic spray-dried powder is a useful means of probiotic addition to dairy products, as this example for Cheddar cheese manufacture shows. According to O' Riordan *et al*. (2001), *Bifidobacterium* PL1 was encapsulated with starch by spray drying at inlet- and outlet air temperature of 100 ºC and 45 ºC, respectively. It was observed that the *Bifidobacterium* cells declined by less than 1 log after spray drying. However, the starch-coated cells did not display any enhanced viability compared with free cells during storage in two dry food preparations (dry malted beverage powder and commercial muesli) over 20 day storage at ambient temperature. Recently, the effect of growth phase and inclusion of a prebiotic substance in the feed media on probiotic viability during spray drying of *L. rhamnosus* GG at an outlet temperature of 85–90 ºC was examined. Results showed that stationary phase cultures survived best (31–50 %) in both feed media (reconstituted skim milk (RSM) (20 % w/v) or mixture of RSM (10 % w/v) and polydextrose (PD) (10 % w/v). Stationary phase *L. rhamnosus* GG was subsequently spray-dried in the presence of the prebiotic inulin in the feed media, composed of RSM (10 % w/v) and inulin (10 % w/v) and survival following spray-drying was 7.1–43 %. However, survival of the *L. rhamnosus* GG after spray drying in powders produced using PD (20 % w/v) or inulin (20 % w/v) as the feed media was only 0.011–0.45 % (Corcoran *et al*., 2004).

4. Survival of lactic acid bacteria during storage

The storage condition will influence the shelf life of the probiotic products and the suitable conditions are essential to maintain viable populations of probiotics at sufficiently high levels to assure their therapeutic activity throughout shelf life. It was found that oxygen, moisture content, light, microbial contamination and elevated temperatures can greatly affect the viability and stability of probiotic during storage. There has been shown that survival of spray-dried cells was inversely related to temperature (Desmond *et al*., 2002; Wang *et al*., 2004; Teixeira *et al*., 1995). Ananta

et al. (2005) demonstrated that the loss of viability of spray-dried *L. rhamnosus* GG in reconstituted skim milk with or without prebiotic substances was accelerated at higher storage temperature. In agreement with Wang *et al*. (2004), a percent of survival of spray-dried *S. thermophilus* and *B. longum* in fermented soymilk was higher at storage temperature of 4 °C than 25 °C. Among all the packaging materials and storage temperatures test, *S. thermophilus* and *B. longum* were most stable in the dried fermented soymilk held in laminated pouch and stored at 4 °C. At this storage condition, *S. thermophilus* and *B. longum* in the spray-dried fermented soymilk showed a survival percent of 29.5 % and 57.7 %, respectively after 4 months of storage. Meanwhile, at storage temperature of 25 °C, *S. thermophilus* and *B. longum* in the spray-dried fermented soymilk showed a survival percentage of 22.7 % and 37.7 %, respectively. Similarly, Teixeira *et al*. (1995) reported that the total number of spray-dried *L. delbrueckii* ssp. *bulgaricus* in skim milk decreased as the storage temperature increased. According to Corcoran *et al*. (2004), spray-dried various growth phase *L. rhamnosus* GG in 20 % RSM were placed in polythene bags and stored at 4, 15 or 37 °C. Optimal survival spray-dried stationary phase *L. rhamnosus* GG occurred during storage at 4 °C, while there was a 30-fold reduction in probiotic numbers in powders stored at 37 °C after 8 weeks. Spray-dried lag phase *L. rhamnosus* GG survived well at the lower storage temperatures, whereas the highest losses was observed at 37 °C with 40-fold reduction in viable numbers. Storage of spray-dried early log phase cultures yielded highest viability losses at 37 °C, while lower death rates (16-fold decrease) occurred at 15 °C.

Generally, the addition of protective agents to starter cultures is a common means to protect cells during drying and storage. There has been reported that the viability of vacuum-dried *L. acidophilus* in the presence of trehalose and borate (19.5 %) was higher than that of trehalose alone (11.4 %) after storage for 34 days, whereas the viability of cells without protective substance decreased to 4.5 % after 16 days (Santivarangkna *et al*., 2007). The addition of protective substance of gum acacia found to increase the survival of probiotic lactobacilli during storage. Desmond *et al*. (2002) demonstrated that the survival of probiotic lactobacilli in gum acacia-containing powders was dramatically better than untreated cultures during storage at

4–30 °C for 4 weeks. The addition of protective substances protected the viability of lactobacilli better at 4 °C than 15 and 30 °C. At 4 °C survival of the probiotic culture in GA-containing powders showed 20-fold compared to control while, at 15 and 30 °C, greater than 1000-fold higher survival was obtained. According to Hamsupo (2005), the spray-dried *L. reuteri* KUB-AC5 in presence of monosodium glutamate (MSG) gave the highest survival both after storage at 4 °C and 30 °C for 3 months. The low viable cell number was obtained from the spray-dried *L. reuteri* KUB-AC5 in presence of ascorbic acid or soluble starch.

Because bifidobacteria are anaerobic microorganisms, oxygen toxicity is an important and critical problem. Simpson *et al*. (2005) found that the viability reduction of *Bifidobacterium* species is correlated with heat and oxygen tolerance. At 25 °C, the viability of all spray-dried *Bifidobacterium* species in skim milk was significant reductions after only 30 days of storage. The moderate or high tolerance to heat and oxygen of *B. animalis* ssp. *animalis*, *B. animalis* ssp. *lactis* and *B. pseudolongum* ssp. *pseudolongum* retained approximately 30 %, 5 % and 20 % viability, respectively, compared with 0.01–0.2 % viability for the other species. According to Shah (2000), the increase in numbers and survival of *L. acidophilus* during storage were directly affected by the dissolved oxygen content which was shown to be higher in yogurts made in plastic containers than glass. The viable cell count of *L. acidophilus* stored in glass bottles remained higher than those stored in plastic cups. Bifidobacteria multiplied better in glass bottles than in plastic cups. The initial counts of the bifidobacteria population were 1.6-fold higher in yogurt prepared in glass bottles than in plastic cups.

There has been reported that ascorbic acid (vitamin C) can act as an oxygen scavenger and resulted in an enhanced survival of probiotic bacteria during storage. The viability of yoghurt and probiotic bacteria was assessed during 35 days storage of yoghurt supplemented with four levels of ascorbic acid using four commercial starter cultures. Results showed that the viable cell of *S. thermophilus* were lower, whereas those of *L. delbrueckii* ssp. *bulgaricus* were higher, with increasing concentration of ascorbic acid. This indicated that ascorbic acid may lower redox potential by

scavenging oxygen, thus affecting the growth of *S. thermophilus*. However, Teixeira *et al*. (1995) found that the death rate of *L. delbrueckii* ssp. *bulgaricus* was higher in the presence of ascorbic acid and monosodium glutamate (MSG) during storage at 20 °C. Meanwhile, at 4 °C, survival of *L. delbrueckii* ssp. *bulgaricus* was higher in the presence of ascorbic acid and MSG than in the control. This was due to ascorbic acid can also act as pro-oxidant which generates hydroxyl radicals that can attack and oxidize biological molecules. The pro-oxidation reaction in ascorbic acid protein mixtures seems to be temperature-dependent (Santivarangkna *et al*., 2007).

MATERIALS AND METHODS

Materials

1. Microorganisms

Three strains were used: *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 (Bangkok MERCEN, Thailand Institute and Science and Technological Research, TISTR), isolated from intestines of healthy chicken and *L. plantarum*, isolated from fermented vegetable. The strains were stored at -80 °C in MRS broth (Merck, Darmstadt, Germany) with 20 % glycerol. The culture was two times transferred in MRS broth (for 24 h, at 37 °C) prior to use as the inoculum for the experiments.

2. Chemical and media

2.1 Microbiological media

MRS medium, initially proposed by de Man *et al.,* (1960) and nutrient broth were prepared according to the manufacturer's instructions (Difco Laboratory, Detroit Michigan, USA). Agar was from Merck Darmstadt Germany.

2.2 Miscellaneous chemicals

- Acetonitrile (Lab-Scan, Ireland)
- Calcium carbonate ($CaCO_3$; Univar, Ajak, NSW, Australia)
- Calcium hydroxide ($Ca(OH)_2$; Ajax Finechem, NSW, Australia)
- Copper sulphate pentahydrate ($CuSO_4.5H_2O$; Ajak, NSW, Australia)
- 95% Ethyl alcohol (Solvent solution Grade A; Commercial grade)
- Galactose (Univar, APS, NSW, Australia)
- Glucose (Univar, Ajax Finechem, NSW, Australia)
- Glycerol (Carlo Erba reagent, MI, U.S.A.)

- Lithium lactate (Sigma, Japan)
- Maltodextrins (Commercial grade)
- p-hydroxydiphenyl (Fluka, Sigma, Steinheim, Germany)
- Phenol (Amresco, Ohio, U.S.A.)
- Sulfuric acid (H_2SO_4; Lab-Scan, Ireland)
- Sodium hydroxide (NaOH; Merck, Germany)
- Sucrose (Univar, Ajak, NSW, Australia)
- Sodium chloride (NaCl; Univar, Ajak, NSW, Australia)

3. Apparatus

- Atomic Absorption Spectrophotometer (AVANA, IL, USA)
- Autoclave (Hirayama, Model HA-240M, Japan)
- Balance 2 digits (Alsep, Tokyo, Japan and Ohaus, NJ, USA)
- Centrifuge (Hermle, Germany)
- Centrifuge (Sigma, Germany)
- Hot Air Oven (Memmert, Germany)
- Hot Plate (Harmony, LMS Laboratory&Medical supplies, VA, USA)
- HPLC system (Shimadsu, Kyoto, Japan)
- Incubator (Binder, Germany)
- pH meter (Schott, Germany)
- Pilot Scale Spray Dryer (Mobile Minor 2000, Niro, Denmark)
- Spectrophotometer (Thermospectronic, Helios Gamma, England)
- Vortex mixer (Genie, Scientific Industries, USA)
- Water Bath (Digital Heat, SPC Group, Thailand)

Methods

4. Screening of lactic acid bacteria

4.1 Lactic acid production

Single colony of LAB was transfered to 5 ml MRS broth and incubation at 37 °C for 24 h. The culture was then centrifuged at 14,000 *g* for 15 min. Supernatant was used for further analysis to the amount of lactic acid by Barker and Summerson (1941) method.

4.2 Tolerance of lactic acid bacteria to simulated gastric and bile juices

Tolerances of LAB to simulated gastric and bile juices were determined according to the procedures described by Michida *et al*. (2006). Each strain was grown in MRS broth at 37 °C for 24 h. The culture was centrifuged at 14,000 *g* for 15 min. The pellet was washed twice with 0.5 % (w/v) sterile saline solution and resuspended in the same solution. The suspension of free cell (0.2 ml) was mixed with 0.5 % (w/v) sterile saline (0.3 ml) and finally mixed with 1.0 ml of simulated gastric (pH 2.0) or bile juice (pH 8.0). Subsequently, the mixture was incubated at 37°C. For gastric tolerance, the samples were taken at 0, 30, 60, 90 and 180 min to determine the viable cell count. For bile tolerance, the samples were taken at 0 and 240 min. All tests were carried out in duplicate.

For the spray-dried cultures, the fermented soymilk powders were rehydrated with 0.5 % NaCl and vigorous shaking for 2 min. The dispersion of the spray-dried cultures was obtained and determined the tolerances to simulated gastric and bile juices as the procedure described by free cell.

5. Fermentation of soymilk

5.1 Preparation of fermented soymilk

Soymilk was prepared according to the procedures described by Wang *et al*. (2002). Soybeans were washed and soaked in distilled water. The soaked soybeans were mixed with distilled water (bean:water = 1:10 w/v) and ground by a blender for 3 min. The slurry was then filtered through double layer cheesecloth to yield soymilk. Soymilk was sterilized at 121 °C for 15 min.

LAB were cultured in MRS broth at 37 °C for 24 h. The culture was centrifuged at 14,000 *g* for 15 min. The cell pellet was washed twice with 0.85 % NaCl and resuspended in distilled water. The sterilized soymilk was then inoculated with 5 % (v/v) of cell suspension. Fermentation was conducted at 37 °C for 48 h. The samples were withdrawn aseptically at 0, 12, 24, 36 and 48 h fermentation for duplicated analysis of cell growth, pH, organic acid production, residual sugar concentration, soluble calcium content and oligosaccharides content.

In order to evaluate the effect of sugar addition, glucose, galactose or sucrose were added to soymilk before fermentation.

5.2 Determination of soluble calcium

Aliquots were centrifuged at 14,000 *g* for 30 min. After centrifugation, sample was separated into 3 portions: the upper fat layer, an intermediate layer and the sediment. The intermediated layer was then filtered through a 0.22 μm membrane before measurement of soluble calcium content by atomic absorption spectrophotometry.

5.3 Determination of oligosaccharide

Samples were centrifuged at 14,000 *g* for 30 min. Supernatant portion was filtered through a 0.22 μm membrane before analyses. The volume injection of 50 μl of samples was analyzed on a HPLC system consisting of Prevail Carbohydrate ES column (250 x 4.6 mm) and a refractive index detector was used. The mobile phase consisted of 65 % acetonitrile, at a flow rate of 2 ml/min. The column temperature was 25 °C. Sucrose, raffinose and stachyose were used as standards.

6. Spray drying of fermented soymilk

The 5 % (v/v) inoculum of LAB in distilled water was inoculated into soymilk and incubated at 37 °C for 48 h. The fermented soymilk was mixed with 10 % (w/v) maltodextrin and then spray-dried by pilot scale spray drier. The mixture was flowed into atomizer by rotary pump at 10-12 rpm. The inlet air temperature was 130 °C. The outlet air temperature of 70 °C was controlled by adjusting the flow rate of the feed solution. The dried sample was collected from the base of the cyclone. Each sample of spray-dried LAB was analyzed for survival percentage and moisture content in duplicate.

7. Storage of spray-dried fermented soymilk

The spray-dried fermented soymilk powder was placed in aluminum foil bag and kept at 4 °C and 30 °C for 6 months. The viable cell and moisture content of the strain were determined for their stability at time intervals.

8. Analytical procedures

8.1 Enumeration of viable cell numbers

According to dried product, each sample of dried soymilk containing lactic acid bacteria (1 g) was rehydrated with 0.85 % NaCl solution (9 ml) and vigorous

shaking for 2 min. Each sample was serially diluted (1:10) in 0.85 % NaCl solution and plate count was performed using MRS agar by pour plate method. The duplicate plates were done for each dilution. Plates were incubated at 37 °C for 48 h. The viable cells were expressed as log CFU/ml of soymilk.

8.2 Viability determination

Viable cell count was determined and calculated as colony-forming units (CFU) per milliliter. The results were expressed as log values. The survival rate was calculated as follow: survival rate (%) = [logN/logN_0] ×100, where N_0 is the viability of LAB before spray drying and N is the viability after spray drying.

8.3 Moisture content of spray-dried fermented soymilk

Moisture content of spray-dried fermented soymilk containing lactic acid bacteria was determined according to AOAC Official method 960.18 (AOAC, 2000).

8.4 Measurement of organic acids

Lactic acid content was determined using the method of Barker and Summerson (1941). For the method with modification of William (2000) was used to determined acetic acid concentration. The description of these methods was shown in Appendix A.

8.5 Measurement of sugar concentration

Residual sugar concentration in cultivated culture was determined by use of phenol-sulfuric acid reaction (Dubois *et al.,* 1956). The description of this method was shown in Appendix A.

8.6 Statistical analysis

The measurements were determined in duplicate and subject to analysis of variance (ANOVA) using the statistical software. Treatment means were compared by Tukey's test, at the 5 % level of significance.

9. Places and duration

Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Thailand.

The experiments were carried out from June 2006 to May 2008.

RESULTS AND DISCUSSION

1. Screening of lactic acid bacteria

1.1 Lactic acid production by lactic acid bacteria

As shown in Figure 2, three strains of LAB produced high amounts of lactic acid in MRS medium after 24 h of incubation at 37 °C. The amount of lactic acid from *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49 were 12.32, 10.38 and 18.52 g/l, respectively.

The production of organic acid-mainly lactic acid contributes to the flavor of the final product. It is clear that organic acids are one of factors to prolong shelf life of the final product. The inhibition of pathogenic and spoilage flora is dependent on concentration of organic acids (Ammor and Mayo, 2007).

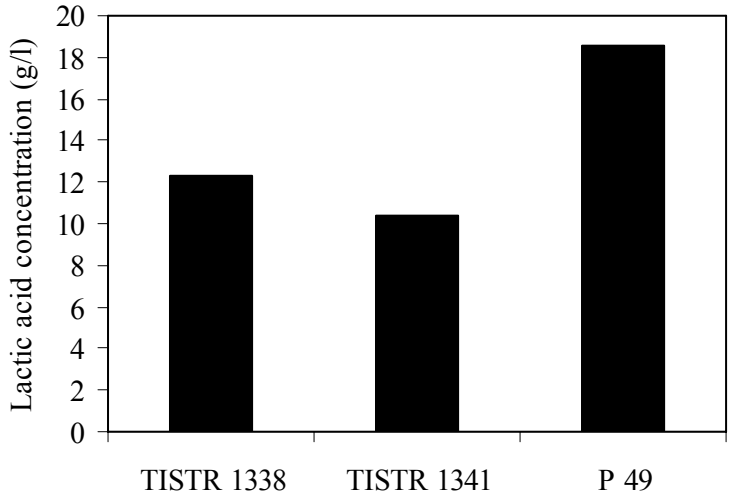


Figure 2 Lactic acid concentration produced by *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49 after incubation at 37 °C for 24 h

1.2 Survival of lactic acid bacteria under gastrointestinal conditions

To optimally fulfill of a potential probiotic bacteria, the one criteria of evaluation of survival in the intestinal ecosystem should be performed (Truelstrup Hansen *et al*., 2002). The pH of pure gastric juice ranges from 1.3 to 2.5 and of the small intestine is about 7.43 (Truelstrup Hansen *et al*., 2002; Krasaekoopt *et al*., 2004). Hence, the present study was to investigate the survival of the test strains under simulated gastric (pH 2.0) and bile juice (pH 8.0). As shown in Figure 3, the initial cell number of test strains ranged from 10^7-10^8 CFU/ml and the viable cell of all strains rapidly decreased to 2 log CFU/ml after 30 min exposure to low pH in simulated gastric juice at 37 °C. After 60 min incubation, the viable cell number of the strains decreased to an undetectable level. It was probably due to their low acid resistance and the ingestion of unprotected LAB would result in reduced viability.

Similar result was obtained by Michida *et al*. (2006). They found that the viable cell of free *L. plantarum* decreased dramatically from 7.24 to 1.92 log CFU/ml during 30 min exposure to gastric juice. On the other hand, higher survival was reported by Chandramouli *et al*. (2004). The viable number of free *L. acidophilus* CSCC 2400 was decreased from 10^9 to 10^4 CFU/ml (5 log decreasing) after incubation at low pH (pH 2) for 3 h. Mandal *et al*. (2006) reported that free cells of *L. casei* NCDC 298 survived better with remained at 4.24 and 3.38 log CFU/ml at the end of 1 and 3 h exposure to simulated gastric solution (pH 1.5). The ability of free cell of all strains to survive exposure to low pH in simulated gastric juice varied markedly among species and strains. These results agreed with previous reports of Truelstrup Hansen *et al*. (2002). They found that acid resistance of free cell of Bifidobacterium varies among strains within a species. The population of the most resistant strain of *B. lactis* Bb-12 decreased from 9.5 to 8.2 log CFU/ml after exposure to simulated gastric juice at pH 2.0 for 2 h. Meanwhile, the viable cell numbers of the less resistant strains of *B. adolescentis* 15703, *B. breve* 15700 and *B. longum* Bb-46 were reduced by 3-4 log CFU/ml. All other strains of *Bifidobacterium* spp. tested displayed an even lower tolerance to simulated gastric juice at pH 2.0.

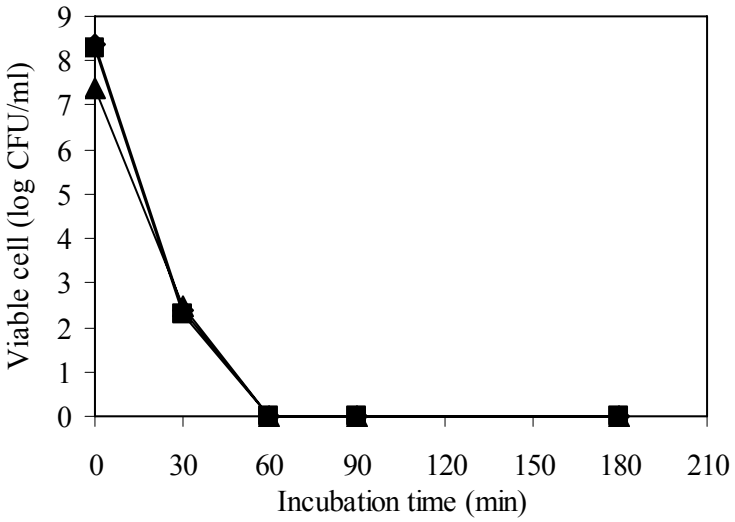


Figure 3 Change in viable cell of *L. acidophilus* TISTR 1338 (♦), *L. casei* subsp. *tolerans* TISTR 1341 (■) and *L. plantarum* P49 (▲) during exposure to simulated gastric juice at pH 2.0 for 180 min

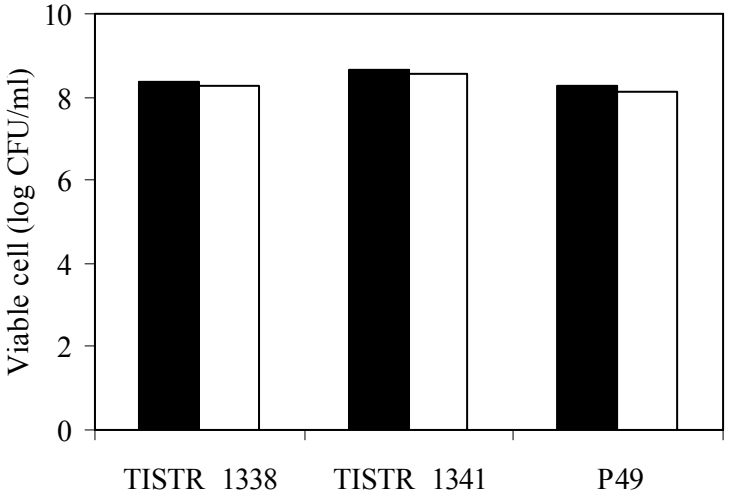


Figure 4 Viable cell of *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49 after 240 min (□) exposure to simulated bile juice at pH 8.0 compared with control (■)

Tolerance of LAB to simulated bile juice containing 4.5 % bile salts was also investigated. Results were shown in Figure 4. The viable cell number of all test strains remained constant of ~10^8 CFU/ml with reduction of 0.08-0.14 log CFU/ml after 240 min incubation. This result was similar to the result obtained by Lee *et al*. (2004). The population of *L. bulgaricus* KFRI 673 was fully maintained in simulated intestinal juice (pH 7.4) after 180 min incubation. Similarly, Rao *et al*. (1989) showed that survival of *B. pseudolongum* at pH 6.06 and 7.13 was fully maintained.

Krasaekoopt *et al*. (2004) concluded that the survival of *L. acidophilus*, *B. bifidum*, and *L. casei* decreased proportionately with the time of exposure to bile salt solutions. However, bile resistance of free cells varies greatly among strains within a species and among species (Truelstrup Hansen *et al*. 2002).

2. Fermentation of soymilk by lactic acid bacteria

2.1 Microbial growth of LAB and pH change during fermentation

The growth of LAB was presented in Figure 5. When *L. plantarum* P49 was used as starter culture, the viable cell number was increased from $4.1x10^6$ to $4.6x10^7$ CFU/ml after 48 h fermentation. Whereas, the viable cell number of *L. acidophilus* TISTR 1338 and *L. casei* subsp. *tolerans* TISTR 1341 remained constant from $1.08x10^8$ and $1.24x10^8$ CFU/ml to $1.64x10^8$ and $1.50x10^8$ CFU/ml, respectively. Noticeably, the viable cell of three test strains was remained at high level even a long time of fermentation of 48 h. During fermentation of soymilk, decrease in pH of all test strains was shown in Figure 6. After 12 h fermentation, the dramatical decrease in pH was found in soymilk fermented with *L. plantarum* P49. The pH value decreased from 6.60 to 4.50. Meanwhile, the slightly decrease in pH was found in soymilk fermented with *L. acidophilus* TISTR 1338 and *L. casei* subsp. *tolerans* TISTR 1341. At 48 h fermentation, the pH value of 6.01, 6.29 and 4.48 were obtained in soymilk fermented with *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49, respectively.

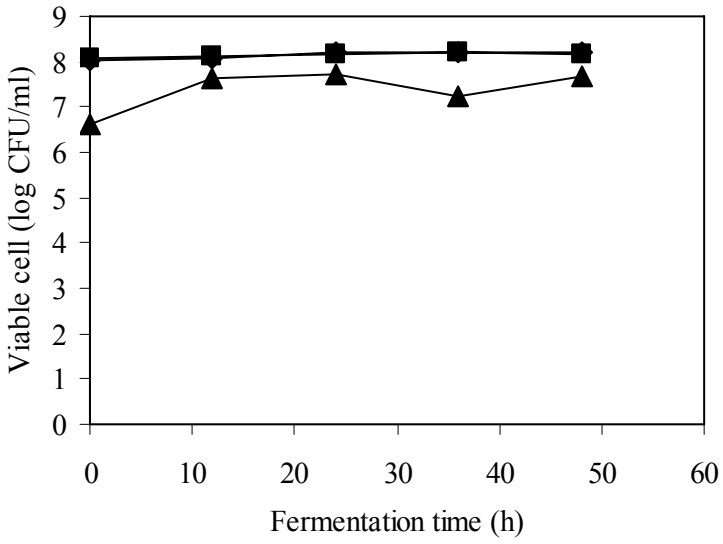


Figure 5 Viable cell of *L. acidophilus* TISTR 1338 (♦), *L. casei* subsp. *tolerans* TISTR 1341 (■) and *L. plantarum* P49 (▲) during fermentation of soymilk for 48 h

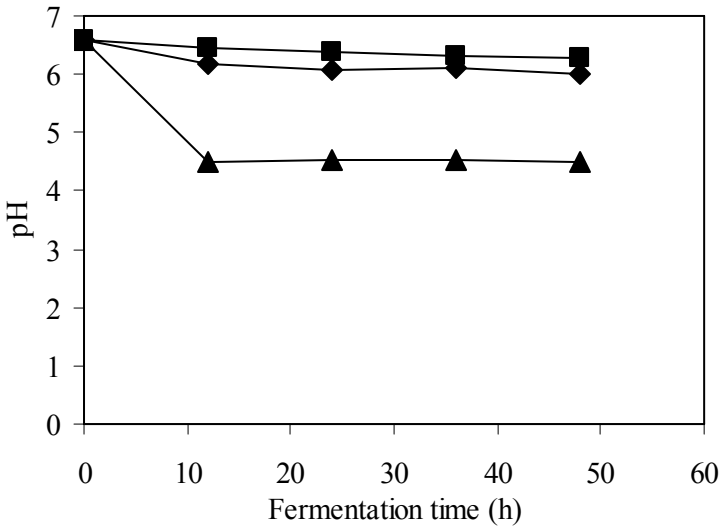


Figure 6 Change in pH during fermentation of soymilk by *L. acidophilus* TISTR 1338 (♦), *L. casei* subsp. *tolerans* TISTR 1341 (■) and *L. plantarum* P49 (▲) for 48 h

A similar result was reported by Božanić *et al*. (2008). The growth of *L. acidophilus* La5 in soymilk was very poorly and *L. casei* Lc-01 was grown in soymilk but pH value decreased with around 5.7. It is well known that lactobacilli required complex nutrients for their growth. Soymilk contents almost all that requirements except iron which might be the reason for poor growth of *L. acidophilus*. Moreover, vigorous stimulators of growth of *L. acidophilus* increased amount of thiol groups, whereas peptone and trypsin stimulate its acid production. Also, soymilk is scarce in amino acids containing sulphur and this might be more reason for their poor growth (Božanić *et al*., 2008).

2.2 Organic acid production

During soymilk fermentation by *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49, lactic acid and acetic acids were determined. Result showed that lactic acid was the dominant organic acid found in this experiment (Figure 7), whereas acetic acid was not detectable.

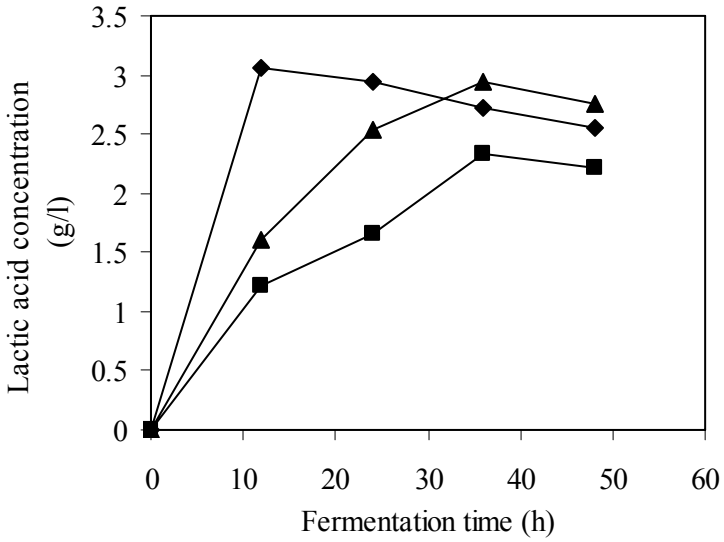


Figure 7 Lactic acid concentration during fermentation of soymilk by *L. acidophilus* TISTR 1338 (♦), *L. casei* subsp. *tolerans* TISTR 1341 (■) and *L. plantarum* P49 (▲) for 48 h

As shown in Figure 7, *L. acidophilus* TISTR 1338 was able to produce lactic acid faster than *L. plantarum* P49 and *L. casei* subsp. *tolerans* TISTR 1341. Lactic acid concentration was reached maximum value of 3.06 g/l after 12 h of fermentation and gradually decreased to 2.56 g/l at the end of fermentation. In other hand, *L. acidophilus* TISTR 1338 and *L. plantarum* P49 produced lower rate of lactic acid and final concentration were 2.22 and 2.76 g/l, respectively. Decreasing in the lactic acid contents could be due to lactic acid used as energy source after the exhaustion of sugar by the cell (Ragout *et al*., 1994; Pintado *et al*., 1999; Pintado *et al*., 2005)

2.3 Oligosaccharide reduction

Change in concentration of raffinose and stachyose in soymilk fermented with *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49 was presented in Figures 8 and 9. It was found that the content of raffinose and stachyose in soymilk fermented with single culture of the strains decreased as the fermentation time increased. After 48 h fermentation, the higher reduction of raffinose was found in soymilk fermented with single strain of *L. acidophilus* TISTR 1338 and *L. plantarum* P49 and the concentration of raffinose was reduced from 0.74 to 0.42 and 0.44 mM, respectively. Meanwhile, the concentration of raffinose in soymilk fermented with *L. casei* subsp. *tolerans* TISTR 1341 was reduced from 0.74 to 0.46 mM. The higher reduction of stachyose concentration was found in soymilk fermented with *L. plantarum* P49. Stachyose concentration was reduced from 2.96 to 2.60 mM.

Reduction of raffinose and stachyose was caused by hydrolysis of α-galactosidase (LeBlanc *et al*., 2004; Mital *et al*., 1973; Garro *et al*., 1996; Donkor *et al*., 2007). According to LeBlanc *et al*. (2004), the levels of α-gal activity in lactic acid bacteria are different among strains. In this experiment, *L. plantarum* P49 metabolized raffinose and stachyose more efficiently than other strains. This may indicated that *L. plantarum* P49 has a higher α-gal activity than *L. acidophilus* TISTR 1338 and *L. casei* subsp. *tolerans* TISTR 1341.

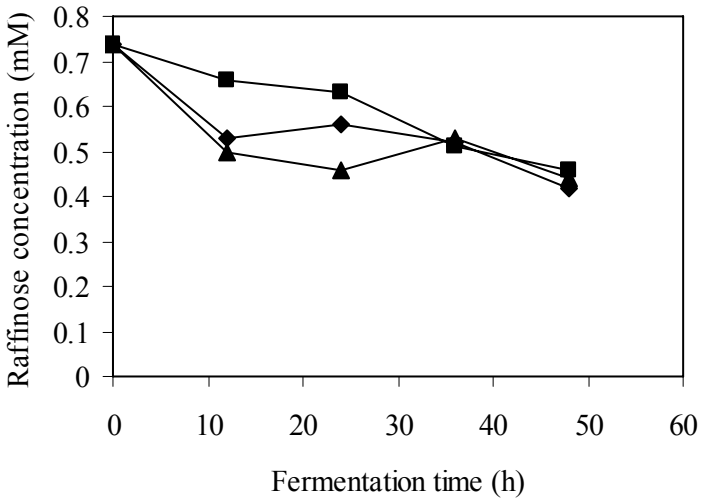


Figure 8 Change in raffinose concentration during fermentation of soymilk by *L. acidophilus* TISTR 1338 (♦), *L. casei* subsp. *tolerans* TISTR 1341 (■) and *L. plantarum* P49 (▲) for 48 h

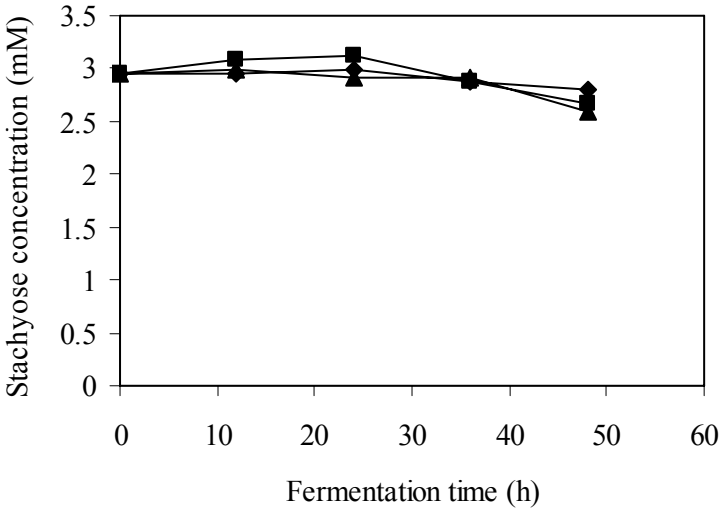


Figure 9 Change in stachyose concentration during fermentation of soymilk by *L. acidophilus* TISTR 1338 (♦), *L. casei* subsp. *tolerans* TISTR 1341 (■) and *L. plantarum* P49 (▲) for 48 h

Therefore, *L. plantarum* P49 was selected for further study. Effect of sugar addition on growth and lactic acid production was carried out in order to enhance calcium solubility and oligosaccharide reduction in soymilk.

2.4 Calcium solubility

As shown in Figure 10, the initial soluble calcium content in soymilk was 8.63 mg/l. After 12 h fermentation, increased in soluble calcium was observed only in *L. plantarum* P49. While, soluble calcium in soymilk fermented with *L. acidophilus* TISTR 1338 and *L. casei* subsp. *tolerans* TISTR 1341 was slightly decreased. After 48 h fermentation, the soluble calcium level was 7.32 and 5.86 mg/l, respectively. Decrease in soluble calcium may be attributed to the uptake of minerals into the cell of LAB (Bergqvist *et al*., 2005). Tang *et al*. (2007) also found that soluble calcium in calcium-fortified soymilk fermented with *L. acidophilus* ATCC33200, *L. acidophilus* ATCC4356, *L. acidophilus* ATCC4461 and *L. casei* ASCC290 was decreased after 12 h of fermentation.

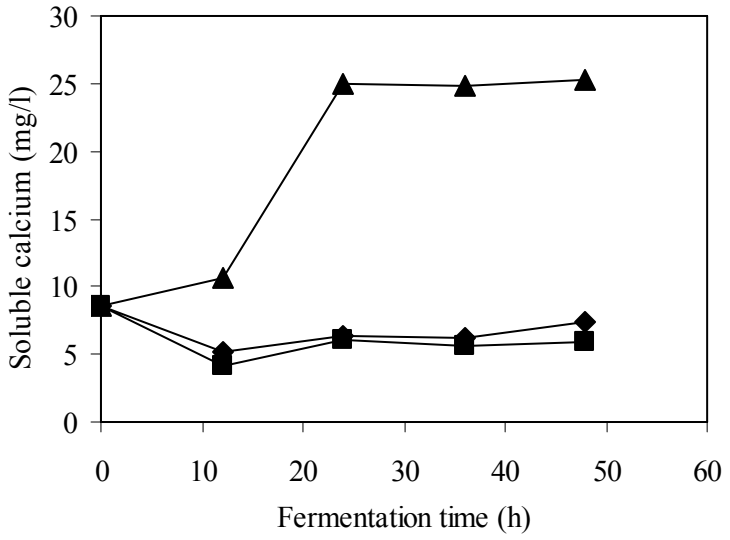


Figure 10 Change in soluble calcium concentration during fermentation of soymilk by *L. acidophilus* TISTR 1338 (♦), *L. casei* subsp. *tolerans* TISTR 1341 (■) and *L. plantarum* P49 (▲) for 48 h

The largest increase in soluble calcium was found in soymilk fermented with *L. plantarum* P49. The soluble calcium content was 25.24 mg/l after 48 h fermentation. Many reports indicated that pH reduction and the production of organic acids during fermentation contributed to calcium solubility (Tang *et al*., 2005; Lopez *et al*., 2000). As the explanation of the growth correlated with soluble calcium, the large change in pH was occurred in 12 h fermentation, whereas soluble calcium is vastly changes in between 12 h to 24 h fermentation. It seemed to well correlate with lactic acid productions, soluble calcium slightly increased during 12 h fermentation and vastly increased after 12 h to 24 h fermentation and seem to be stable throughout 48 h fermentation, also lactic acid concentration is continued increase during 24 h fermentation and less accumulation throughout the end of fermentation.

There are several mechanisms correlated with microbial activities affected on an increase of mineral solubility including pH decrease, degradation of the insoluble metal-binding ligands such as phytate and production of the soluble metal-chelating compounds such as organic acids (Bergqvist *et al*., 2005). According to Tang *et al*. (2007), they reported that the ionization of the calcium salts was occurred due to pH lowering resulted in an increase of calcium solubility. Its chelating properties of some organic acids such as tartaric, succinic and propionic can probably accounted for the formation of soluble acid-mineral (iron) complexes (Salovaara *et al*., 2003). Lopez *et al*. (2000) showed that fermentation of *Leu. mesenteroides* strain 38 in whole wheat flour medium established the degradation of phytic acid and the production of lactic acid lead to greater calcium solubility than in control medium.

3. Effect of sugar addition on oligosaccharide content and calcium solubility in soymilk fermented by *L. plantarum* P49

3.1 Effect of sugar addition on microbial growth and pH change

As shown in Figure 11, the poor growth of the strain was observed with viable cell number of 1.44×10^7 CFU/ml when galactose was added. The addition of sucrose in soymilk exhibited a highest viable cell number of 7.58×10^7 CFU/ml.

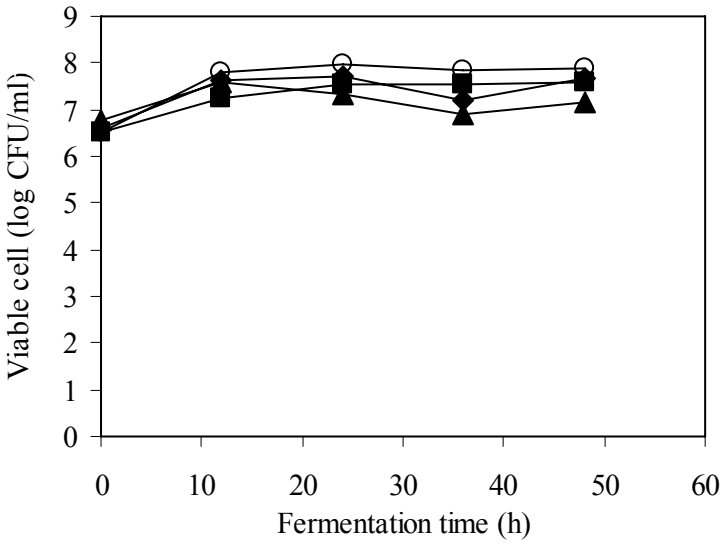


Figure 11 Viable cell of *L. plantarum* P49 during fermentation of soymilk (♦) and soymilk with glucose (■), galactose (▲) and sucrose (○) addition for 48 h

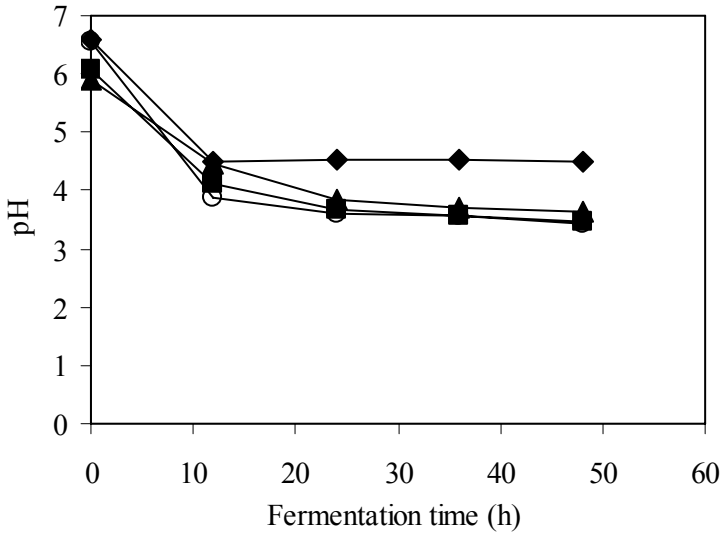


Figure 12 Change in pH during fermentation of soymilk (♦) and soymilk with glucose (■), galactose (▲) and sucrose (○) addition by *L. plantarum* P49 for 48 h

However, glucose and galactose addition did not improve the viable cell number of the strain compared to soymilk without sugar addition. Meanwhile, sucrose addition resulted in higher viable cell than without sugar addition.

During fermentation of *L. plantarum* P49, pH values in soymilk with and without sugar addition largely decreased during 12 h fermentation, and quite stable only in soymilk without sugar addition until the end of fermentation (Figure 12). Whereas soymilk with sugar addition, slightly decrease in pH was occurred after 24 h fermentation and continuous until the end of fermentation. At 48 h fermentation, the lowest pH value was obtained in soymilk with sucrose addition. Cultivation of *L. plantarum* P49 in soymilk and soymilk with glucose, galactose and sucrose addition exhibited the final pH values of 4.48, 3.47, 3.63 and 3.43, respectively. The pH value in soymilk with each three types of sugar addition was significantly higher difference than in soymilk without sugar addition.

The results obtained in this study indicated that sucrose was a suitable carbon source for growth lactobacilli. Similarly, Garro *et al*. (1998) found that *L. casei* could utilize sucrose from soymilk for growth and lactic acid production with pH value largely decreased to 4 after 24 h fermentation. Consistent with Kwon *et al*. (2002), bifidobacteria used sucrose as a main carbon source during fermentation, notable minor stachyose, and negligible little fructose and raffinose.

3.2 Effect of sugar addition on organic acid production

Lactic and acetic acids production of the strain in soymilk with and without sugar addition were shown in Figures 13 and 14. After 12 h fermentation, the levels of lactic and acetic acids increased in both soymilk and soymilk without sugar addition. The highest lactic acid production was observed in soymilk with glucose addition. The amount of lactic acid was 8.04 g/l after 48 h fermentation. At this time, the addition of glucose and sucrose gave the significantly higher lactic acid concentration than without sugar addition.

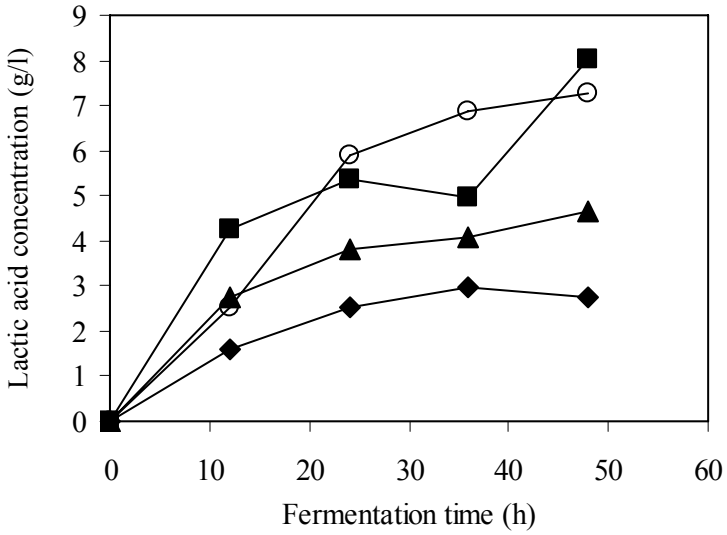


Figure 13 Lactic acid concentration during fermentation of soymilk (♦) and soymilk with glucose (■), galactose (▲) and sucrose (○) addition by *L. plantarum* P49 for 48 h

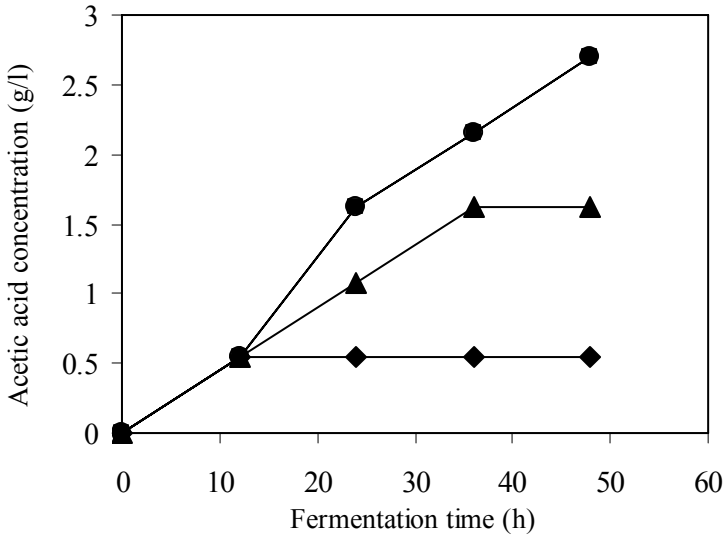


Figure 14 Acetic acid concentration during fermentation of soymilk (♦) and soymilk with glucose (■), galactose (▲) and sucrose (○) addition by *L. plantarum* P49 for 48 h

For acetic acid production, the highest acetic acid concentration was obtained in soymilk with glucose and sucrose addition (Figures 14). At the end of fermentation, acetic acid content in soymilk with glucose and sucrose addition was the same at 2.70 g/l. As lactic acid production, the addition of each three sugars gave the significantly higher acetic acid concentration than without sugar addition. However, the amount of lactic acid was higher than acetic acid in all samples at the end of fermentation.

3.3 Effect of sugar addition on oligosaccharide reduction

Figures 15 and 16 showed the decrease of raffinose and stachyose, respectively in soymilk and soymilk with glucose, galactose and sucrose addition during fermentation by *L. plantarum* P49. Results showed that the addition of sugar rapidly decreased the level of raffinose within 12 h and after that the level remained constant throughout the end of fermentation. After 48 h fermentation, the highest reduction of raffinose from 0.74 to 0.24 mM was found from galactose addition followed by sucrose and glucose. It was possible that galactose could be the most effective inducer for α-galactosidase activity of *L. plantarum* P49. According to Garro *et al*. (1996), hydrolyze of α-galactosides of raffinose and stachyose needs α-galactosidase and the level of the enzyme was affected by other growth conditions such as the carbon source. Results from in Figure 16 showed that sugar addition slightly decreased the level of stachyose. Glucose and galactose addition decreased stachyose concentration from 2.96 to 2.49 and 2.58 mM, respectively. In addition, sucrose addition did not decrease the level of stachyose compared to control.

3.4 Effect of sugar addition on calcium solubility

Figure 17 showed fermentation of soymilk with glucose, galactose and sucrose by *L. plantarum* P49. It was found that the addition of sugar enhanced the calcium solubility. The maximum increase in calcium solubility of all treatments was reached at 24 h fermentation. The largest increase in soluble calcium (64.2 mg/l) was found in soymilk with sucrose addition which was 2.5-fold comparing to control.

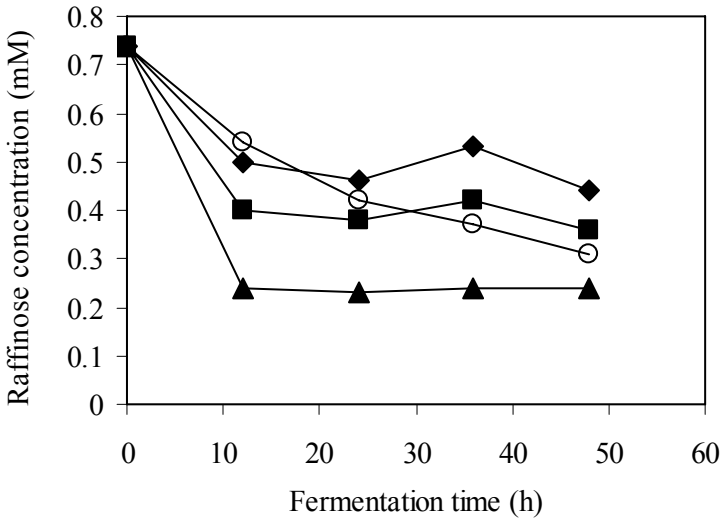


Figure 15 Change in raffinose concentration during fermentation of soymilk (♦) and soymilk with glucose (■), galactose (▲) and sucrose (○) addition by *L. plantarum* P49 for 48 h

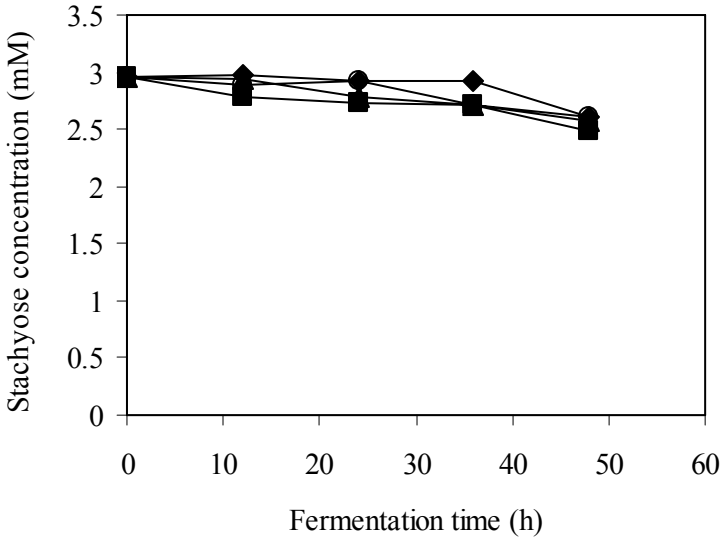


Figure 16 Change in stachyose concentration during fermentation of soymilk (♦) and soymilk with glucose (■), galactose (▲) and sucrose (○) addition by *L. plantarum* P49 for 48 h

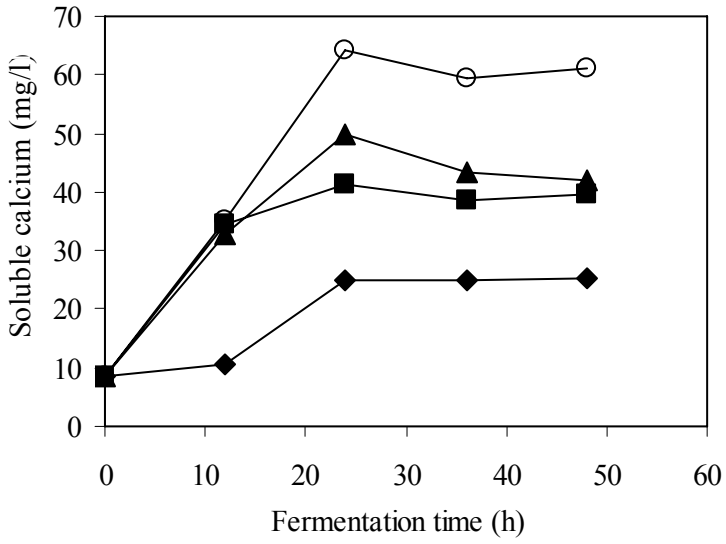


Figure 17 Change in soluble calcium concentration during fermentation of soymilk (♦) and soymilk with glucose (■), galactose (▲) and sucrose (○) addition by *L. plantarum* P49 for 48 h

According to Tang *et al*. (2007), it was assumed that lowered pH associated with production of organic acids during fermentation was the main factor increase soluble calcium concentration. Calcium solubility was measured directly after addition of acid to calcium-fortified soymilk to alter pH. Calcium solubility gradually increased as pH decreased. Greatest calcium solubility occurred at pH 1.0. This pH value enhanced calcium solubility of 93.8%. Consistency with the result obtained in this study, fermented soymilk with sucrose addition exhibited the lowest pH related with the highest organic acid production, resulted to the highest soluble calcium content (Figure 12, 13, 17).

These results suggested that sucrose could be a suitable carbon source for *L. plantarum* P49. Meanwhile, the smaller increase in calcium solubility was obtained from the addition of galactose followed by glucose.

4. Production of fermented soymilk by spray drying

4.1 Viability of lactic acid bacteria during spray drying

Fermented soymilk containing single strain of *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49 with initial cell number of $2.06x10^9$, $1.69x10^9$ and $1.08x10^9$ CFU/g, respectively, was spray-dried at inlet air temperature of 130 °C and outlet air temperature of 70 °C. After spray drying, viable cell of *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49 were $8.7x10^9$, $8.8x10^9$ and $2.5x10^8$ CFU/g, respectively (Figure 18). It was found that *L. acidophilus* TISTR 1338 and *L. casei* subsp. *tolerans* TISTR 1341 exhibited high resistant to heat with high survival of 106.77 % and 107.69 %, respectively. While *L. plantarum* P49 was more sensitive to heat with survival of 93.02 %. The loss in viability was most likely due to dehydration and thermal damage of cell structures and cell components during spray drying (Hamsupo, 2005). In addition, thermal shock may occur during the introduction of spray droplets into the hotter inlet air and affect to cell survival (Kim and Bhowmik, 1990).

It was obviously seen that viability of all test strains was very high. Comparison to To and Etzel (1997), the residual viability of *L. casei* subsp. *pseudoplantarum* UL137 was 14.7 and 13 % after spray drying at outlet air temperature of 65 and 70 °C, respectively. This might be due to the protective ability of maltodextrins as a protective substance used in this study. Maltodextrins were high efficient to protect the cells during spray drying (Teixeira *et al*., 1995). The amorphous form of maltodextrins is able to prevent protein unfolding during drying process (DePaz *et al*., 2002). It was found that the high T_g of sugar could stabilize lipid bilayer during dehydration (Santivarangkna *et al*., 2007). In addition, soymilk was able to protect cell due to the protein of soymilk which could prevent cellular injury by stabilizing cell membrane constituents (Castro *et al*., 1995).

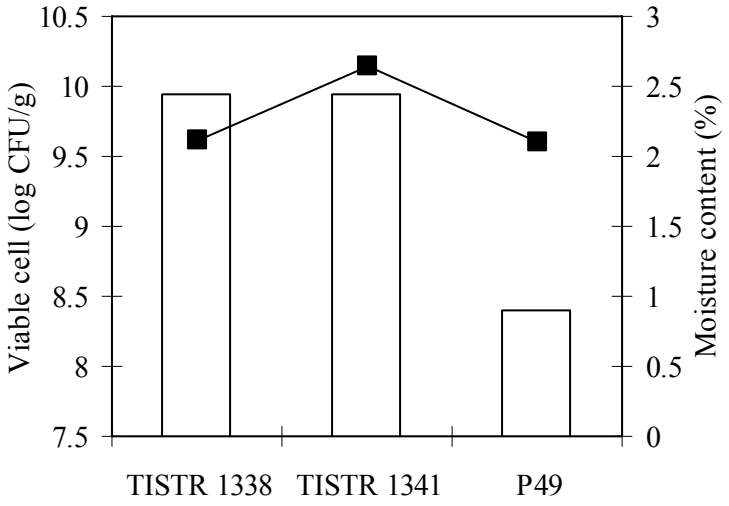


Figure 18 Viable cell (□) and moisture content (■) of spray-dried *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49 after spray drying

Generally, the desired level of water content in the product is achieved in between 1 and 3 % after spray drying (Vega and Roos, 2006). During spray drying, the residual moisture content dependent on the composition of the fluid in which each microorganisms are dried and the species of microorganisms (Scott, 1958). As expected, the moisture content of spray-dried *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49 was variable after spray drying. The spray-dried *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49 exhibited moisture content of 2.11, 2.64 and 2.10 %, respectively (Figure 18).

4.2 Survival of spray-dried lactic acid bacteria under gastrointestinal conditions

The spray-dried cells of *Lactobacilli* spp. were evaluated the tolerant to simulated gastric juice and simulated intestinal juice. The viable cell number of spray-dried of all three strains was quite stable with reduction of 0.02-0.84 log CFU/ml after

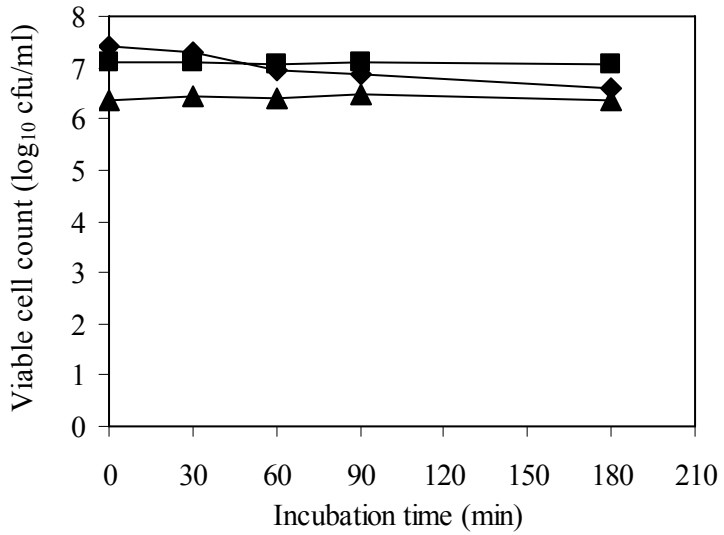


Figure 19 Viable cell of spray-dried *L. acidophilus* TISTR 1338 (♦), *L. casei* subsp. *tolerans* TISTR 1341 (■) and *L. plantarum* P49 (▲) during exposure to simulated gastric juice at pH 2.0 for 180 min

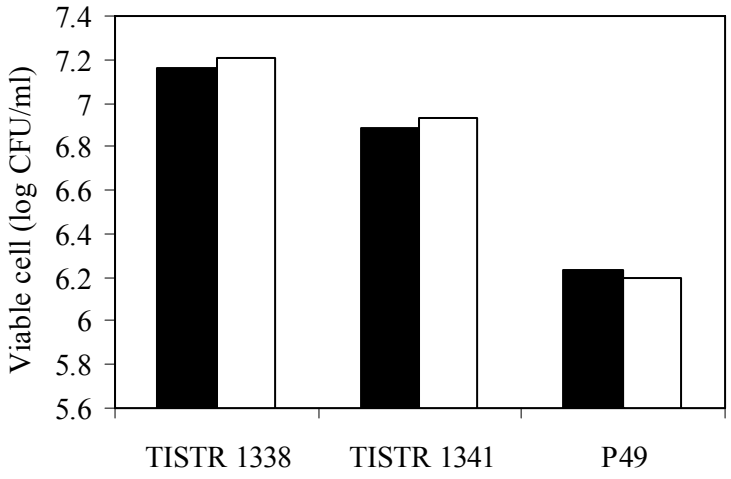


Figure 20 Viable cell of spray-dried *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49 after 240 min (□) exposure to simulated bile juice at pH 8.0 compared with control (■)

180 min exposure to simulated gastric juice at pH 2 (Figure 19). According to simulated bile juice tolerance (Figure 20), the spray-dried *L. acidophilus* TISTR 1338 and *L. casei* subsp. *tolerans* TISTR 1341 slightly increased from 7.16 to 7.21 log CFU/ml and 6.89 to 6.93 log CFU/ml, respectively, during 240 min incubation. Meanwhile, the viable cell of spray-dried *L. plantarum* P49 quite stable with 0.03 log reduction after 240 min incubation.

It was observed that cell encapsulated with soymilk by spray drying was more tolerance to simulated gastrointestinal juice than free cell. According to Michida *et al*. (2006), the addition of malt and barley extracts significantly improved the viability of *L. plantarum*. This was probably due to sugar content in the cereal extracts and the present of cereal extracts had a synergistic effect on the gastrointestinal tolerance. In addition, many reports have been proposed that survival of probiotic LAB was enhanced in simulated gastrointestinal conditions using microencapsulation techniques (Krasaekoopt *et al*., 2004; Truelstrup Hansen *et al*., 2002; Mandal *et al*., 2006; Picot and Lacroix, 2003).

Comparing of free cell, the spray-dried cell of the test strains largely improved survival under low acidity of stomach. Meanwhile, the spray-dried of the test strains slightly improved survival in the presence of pancreatin and bile salts compared to free cell of the strains tested. It was probably from the concept of microencapsulation allows the active core ingredient, or substrate, to be separated from its environment by a protective film or coating (Picot and Lacroix, 2004). This will protect encapsulated cells from interacting with the gastric and bile juices.

5. Stability of spray-dried lactic acid bacteria during storage

In this study, spray-dried powder of fermented soymilk containing *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49 was obtained from the pilot scale spray dryer. The spray-dried powders were kept at 4 °C and 30 °C for 6 months. Evaluation of viability of the test strains was shown in Figure 21.

Initial viable cell of *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49 were $8.7x10^9$, $8.8x10^9$ and $2.5x10^8$ CFU/g, respectively. It was observed that the viable cell of all three strains in the sample was quite stable during storage at 4 °C for 6 months. The total number of viable cell of *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49 was ~10^8-10^9 CFU/g, after 6 months of storage, which meet the number required for use as probiotic functional food (>10^7 CFU/g). The viable cell of the strains decreased as the storage temperature increased. After 3 months of storage, viable cell of *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49 decreased to 10^4 CFU/g and there were no viable after 4 months of storage at 30 °C. While, the viable cell of *L. acidophilus* TISTR 1338 absolutely lost just the first month of storage.

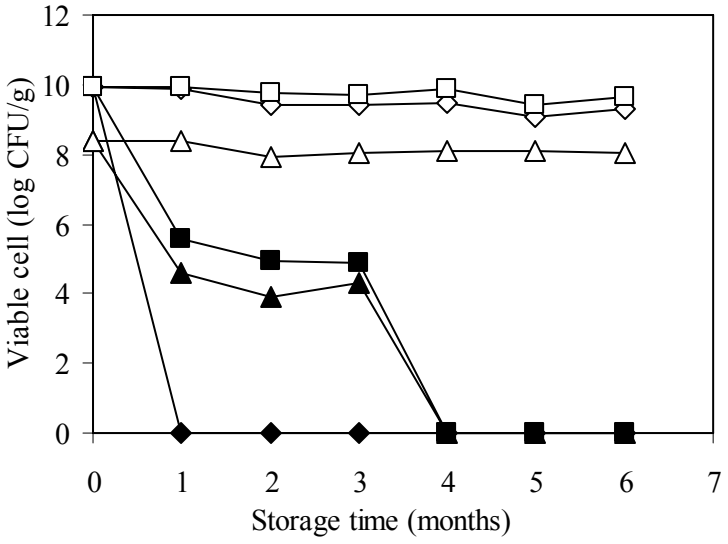


Figure 21 Viability of spray-dried *L. acidophilus* TISTR 1338 at 4 °C (◊) and 30 °C (♦), *L. casei* subsp. *tolerans* TISTR 1341 at 4 °C (□) and 30 °C (■) and *L. plantarum* P49 at 4 °C (Δ) and 30 °C (▲) during 6 months of storage

According to Selmer-Olsen *et al*. (1999), survival of dried LAB was strongly dependent on the storage temperature and the storage stability increased with increasing temperature. Similarly, Espina and Packard (1979) showed that percentage

of survival decreased immediately (about 50 % loss) after storage of spray-dried *L. acidophilus* at 4 °C for 30 days. Gardiner *et al*. (2002) also reported that probiotic viable cell number at 4 °C and 15 °C was maintained at about 10^8 CFU/g for up to 49 days, while viable cell number at 30 °C declined to 10^6 CFU/g during 49 days of storage. Moreover, the spray-dried *L. reuteri* KUB-AC5 showed high viable cell during storage at 4 °C for 4 months, but the total number of the strain decreases as the storage temperature increased to 30 °C (Hamsupo *et al*., 2005).

Furthermore, results indicated that the temperature of storage was a critical parameter affecting the survival of spray-dried *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49. The mortality of the strains was increased with decreasing temperature of storage. This was probably because inactivation during storage is related to formation of radicals in the presence of oxygen, fatty acid oxidation and DNA damage. Changes in unsaturated lipids degree of lipid oxidation contributed to a further increase in permeability and also affected enzymatic activities associated with membrane (Castro *et al*., 1996). Wang *et al*. (2004) investigated the viability of LAB and bifidobacteria in fermented soymilk during storage. They also found that the storage temperature was the main factor detrimental to the dried cultures. At 4 °C, survival of *S. thermophilus* ranging from 89 % to 92 % depend on packaging materials, while survival percentage ranging from 86 % to 91 % was noted for 25 °C after 4 months of storage.

Moisture content (or water activity) in dried product is one important factor affecting the viability of the cells during storage. Microorganism could be stabilized better in the low-water activity product which was appropriate to such microorganisms. (de Valdez *et al*., 1985). Moisture content of food powder product required for Thai Industrial Standard was lower than 7 % (Chotigo, 2009).

Figure 22 showed the moisture content of spray-dried cell; *L. acidophilus* TISTR, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49 in fermented soymilk powder during storage at 4 °C and 30 °C for 6 months. The moisture content

of all samples seemed to be gradually increased as the storage time increased. Initially, the moisture contents of spray-dried fermented soymilk were 2.11, 2.64 and 2.10 %, respectively of *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49. After 6 months of storage, the moisture contents increased to 3.95, 4.02 and 3.64 %, respectively of *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 and *L. plantarum* P49.

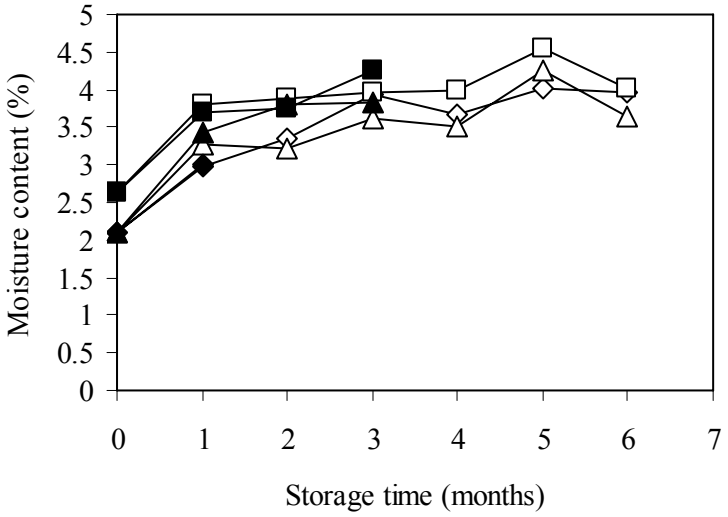


Figure 22 Moisture content during 6 months of storage of spray-dried *L. acidophilus* TISTR 1338 at 4 °C (◊) and 30 °C (♦), *L. casei* subsp. *tolerans* TISTR 1341 at 4 °C (□) and 30 °C (■) and *L. plantarum* P49 at 4 °C (Δ) and 30 °C (▲)

CONCLUSION

Three strains of lactic acid bacteria, *L. acidophilus* TISTR 1338, *L. casei* subsp. *tolerans* TISTR 1341 isolated from the chicken intestine and *L. plantarum* P49 isolated from fermented cabbage were tested for their capabilities to use for the development of functional foods. Results showed that all test strains have ability to produce high amounts of lactic acid ranged from 10.38-18.52 g/l and they exhibited high tolerant to simulated bile juice with the viable cell numbers of ~10^8 CFU/ml after 240 min incubation. However, the strains survived in low pH for only 30 min incubation with the viable cell remained of ~10^2 CFU/ml. There was no viable cell of all strains after 60 min incubation.

During soymilk fermentation at 37 ^oC for 48 h, *L. acidophilus* TISTR 1338 and *L. casei* subsp. *tolerans* TISTR 1341 did not show any growth while *L. plantarum* P49 exhibited slightly growth. However, they could survive in soymilk throughout the end of fermentation with the viable cell number of ~10^8 CFU/ml. Dramatic decrease in pH was found in soymilk fermented with *L. plantarum* P49. Consequently, there was the highest increase in soluble calcium from 11.91 to 34.82 % after 48 h. It was observed that the increase in calcium solubility related to lowered pH associated with the production of organic acids. In addition, single culture fermentation of three test strains decreased the content of raffinose and stachyose in soymilk. Raffinose and stachyose contents decreased as the fermentation time increased. Moreover, results revealed that *L. plantarum* P49 was ability to exploit these raffinose and stachyose as substrates more efficiently than other two strains.

The addition of sugar in soymilk fermented with *L. plantarum* P49 was found to improve calcium solubility and reduction of raffinose and stachyose. The fermented soymilk with sucrose addition contained 2.5-fold and 7-fold higher in soluble calcium compared to fermented soymilk without sugar addition and non-fermented soymilk, respectively. The addition of galactose in soymilk fermented by *L. plantarum* P49 exhibited highest decrease in the level of raffinose. Meanwhile, the highest decrease in the level of stachyose was obtained by glucose addition.

The present study has shown that all test strains in fermented soymilk were not susceptible to spray drying. The high survival of all strains was obtained after spray drying. A higher survival of all strains could be achieved by placing the dried fermented soymilk in aluminum foil bag at 4 °C compared to 30 °C. This finding suggested that the temperature of storage was a critical parameter affecting the survival of spray-dried cells. Spray drying may be one of the adequate dehydration processes for the fermented soymilk product to facilitate storage, transportation and to maintain high viability of LAB. Furthermore, the spray-dried cells of *Lactobacilli* were found to largely improve survival under low acidity of stomach. The viable cell of all three strains in spray-dried powder was quite stable during 180 min exposure to simulated gastric juice. In the presence of pancreatin and bile salts survival of the test strains in spray-dried powder was also improved. Viable cell of the spray-dried *L. acidophilus* TISTR 1338 and *L. casei* subsp. *tolerans* TISTR 1341 slightly increased during 240 min incubation. For the spray-dried *L. plantarum* P49, the viable cell was quite stable after 240 min incubation.

In conclusion, fermentation of soymilk by *L. plantarum* P49 could potentially enhance calcium availability and oligosaccharide reduction. The spray-dried fermented soymilk containing high viable cell of potentially probiotic lactic acid bacteria could be healthy beverage with regard to a possible role of probiotics and prebiotics in improvement of functions and reducing the risk of diseases. In addition, soymilk microencapsulated *L. plantarum* P49 exhibited high tolerance to simulated gastrointestinal juice.

Finally, further research should be aimed at a large scale production of the probiotic fermented soymilk product and consumer acceptability should be assessed.

LITERATURE CITED

Alm, L. 1982. Effect of fermentation on lactose, glucose and galactose content in milk and suitability of fermented milk products for lactose-deficient individuals. **J Dairy Sci**. 65: 346–352.

Ammor, M. S. and B. Mayo. 2007. Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. **Meat Sci.** 76: 138-146.

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

Barker, S. B. and W. H. Summerson. 1941. The colorimetric determination of lactic acid in biological material. **J. Biol. Chem.** 138: 535-554.

Bergqvist, S. W., A. S. Sandberg, N. G. Carlsson and T. Andlid. 2005. Improved iron solubility in carrot juice fermented by homo– and hetero– fermentative lactic acid bacteria. **Food Microbiol.** 22: 53-61.

Caplice, E. and G. F. Fitzgerald. 1999. Food fermentations: role of microorganisms in food production and preservation. **Int. J. Food Microbiol.** 50: 131–149.

Carroll, K. K., A. Elzbieta and M. Kurowska. 1995. Soy Consumption and Cholesterol Reduction: Review of Animal and Human Studies. **American Institute of Nutrition.** 594S-597S.

Castro, H., P. Teixeira and R. Kirby 1995. Storage of lyophilized cultures of *Lactobacillus bulgaricus* under different relative humidities and atmospheres. **Appl. Microbiol and Biotech**. 44: 172–176.

Chandramoulia, V., K. Kailasapathya, P. Peirisb and M. Jones. 2004. An improved method of microencapsulation and its evaluation to protect Lactobacillus spp. in simulated gastric conditions. **J. Microbiol. Methods.** 56: 27– 35.

Chien, H. L., H. Y. Huang and C. C. Chou. 2006. Transformation of isoflavone phytoestrogens during the fermentation of soymilk with lactic acid bacteria and bifidobacteria. **Food Microbiol.** 23: 772-778.

Conrad, P. B., D. P. Miller, P. R. Cielenski and J. J. de Pablo. 2000. Stabilization and preservation of *Lactobacillus acidophilus* in saccharide matrices. **Cryobiology.** 41(1): 17-24.

Conway, P. L. 1996. Selection criteria for probiotic microorganisms. **Asia Pacific J Clin Nutr.** 5: 10-14.

Corcoran, B.M., R.P. Ross, G.F. Fitzgerald and C. Stanton. 2004. Comparative survival of probiotic lactobacilli spray-dried in the presence of prebiotic substances. **J. Appl. Microbiol.** 96: 1024–1039.

DePaz, A. R., D. A. Dale, C. C. Barnett, J. F. Carpenter, A. L. Gaertner and T. W. Randolph. 2002. Effects of drying methods and additives on the structure, function, and storage stability of subtilisin: role of protein conformation and molecular mobility. **Enzyme Microb. Technol.** 31: 765-774.

Desmond, C., C. Stanton, G. F. Fitzgerald, K. Collins and R. P. Ross. 2002. Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying. 12(2): 183-190.

Desmond, C., R. P. Ross, E. O'Callaghan, G. Fitzgerald and C. Stanton. 2002. Improved survival of *Lact. paracasei* NFBC 338 in spray-dried powders containing gum acacia. **J. Appl. Microbiol.** 93: 1003–1011.

de Valdez, G.F., G.S. de Giori, A.P. Holgodo and G. Oliver. 1985. Effect of drying medium on residual moisture content and viability of freeze-dried lactic acid bacteria. **AEM.** 49: 413-415.

Dinakar, P. and V. V. Mistry. 1994. Growth and Viability of *Bifidobacterium bifidum* in Cheddar Cheese. **J. Dairy Sci**. 77: 2854-2864.

Donkor, O. N., A. Henriksson, T. Vasiljevic and N.P. Shah. 2007. α-Galactosidase and proteolytic activities of selected probiotic and dairy cultures in fermented soymilk. **Food Chem.** 104(1): 10-20.

Espina, F. and V. S. Packard. 1979. Survival of *Lactobacillus acidophilus* in a spray-drying process. **J. Food Prot.** 42(2): 149-152.

Fávaro Trindade, C. S., S. C. Terzi, L. C. Trugo, R. C. Della Modesta and S. Couri. 2001. Development and sensory evaluation of soy milk based yoghurt. **Archivos Latinoamericanos De Nutricion.** 51(1): 100-104.

Fernández, M.F., S. Boris and C. Barbés. 2003. Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. **J. Appl. Microbiol.** 94: 449–455.

Fuller, R. 1989. Probiotics in man and animals. **J. Appl. Bacteriol.** 66: 365-378.

_____. 1992. History and development of probiotics, pp. 1 - 8. In R. Fuller (ed.). **Probiotics: The Scientific Basis.** Chapman & Hall, London.

Gardiner, G., C. Stanton, P. B. Lynch, J. K. Collins, G. Fitzgerald and R. P. ROSS. 1999. Evaluation of Cheddar Cheese as a Food Carrier for Delivery of a Probiotic Strain to the Gastrointestinal Tract. **J. Dairy Sci.** 82: 1379–1387.

Gardiner, G. E., P. Bouchier, E. O'Sullivan, J. Kelly, J. K. Collins, G. Fitzgerald, R. Paul Ross and C. Stanton. 2002. A spray-dried culture for probiotic Cheddar cheese manufacture. **Int. Dairy J.** 12: 749–756.

Garneau, S., N. I. Martin, J. C. Vederas. 2002. Two-peptide bacteriocins produced by lactic acid bacteria. **Biochimie.** 84: 577–592.

Garro, M.S., G.F. de Valdez, G. Oliver and G. S. de Giori. 1996. Influence of carbohydrates on the α-galactosidase activity of *Lactobacillus fermentum*. **Current Microbiology.** 33: 302-305.

Hamsupo, K. 2005. **Production and formulation of lactic acid bacteria producing antimicrobial substances as chicken probiotic adjuncts.** Graduate School, Kasetsart University.

Hamsupo, K., P. Sukyai, S. Nitisinprasert and P. Wanchaitanawong. 2005. Different growth media and growth phases affecting on spray drying and freeze drying of *Lactobacillus reuteri* KUB-AC5. **Kasetsart J.** 39: 718–724.

Holzapfel, W. H., P. Haberer, R. Geisen, J. Björkroth and U. Schillinger. 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. **Am J Clin Nutr.** 73: 365S-73S.

Hou, J. W., R. C. Yu and C. C. Chou. 2000. Changes in some components of soymilk during fermentation with bifidobacteria. **Food Res. Int.** 33: 393-397.

Hugas, M. 1998. Bacteriocinogenic Lactic Acid Bacteria for the Biopreservation of Meat and Meat Products. **Meat Sci.** 49: S139-S150.

Isolauri, E., T. Arvola, Y. Sütas, E. Moilanen and S. Salminen. 2000. Probiotics in the management of atopic eczema. **Clin. Exp. Allergy.** 30: 1605–1610.

Jacobsen, B. K., S. F. Knutsen and G. E. Fraser. 1998. Does high soy milk intake reduce prostate cancer incidence? The Adventist Health Study (United States). **Cancer Causes and Control.** 9: 553-557.

Jay, J. M. 1982. Antimicrobial Properties of Diacetyl. **AEM.** 44(3): 525-532.

Johnson, J.A.C. and M. R. Etzel. 1995. Properties *of Lactobacillus helveticus* CNRZ-32 Attenuated by Spray-Drying, Freeze-Drying, or Freezing. **J. Dairy Sci.** 78: 761-768.

Karovičová, J. and Z. Kohajdová. 2005. Lactic acid-fermented vegetable juices-palatable and wholesome foods. **Chem. Pap.** 59(2): 143-148.

Khedkar, C. D., J. M. Mantri, R. D. Garge, S. A. Kulkarni and G. D. Khedkar. 1993. Hypocholesterolemic effect of fermented milks: A review. **Cult. Dairy Prod. J. 28**: 14–18.

Kim, S. S. and S. R. Bhowmik. 1990. Survival of lactic acid bacteria during spray drying of plain yogurt. **J. Food Sci.** 55(4): 1008-1010.

Krasaekoopt, W., B. Bhandari and H. Deeth. 2004. The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria. **Int. Dairy J**. 14: 737–743.

Kwon, B., Y.B. Kim, J. H. Lee, H. J. Lee, D.K. Chung and G.E. JI. 2002. Analysis of Sugars and α-Galactosidase Activity during soymilk fermentation by bifidobacteria. **Food Sci. Biotechnol.** 11(4): 389-391.

LeBlanc, J. G., M. S. Garro and G. S. de Giori. 2004. Sugar and acid contents in soymilk fermented with lactic acid bacteria alone or simultaneously with bifidobacteria. **Food Microbiol.** 20: 333–338.

Lee, C. H. 1997. Lactic acid fermented foods and their benefits in Asia. **Food Control.** 8 (S/6): 259-269.

Lee, Y. K. and S. Salminen. 1995. The coming of age of probiotics. **Trends Food Sci. Tech.** 6: 241-245.

Lee, K.I. and T.R. Heo. 2000. Survival of *Bifidobacterium longum* Immobilized in Calcium Alginated Beads in Simulated Gastric Juices and Bile Salt Solution. **Appl. Environ. Microbiol.** 66: 869-873.

Lee, J. S., D. S. Cha and H. J. Park. 2004. Survival of freeze-dried *Lactobacillus bulgaricus* KFRI 673 in chitosan-coated calcium alginate microparticles. **J. Agric. Food Chem.** 52: 730Õ-7305.

Lian, W. C., H. C. Hsiao and C. C. Chou. 2002. Survival of bifidobacteria after spray drying. **Int. J. Food Microbiol.** 74: 79-86.

Ling, W. H., R. Korpela, H. Mykkanen, S. Salminen and O. Hanninen. 1994. *Lactobacillus Strain GG Supplementation Decreases* Colonie Hydrolytic and Reductive Enzyme Activities in Healthy Female Adults. **American Institute of Nutrition.** 18-23.

Liu, D-M., L. Li, X.-Q. Yang, S.-Z. Liang and J.-S. Wang. 2006. Survivability of *Lactobacillus rhamnosus* during the preparation of soy cheese. **Food Technol. Biotechnol.** 44(3): 417–422.

Liu, K. 1999. Oriental Soyfoods, pp. 139-199. In C. Y. W. Ang, K. Liu and Y. W. Huang (eds). **Asian Foods Science & Technology.** Technomic publication, Pennsylvania, U.S.A.

Liu, J. R. and C. W. Lin. 2000. Production of kefir from soymilk with or without added glucose, lactose, or sucrose. **J. Food Sci**. 65(4): 716-719.

Lopez, H.W., A. Ouvry, E. Bervas, C. Guy, A. Messager, C. Demigne and C. Remesy. 2000. Strains of lactic acid bacteria isolated from sour doughs degrade phytic acid and improve calcium and magnesium solubility from whole wheat flour. **J. Agric. Food Chem.** 48: 2281-2285.

Majamaa, H. and E. Isolauri. 1997. Probiotics: a novel approach in the management of food allergy. **J. Allergy Clin. Immunol.** 99: 179–186.

Mandal, S., A. K. Puniya, K. Singh. 2006. Effect of alginate concentrations on survival of microencapsulated Lactobacillus casei NCDC-298. **Int. Dairy J.** 16: 1190–1195.

Mayra-Mäkinen, A. and M. Bigret. 2004. Industrial use and production of lactic acid bacteria, pp. 175-198. *In* S. Salminen, A. V. Wright and A. Ouwehand (eds). **Lactic Acid Bacteria : Microbiological and Functional Aspects.** Marcel Dekker, Inc., New York.

Michida, H., S. Tamalampudi, S. S. Pandiella, C. Webb, H. Fukuda and A. Kondo. 2006. Effect of cereal extracts and cereal fiber on viability of *Lactobacillus plantarum* under gastrointestinal tract conditions. **Biochem. Eng. J.** 28: 73-78.

Mital, B. K., R. S. Shallenberger and K. H. Steinkraus. 1973. α-Galactosidase activity of lactobacilli. **Appl Microbiol.** 26(5): 783-788.

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

Nguyen, T.T., G. Loiseau, C. Icard-Verniere, I. Rochette, S. Treche and J.P. Guyot. 2007. Effect of fermentation by amylolytic lactic acid bacteria, in process combinations, on characteristics of rice/soybean slurries: A new method for preparing high energy density complementary foods for young children. **Food Chem.** 100: 623–631.

O'Riordan, K., D. Andrews, K. Buckle and P. Conway. 2001. Evaluation of microencapsulation of a Bifidobacterium strain with starch as an approach to prolonging viability during storage. **J. Appl. Microbiol.** 91: 1059-1066.

Otieno, D. O., J. F. Ashton and N. P. Shah. 2007. Isoflavone phytoestrogen degradation in fermented soymilk with selected β-glucosidase producing *L. acidophilus* strains during storage at different temperatures. **Int. J. Food Microbiol.** 115: 79-88.

Parvez, S. K.A. Malik, S. Ah Kang and H.-Y. Kim. 2006. Probiotics and their fermented food products are beneficial for health. **J. Appl. Microbiol.** 100: 1171–1185.

Picot, A. and C. Lacroix. 2004. Encapsulation of bifidobacteria in whey protein-based microcapsules and survival in simulated gastrointestinal conditions and in yoghurt. **Int. Dairy J**. 14: 505–515.

Pintado, J., Guyot, J. P. and M. Raimbault. 1999. Lactic acid production from mussel processing wastes with an amylolytic bacterial strain. **Enzyme Microb. Technol.** 24: 590-598.

_____. 2005. Influence of polysaccharides on oxygen dependent lactate utilization by an amylolytic *Lactobacillus plantarum* strain. **Int. J. Food Microbiol.** 98: 81-88.

Ragout, A., Siñeriz, F. H. Diekmann and G. F. de Valdez. 1994. Effect of environmental pH on the fermentation balance of *Lactobacillus reuteri*. **J. Appl Bacteriol.** 77: 388-391.

Rao, A. V., N. Shiwnarain and I. Maharaj. 1989. Survival of microencapsulated *Bifidobacterium pseudolongum* in simulated gastric and intestinal juices. **Can. Inst. Food Sci. Technol. J.** 22: 345-349.

Rekha, C. R. and G. Vijayalakshmi. 2008. Biomolecules and nutritional quality of soymilk fermented with probiotic yeast and bacteria. **Appl. Biochem. Biotechnol.** 151: 452–463.

Rolfe, R. D. 2000. The Role of Probiotic Cultures in the Control of Gastrointestinal Health. **ASNS.** 396S-402S.

Saito, T. 2004. Selection of useful probiotic lactic acid bacteria from the *Lactobacillus acidophilus* group and their applications to functional foods. **Anim. Sci. J.** 75: 1-13.

Salminen, S., A. C. Ouwehand, and E. Isolauri. 1998. Clinical applications of probiotic bacteria. **Int. Dairy J.** 8:563–572.

Salovaara, S., A. S. Sandberg and T. andlid. 2003. Combined impact of pH and organic acids on iron uptake by CaCO-2 cells. **J. Agric. Food Chem.** 51(26): 7820-7824.

Sanders, M. E. D. C. Walker, K. M. Walker, K. Aoyama and T. R. Klaenhammer. 1996. Performance of commercial cultures in fluid milk applications. **J. Dairy Sci.** 79: 943-955.

Santivarangkna, C., U. Kulozik and P. Foerst. 2007. Alternative drying processes for the industrial preservation of lactic acid starter cultures. **Biotechnol. Prog.** 23: 302-315.

Savard, T., C. Beaulieu, N.J. Gadren, C.P. Champagne. 2002. Characterization of spoilage yeasts isolated from fermented vegetables and inhibition by lactic, acetic and propionic acids. **Food Microbiol.** 19: 363–373.

Scalabrini, P., M. Rossi, P. Spettoli and D. Matteuzzi. 1998. Characterization of *Bifidobacterium* strains for use in soymilk fermentation. **Int. J. Food Microbiol.** 39: 213-219.

Schillinger, U. and F. K. Lucke. 1989. Antibacterial Activity of Lactobacillus sake Isolated from Meat. **AEM.** 55(8): 1901-1906.

Scott, W.J. 1958. The effect of residual water on the survival of dried bacteria during storage. **J. Gen. Microbiol.** 19: 624-633.

Selmer-Olsen, E., T. Sørhaug, S. E. Birkeland and R. Pehrson. 1999. Survival of *Lactobacillus helveticus* entrapped in Ca-alginate in relation to water content, storage and rehydration. **J. Ind. Microbiol.** 23: 79-85.

Setchell, K. D. R. and A. Cassidy. 1999. Dietary Isoflavones: Biological Effects and Relevance to Human Health. **ASNS.** 758S-767S.

Shah, N.P. 2000. Probiotic Bacteria: Selective Enumeration and Survival in Dairy Foods. **J. Dairy Sci.** 83, 894-907.

Shimakawa, Y., S. Matsubara, N. Yuki, M. Ikeda and F. Ishikawa. 2003. Evaluation of *Bifidobacterium breve* strain Yakult-fermented soymilk as a probiotic food. **Int. J. Food Microbiol.** 81: 131– 136.

Shioiri, T., K. Yahagi, S. Nakayama, T. Asahara, N. Yuki, K. Kawakami, Y. Yamaoka, Y. Sakai, K. Nomoto and M. Totani. 2006. The effect of synbiotic fermented milk beverage containing *Lactobacillus casei* strain Shirota and Transgalactosylated oligosaccharides on defecation frequency, intestinal microflora, organic acid concentrations, and putrefactive metabolites of sub-optimal health state volunteers: A randomized placebo-controlled cross-over study. **Biosci. Microflora.** 25(4): 137-146.

Shirai, K., S. Revah-Moiseev, M. Garcia-Garibay and V. M. Marshall. 1994. Ability of some strains of lactic acid bacteria to degrade phytic acid. **Lett. Appl. Microbiol.** 19: 366-369.

Shotigo, A. 2009. **Production of pelleted feed containing *Lactobacillus reuteri* KUB AC-5 by cold pelleting process.** Graduate school, Kasetsart University.

Simon, O., A. Jadamus and W. Vahjen. 2001. Probiotic feed additives-effectiveness and expected modes of action. **J. Anim. Feed Sci.** 10: 51–67.

Simpson, P. J., C. Stanton, G. F. Fitzgerald and R. P. Ross. 2005. Intrinsic tolerance of Bifidobacterium species to heat and oxygen and survival following spray drying and storage. **J. Appl. Microbiol.** 99(3): 493-501.

Sindhu, S. C. and N. Khetarpaul. 2001. Probiotic fermentation of Indigenous food mixture : effect on antinutrients and digestibility of starch and protein. **J. Food Comp. Anal.** 14: 601-609.

Soomro, A. H., T. Masud and K. Anwaar. 2002. Role of lactic acid bacteria (LAB) in food preservation and human health-A review. **Pakistan J. Nutri.** 1(1): 20-24.

Stanton, C., G. Gardiner, P. B. Lynch, J. K. Collins, G. Fitzgerald and R. P. Ross. 1998. Probiotic cheese. **Int. Dairy J.** 8: 491-496.

Sudheer, K. M., R. B. Sridhar, B. S. Kiran, P. M. Bhilegaonkar, A. Shirwaikar and M. K. Unnikrishnan. 2004. Antiinflamatory and antiulcer activities of phytic acid in rats. **Indian J. Exp. Biol.** 42(2): 179-185.

Tang, A.L., N.P. Shah, G. Wilcox, K.Z.Walker and L. Stojanovska. 2007. Fermentation of Calcium-Fortified Soymilk with *Lactobacillus*: Effects on Calcium Solubility, Isoflavone Conversion, and Production of Organic Acids. **J. Food Sci.** 72(9).

Teixeira, P., H. Castro and R. Kirby. 1995. Spray-drying as a method for preparing concentrated cultures of *Lactobacillus bulgaricus*. **J. Appl. Bacteriol.** 78(4): 456-462.

Teixeira, P., H. Castro, C. Mohacsi-Farkas, and R. Kirby. 1997. Identification of sites of injury in *Lactobacillus bulgaricus* during heat stress. **J. Appl. Microbiol.** 83:219–226.

Teixeira, P. C., M. H. Castro, F. X. Malcata, and R. M. Kirby. 1995. Survival of *Lactobacillus delbrueckii* ssp. *bulgaricus* following spray-drying. **J. Dairy Sci.** 78: 1025-1031.

To, B. C. S. and M. R. Etzel. 1997. Spray Drying, Freeze Drying, or Freezing of Three Different Lactic Acid Bacteria Species. **J. Food Sci.** 62(3): 576-585.

Truelstrup Hansen, L., P. M. Allan-Woj tas,Y. L. Jin and A.T. Paulson. 2002. Survival of Ca-alginate microencapsulated *Bifidobacterium* spp. inmilk and simulated gastrointestinal conditions. **Food Microbiol.** 19: 35-45.

Tsuda, H., K. Hara and T. Miyamoto. 2008. Binding of Mutagens to Exopolysaccharide Produced by *Lactobacillus plantarum* Mutant Strain 301102S. **J. Dairy Sci.** 91:2960–2966.

Vega, C. and Y.H. Roos. 2006. *Invited Review*: Spray-dried dairy and dairy-like emulsions compositional considerations. **J. Dairy Sci.** 89: 383-401.

Vucenik, I. and A. M. Shamsuddin. 2003. Cancer inhibition by inositol hexaphosphate (IP6) and inositol: from laboratory to clinic. **J. Nutr.** 133: 3778S-3784S.

Wang, Y. C., R. C. Yu, H. Y. Yang and C. C. Chou. 2003. Sugar and acid contents in soymilk fermented with lactic acid bacteria alone or simultaneously with bifidobacteria. **Food Microbiol.** 20: 333-338.

Wee, Y.-J., J.-N. Kim and H.-W. Ryu. 2006. Biotechnological Production of Lactic Acid and Its Recent Applications.

William, P. 2000. Effect of cooking on the soluble and insoluble oxalate content of some New Zealand foods. **J. Food Compos. Anal.** 12: 201-206.

Yoon, J. H., L. U. Thompson and D. J. A. Jenkins. 1983. The effect of phytic acid on in vitro rate of starch digestibility and blood glucose response. **Am. J. Clin. Nutr.** 38: 835-842.

APPENDICES

Appendix A

Preparation of stock solution and chemical analysis

1. Determination of the amount of lactic acid (Barker and Summerson, 1941)

1.1 Preparation of reagents

1.1.1 4 % and 20 % copper sulphate solution

Dissolved 4 and 20 g copper sulphate in distilled water and adjusted to a final volume of 100 ml.

1.1.2 1.5% p-hydroxydiphenyl

Dissolved 1.5 g p-hydroxydiphenyl in 5 % NaOH and adjusted to a final volume of 100 ml.

1.2 Procedure

Pipetted 1 ml supernatant of each samples (containing appropriated lactic acid concentration at ~100 μg/ml) into tested tube and then 20 % copper sulphate solution ($CuSO_4.5H_2O$) (1 ml) was added and adjusted to a final volume of 10 ml. Added 1 g calcium hydroxide in each tested tube and the tube were shaken. Centrifugation of the mixtured at 4000 rpm/min for 10 min. Pipetted 1 ml supernatant into tube containing 0.005 ml 4 % $CuSO_4.5H_2O$. Placed tested tubes in an ice bath and slowly added 6 ml concentrated sulfuric acid (conc.H_2 SO_4), then shaking. Each tested tube was boiling in boiled water for 5 min and the tubes were suddenly placed in an ice bath. 2 Droplets of 1.5 % p-hydroxydiphenyl was added into tubes and the tubes were shaken. Then, the tubes were stand at 30 ºC for 30 min. The tubes were boiling in boiled water for 90 sec and suddenly placed in an ice bath again. The absorbance of the solution was measured at wave length of 560 nm.

The amount of lactic acid could be determined by using a standard curve of lithium lactate solution as reference.

$$\text{Lactic acid concentration (g/l)} = \frac{\text{(The absorbance of sample at 560 nm) x (Dilution) x 90.08}}{\text{(Slope of standard curve) x 1,000 x 96.01}}$$

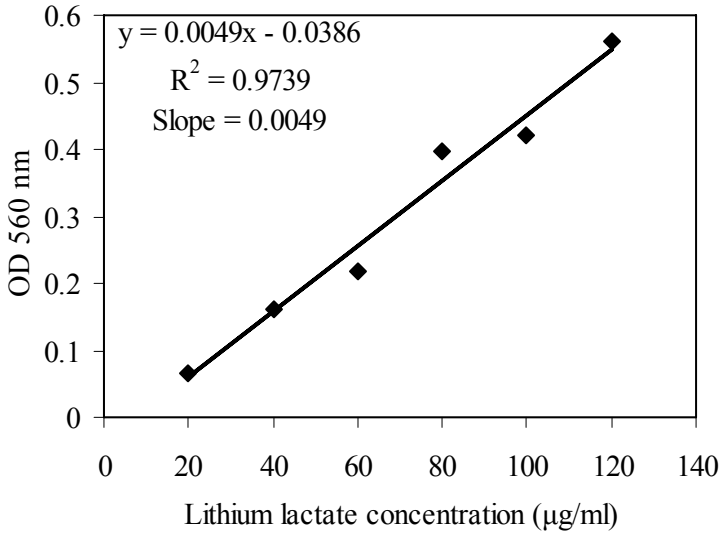
1.3 Plotting of standard curve

1.3.1 Preparation of different lithium lactate concentrations (Appendix Table A1) from 1 mg/ml lithium lactate stock solution.

Appendix Table A1 Preparation of different lithium lactate concentrations for lactic acid determination

Lithium lactate concentrations (μg/ml)	Volume of 1 mg/ml lithium lactate solution (μl)	Distilled water
0	0	1000
20	20	980
40	40	960
60	60	940
80	80	920
100	100	900
120	120	880

1.3.2 Pipetted each lithium lactate concentration into tested tube and followed the procedure described in 3.2. Plotting standard curve of lithium lactate concentration with the absorbance of lithium lactate solution was performed.



Appendix Figure A1 The curve of standard lithium lactate solution

2. Determination of the amount of acetic acid (Modified from William, 2000)

2.1 Preparation of reagents

2.1.1 Free CO_2 water

Preparation of free CO_2 water by boiling of distilled water for 20 min.

2.1.2 Standard solution of 0.1 N NaOH

4 g of NaOH was dissolved in distilled water and adjusted to a final volume of 1 l.

Determination of standard concentration of 0.1 N NaOH by weighing 0.3 g of acid potassium phthalate ($KHC_8H_4O_4$) into 250 ml flask, then 100 ml of free CO_2 water was added. Phenolphthalein (2 droplets) was dropped in acid

potassium phthalate solution which then was titrated with 0.1 N NaOH. The standard concentration of 0.1 N NaOH could be calculated using equation below:

$$\text{Standard concentration (N)} = \frac{KHC_8H_4O_4\ (g) \times 1000}{NaOH\ (ml) \times 204.229}$$

2.1.3 Phenolphthalein solution

Dissolved 1 g phenolphthalein in 95 % alcohol and adjusted to a final volume of 100 ml.

2.2 Procedure

Pipetted 1 ml supernatant of samples into flask (250 ml) and then 40 ml of free CO_2 water was added. Dropped phenolphthalein (2 droplets) into diluted sample solution flask and then titrated with 0.1 N NaOH standard solutions until to the end point (sample solutions became light pink). The amount of acetic acid was calculated as below:

$$\text{The amount of acetic acid (g/100 ml)} = \frac{N \times V \times 60.1 \times 100}{1000 \times 1}$$

By N = Standard concentration of 0.1 N NaOH
V = Volume of 0.1 N NaOH standard solution

3. Total sugar determination by Phenol-sulfuric acid method (Dobois *et al*., 1956)

3.1 Preparation of reagents

3.1.1 95.5 % Sulfuric acid (Concentrated sulfuric acid)

3.1.2 5 % Phenol solution

- Dissolved 5 g of phenol in distilled water and adjusted to a final volume of 100 ml.

3.2 Procedure

3.2.1 Pipetted 1 ml samples (containing appropriated sugar at concentration between 0-80 μg/ml) into each tested tube, then 5 % phenol solution (1 ml) were added.

3.2.2 Rapidly added concentrated sulfuric acid (5 ml) into the mixtured and then shaking.

3.2.3 The mixtured was allowed to stand for 10 min and then shaking. Tested tubes were then placed in water bath at 30 °C for 10 min.

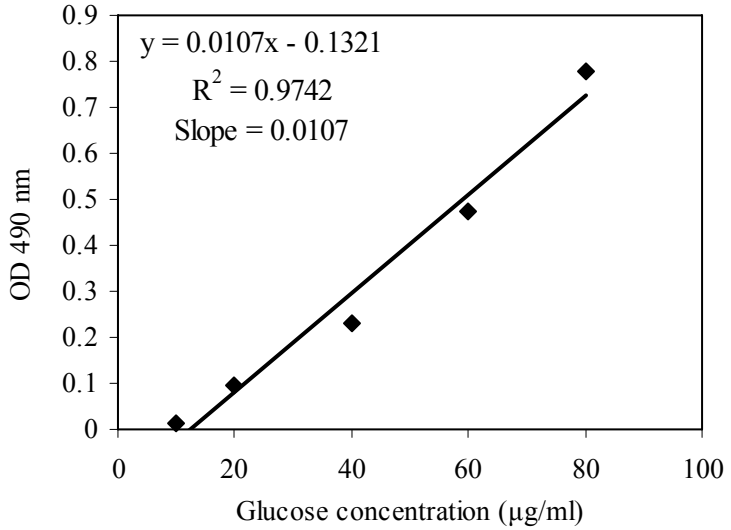
3.2.4 The absorbance of the samples was measured at wave length of 490 nm. The amount of sugar could be determined by using a standard curve of glucose solution as reference.

3.3 Plotting of standard curve

3.3.1 Prepared stock solution of glucose solution by weighing glucose (0.04 g) then dissolved in distilled water and adjusted to a final volume of 100 ml. The final concentration of 400 μg/ml glucose solution was obtained.

3.3.2 Diluted glucose solution to a final concentration of 0, 10, 20, 40, 60 and 80 μg/ml.

Follow the procedure as described in 1.2.



Appendix Figure A2 The curve of standard glucose solution

3.4 Calculation of the amount of sugar

$$\text{The amount of glucose (g/l)} = \frac{\text{(The absorbance of sample at 490 nm) x (Dilution)}}{\text{(Slope of standard curve) x 1,000}}$$

4. Oligosaccharides determination by HPLC method

4.1 Preparation of 65 % acetonitrile

65 ml Acetonitrile was added with 35 ml deionized water, a final concentration of 65 % (v/v) was obtained.

4.2 Preparation of standard solution

200 µl of standard solution was prepared by the mixtured of sucrose, raffinose and stachyose solution as shown in Appendix Table A2.

Appendix Table A2 Preparation of standard solution for HPLC analysis

Standard solution	Volume (µl)			
	50 mM Sucrose stock solution	20 mM Raffinose stock solution	200 mM Stachyose stock solution	Deionized water
1	8	2	2	188
2	16	4	4	176
3	24	6	6	164
4	32	8	8	152
5	40	10	10	140

4.3 Calculation of oligosaccharide content

$$\text{Oligosaccharide concentration (mM)} = \frac{\text{(Area of raffinose)}}{\text{(Slope of standard curve)}}$$

Appendix B

Experimental Result

Appendix Table B1 Sugar concentration of lactic acid bacteria in soymilk without sugar addition during fermentation for 48 h

Lactic acid bacteria strains	Sugar concentration (g/l) during fermentation				
	0 h	12 h	24 h	36 h	48 h
L. acidophilus TISTR 1338	5.14	3.21	2.99	2.88	2.80
L. casei subsp. *tolerans* TISTR 1341	5.14	3.74	3.44	3.40	3.27
L. plantarum P49	5.14	1.74	1.68	1.64	1.61

Appendix Table B2 Sugar concentration of *L. plantarum* P49 in soymilk with various sugar addition during fermentation for 48 h

Type of sugar	Sugar concentration (g/l) during fermentation				
	0 h	12 h	24 h	36 h	48 h
Glucose	39.18	24.45	24.34	25.55	24.37
Galactose	22.06	17.27	13.85	13.50	12.90
Sucrose	40.00	37.21	37.89	33.03	32.47

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