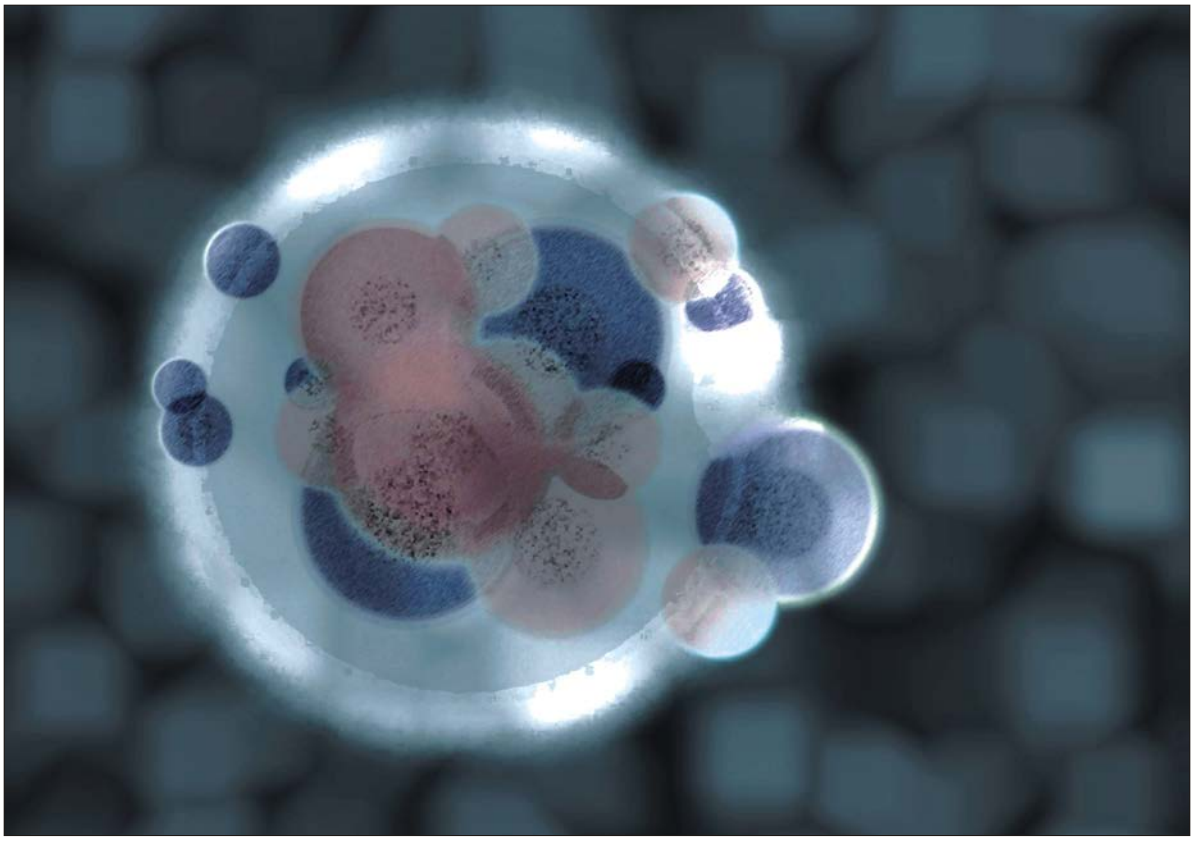

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Stability of Freeze-Dried *Lactobacillus acidophilus* in Banana, Soybean and Pearl Barley Powders

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Abstract: Effect of banana, soybean, pearl barley powders and nonfat dry milk on the viability of freeze-dried *Lactobacillus acidophilus* at 4 and 25°C was studied during 30 days of storage. The survival of freeze-dried *L. acidophilus* at 4°C was greater than that at 25°C. The survival of *L. acidophilus* in banana, pearl barley, soybean powders and nonfat dry milk powder was higher than that in control (0.1% peptone water) at both temperatures indicating that addition of banana, soybean and pearl barley powders improved survival of *L. acidophilus*. The survival of *L. acidophilus* at 25°C in banana, pearl barley, soybean extract and nonfat dry milk was up to 6, 16, 20 and 25 days, respectively, while the organisms survived in samples stored at 4°C. The variation of viability may relate to hygroscopicity of different powders. Similar to nonfat dry milk, soybean powder preserved freeze-dried *L. acidophilus* during storage. The present study showed the potential of banana, soybean and pearl barley powders as cryoprotectants in freeze-dried probiotic preparations.

Key words: *Lactobacillus acidophilus*, banana, soybean and pearl barley, probiotic

INTRODUCTION

Probiotics are live microbial cell preparations that when ingested in certain numbers exert beneficial effect on health (Schrezenmeir and de Vrese, 2001). A concentration of 10 million cells per ml in foods at time of consumption is considered functional. The most commonly used probiotic orgamisms are *Lactobacillus acidophilus* and bifidobacteria. Traditionally, probiotics are supplemented in dairy products such as yogurt and fermented milk. Incorporation of probiotic bacteria in cereal based foods is gaining interests from researchers worldwide. Prebiotic are substances that stimulate the growth and activity of probiotics (Klaenhammer, 2001). The best-known prebiotics are fructooligosaccharides extracted from food sources. The concept of supplementing both probiotics and prebiotics in foods, also known as synbiotics, has attracted enormous attention in recent years to ensure a high level of viable probiotic cells during both product storage and in the body. Synbiotics are used in non-dairy products such as cereal bars (Ouwehand *et al*., 2004) drinks and confectionary (Mattila-Sandholm *et al*., 2002). Plants like chicory, soybean, wheat bran and asparagus contains natural oligosaccharides and have been studied for their prebiotic properties (Klaenhammer, 2001). Extracts of malt, wheat and barley have been reported to improve survival of *L. plantarum*, *L. acidophilus* and *L. reuteri* under acidic conditions (Charalampopoulos *et al*., 2003). The protective effect on the bacteria viability was mainly attributed to carbohydrates present in the cereals. Sugars have been used as cryoprotectants in freeze-drying preservation of bacteria (Carvalho *et al*., 2004). In a tropical country like Thailand, there is a variety of crops available, development of freeze-dried inoculum of probiotic cultures containing prebiotics from cereal crops would reduce the cost of importing these starter cultures from other countries. The present study, we evaluated shelf-life of probiotic inocula containing local fruit, legume and cereal powders (banana, soybean or pearl barley) which potentially contain prebiotic substances at 4 and 25°C.

MATERIALS AND METHODS

Microorganism and growth conditions: This study was conducted in the Department of Food Technology and Nutrition, Mahasarakham University, Thailand in 2005. *L. acidophilus* was obtained from Department of Food Technology and Nutrition, Mahasarakham University's

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culture collection. It was previously isolated from a food product. The strain was revived from frozen glycerol stocks in deMann, Rogosa, Sharpe (MRS) agar (Criterion, Santa Maria, USA) at 42°C for 48 h under aerobic conditions. This was followed by two additional transfers in a similar manner to fully activate the strain prior to use. To prepare *L. acidophilus* cells for survival study, the strain was grown on agar slants and incubated at 42°C for 24 h under aerobic conditions. After incubation, each agar slant was added with 4 mL of peptone (Criterion, Santa Maria, USA) water (0.1%) and mixed with a vortex to obtain approximately 10^9 cfu mL^{-1}.

Preparation of banana, soybean and pearl barley powders: Banana, soybean and pearl barley were purchased from a local store to prepare powders in one batch. Half-ripen bananas were sliced into pieces having a thickness of 0.5 cm and hot-air dried at 60°C for 6 h. The dried banana chips were then powdered in a blender. Soybean and pearl barley were ground with a laboratory hammer mill (Pertern Instruments, Huddinge, Sweden). All powders were screened using 125 micron mesh to eliminate large particles, sterilized in an autoclave at 121°C for 15 min and added with sterile water to obtain the final solid contents of 24%. Reconstituted non-fat dry milk and 0.1% peptone was also included in the study.

Survival study: To study the survival of *L. acidophilus* in freeze-dried powders, *L. acidophilus* cell suspension with a concentration of approximately 10^9 cfu mL^{-1} was added to the banana, soybean pearl barley slurries, peptone water or reconstituted non-fat dry milk to obtain 12% total solids (1:1 w/w) in each treatment except for peptone water (control). One gram of aliquot was frozen in test tubes at -32°C for 2 h and then freeze-dried in a freeze-dryer (Labcono, FreeZone12, Kansas City, USA) at -40°C for 18 h. To obtain accelerated conditions during storage, the test tubes containing dried cells and powders were not sealed but covered with plastic caps to prevent contamination. After freeze-drying, the samples were stored for 0, 3, 6, 9, 12, 16, 20, 25 and 30 days at 4 and 25°C, rehydrated by adding 2 mL of peptone water, serially diluted with peptone water and enumerated by spread plating on MRS agar to determine viability. Plates were incubated at 42°C under aerobic conditions for 48 h. Plates with 25-250 cfu were counted and used to calculate cfu g^{-1}.

Chemical analyses: Banana, soybean and pearl barley powders were extracted by shaking with water (1:4; w/v) for 30 min at 100 rpm and centrifuged at 6,000 x g for 30 min at room temperature. The supernatants (extracts) were used for total soluble sugar, reducing sugar, free amino nitrogen and starch analyses. Total soluble sugars and reducing sugars were determined using phenol sulphuric acid method (Dubois *et al*., 1956) and dinitrosalicylic method (Miller, 1959), respectively. A set of glucose solutions was used to determine a standard curve. Free Amino Nitrogen (FAN) was determined using ninhydrin colorimetric method (Charalampopoulos *et al*., 2003). Starch was determined using polarimetric method (Anonymous, 1999). pH was measured by a pH meter (Mettler, Delta 320, Shanghai, China). Buffering capacity of each extracts was determined by titrating 100 mL of the extract with 1 N HCl. The values were expressed as mmole of HCl required to lower one pH of 1 L extract.

Microscopic analysis: Freeze-dried samples were mounted on aluminum stubs with double sided tape, coated with gold and palladium for 10 min in a sputter coater and viewed with a scanning electron microscope (JEOL, JSM-6464 VL, Tokyo, Japan) operated at 7 kV. Photomicrographs were taken at magnification of 5,000-10,000x in BMP format with a resolution of 1,280x960 pixels.

Statistical analysis: The same frozen stock cultures and equipment were used in all replicates. Data were analyzed with SAS software (SAS Institute, Cary, NC) using PROC ANOVA and GLM. Significant differences between means were determined using Least Significant Difference (LSD) test. Significance was determined by least square means at $p = 0.05$.

RESULTS AND DISCUSSION

pH of the slurries (banana, soybean and pearl barley powders and water), reconstituted nonfat dry milk and peptone water before addition of cell suspension varied from 5.86-7.05 (Table 1). After addition of the banana, soybean and pearl barley slurries and reconstituted nonfat dry milk, pH of the cell suspension significantly increased ($p<0.05$). After addition of *L. acidophilus* cells to banana, soybean, pearl barley slurries and reconstituted nonfat dry milk, the final pH of mixtures was 5.12, 5.81, 5.67 and 6.04, respectively. These pH values are in the optimal pH range for the growth of *L. acidophilus* growth of 5.5-6.0 (Gomes and Malcata, 1999) except for that of banana slurry. Addition of peptone water (control) to the cell suspension was merely diluting the cell density and did not affect pH value of the cell suspension. The low pH and high acidity could result in cellular damages (Doyle *et al*., 1997) and thus low survival of *L. acidophilus* after freeze-drying. Although the pH of soybean slurry and reconstituted nonfat dry milk before addition of the probiotic cells was lower than that of pearl barley slurry, the pH after addition of the probiotic cells was higher than that of pearly barley slurry. This may be

Table 1: pH of banana, soybean and pearl barley slurries, reconstituted nonfat dry milk and peptone water (0.1%)

Samples	Banana slurry	Soybean slurry	Pearl barley slurry	Reconstituted nonfat dry milk	Peptone water
Before addition of *L. acidophilus* cells	5.86[e]	6.71[c]	7.05[a]	6.95[b]	6.34[d]
L. acidophilus cell suspension in peptone water			4.77		
After addition of *L. acidophilus* cells	5.12[d]	5.81[b]	5.67[c]	6.04[a]	4.63[e]

[a, b, c, d, e]: Means in rows with no common superscript letter differ ($p<0.05$)

Table 2: Chemical composition of powders from banana, soybean and pearl barley powders and nonfat dry milk

Constituents	Banana extract	Soybean extract	Pearl barley extract	Reconstituted nonfat dry milk
Total soluble sugars (g/100 g)	1.95±0.39[1]	1.96±0.01	2.81±0.11	3.83±0.22
Reducing sugars (g/100 g)	1.92±0.11	0.83±0.08	2.10±0.02	2.69±0.16
Difference (Total-Reducing Sugars) (g/100 g)	0.03	1.13	0.71	1.14
Free amino nitrogen (mg/100 g)	19.54±0.07	209.90±1.08	31.89±1.29	43.02±0.72
Starch (g/100 g)	7.55±0.09	4.55±0.08	12.86±0.00	ND[3]
Buffering capacity[2] (mmol/pH/L)	17.41	43.27	4.03	37.43

[1]: ±Standard deviation; [2]: Buffering capacity is mmole of 1 N HCl required to change the 1 pH of 1 L extracts; [3]: ND = Not Determined

the effect of buffering capacity of proteins in soybean slurry and reconstitute nonfat dry milk (Table 2).

Selected chemical components of banana, soybean and pearl-barley powders were determined. Non-gelatinized water extracts of the banana, soybean and pearl barley powders were used for the analyses of total soluble sugars, reducing sugars, free amino acid nitrogen, starch and buffering capacity (Table 2). Cereals and legumes have long been used as substrates for lactic acid fermentation in Africa and Asia for the production of foods in different forms such as beverages, baked goods and porridge (Salminen and Wright, 1993). Carbohydrates including β-glucan, oligosaccharides and resistant starch in fruits, legumes and cereals were reported to have prebiotic properties (Charalampopoulos *et al.*, 2002). In this study, soybean and milk powders showed a large difference between total soluble sugars and reducing sugars. The difference could be attributed to the presence of several soluble oligosaccharides. These prebiotic oligosaccharides can be beneficial substrates for growth of *L. acidophilus* when an inoculum containing such substrates is used to start the process of fermentation. In this study, the inoculum system containing soybean had the highest buffering capacity (43.24 mmol/pH), therefore it potentially protects the bacteria from undesirable acidic conditions.

After freeze-drying, *L. acidophilus* numbers dropped by 1-2 log. Control (0.1% peptone) and samples with banana powder showed a low level of protection while soybean and milk exhibited cryo-protection property. The survival of *L. acidophilus* at 25°C in banana, pearl barley, soybean and nonfat milk powders was at least 6, 16, 20 and 25 days, respectively (Fig. 1). Nonfat dry milk is often used as a cryoprotectant. The American Type Culture Collection (ATCC) has been successful in long-term preservation of many different bacteria by using 10% (wt/v) skim milk or 12% (wt/v) sucrose (final concentration) (Gerhardt *et al.*, 1994). Similar to milk, soybean powder contained high FAN, soluble non-

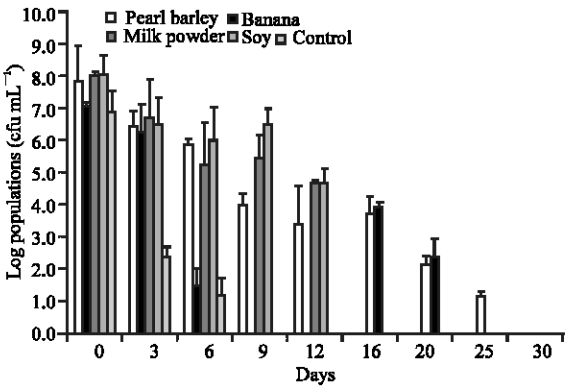

Fig. 1: Survival of *Lactobacillus acidophilus* in freeze-dried pearl barley, banana, soybean, nonfat dry milk powders and control (0.1% peptone) after 30 day storage at 25°C

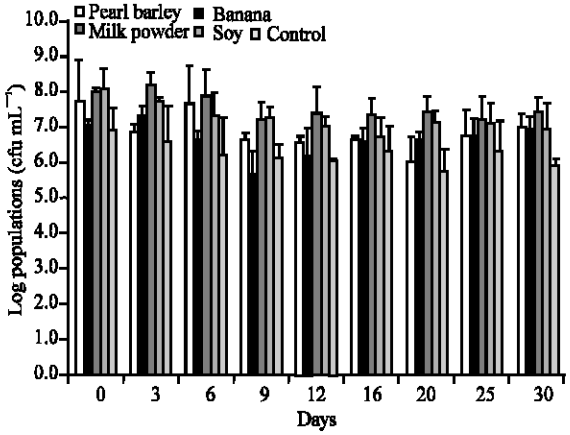

Fig. 2: Survival of *Lactobacillus acidophilus* in freeze-dried pearl barley, banana, soybean, nonfat dry milk powders and control (0.1% peptone) after 30 day storage at 4°C

reducing sugars (difference in total sugars and reducing sugars) and materials responsible for high buffering

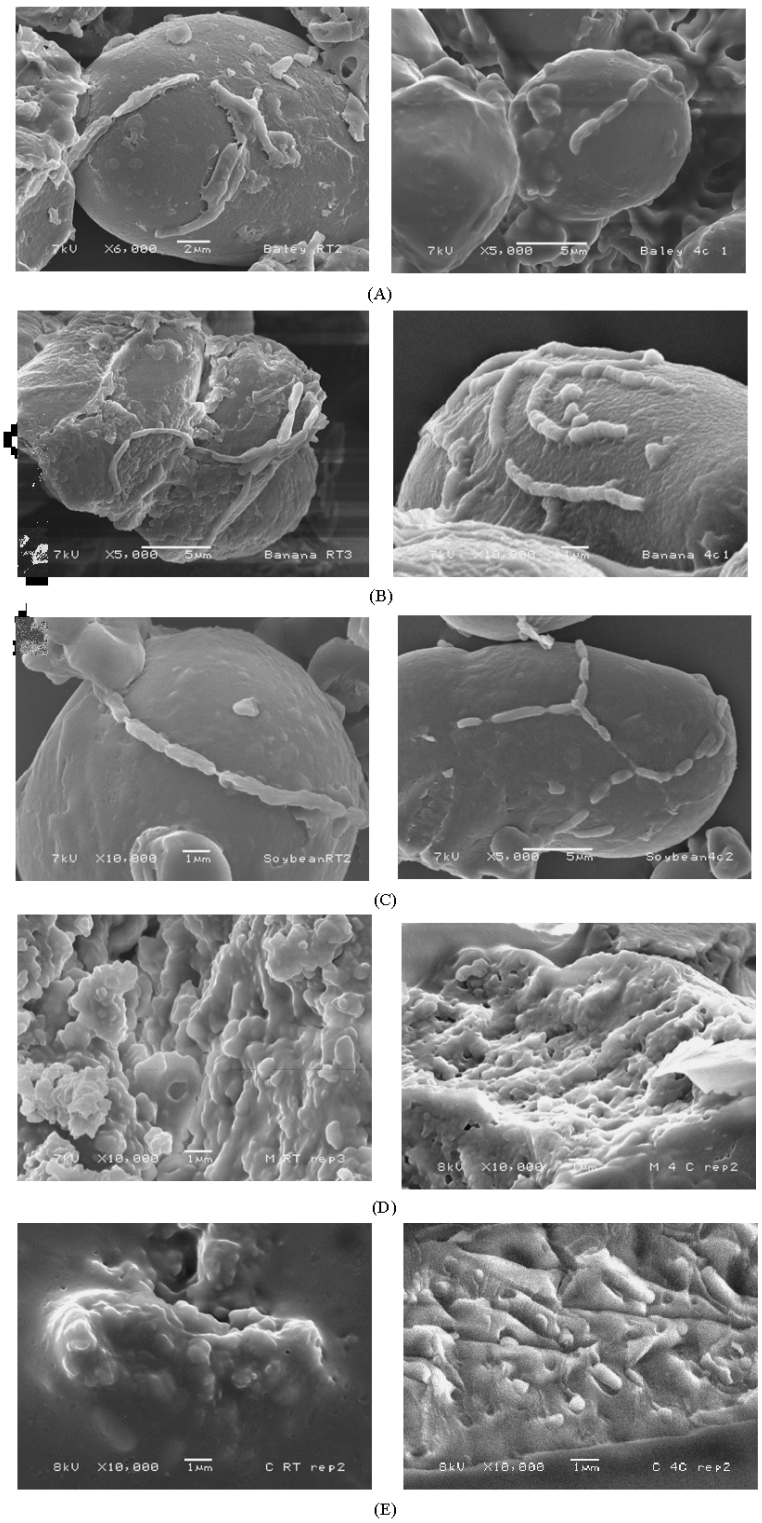

Fig. 3: Photomicrographs of freeze-dried *Lactobacillus acidophilus* with pearl barley (A), banana (B), soybean (C) powders, nonfat dry milk powder (D) and control (E) after 30 day storage at 25°C (left column) and 4°C (right column). Control contained 0.1% peptone

capacity (Table 2) which could help improve survival of *L. acidophilus* during cryopreservation and storage. *L. acidophilus* could not be recovered in control with 0.1% peptone after 9 days at 25°C. This may be due to rehydration of peptone during storage. Chemical composition of banana, soybean and pearl barley powders could affect their hygroscopicity. Powders with high hygroscopicity attracted atmospheric moisture resulting in low survival of *L. acidophilus* cells in freeze-dried samples.

Survival of freeze-dried *L. acidophilus* in powders at 4°C was greater than that at 25°C (Fig. 1, 2). At 25°C, *L. acidophilus* numbers reduced with time while these numbers slightly changed at 4°C. Low temperature affects growth kinetics and preserves cells (Montville, 2001). Vials for storage of freeze-dried cultures should be sealed or double sealed to prevent rehydration which lowers viability of the long-term preserved cultures (Gerhardt *et al*., 1994). In this study, we intentionally did not seal the test tubes and this obviously affected viability of the bacteria. We therefore observed different degrees of protection during storage, especially at 25°C, provided by different powders.

Figure 3 show cells of *L. acidophilus* in different samples. *L. acidophilus*, often in chains, attached to non-gelatinized starch granules in samples with pearl barley, banana and soybean and may be covered with a thin layer of water soluble materials. These materials may play an important role in protection the bacterial cells from cryopreservation process and storage. Nonfat dry milk powder containing mostly water soluble materials (Fennema, 1996) that covered cells of *L. acidophilus* (Fig. 3D) thus providing a more efficient protection during cryopreservation. Peptone water is used as a nitrogen source and contains amino acids, FAN (Anonymous, 1998) and also water soluble thus resulting in the similar embedded cells as seen in the samples containing dry milk powder. As seen under the SEM, *L. acidophilus* cells attached on soybean powder in a similar manner to those on pearl and banana powders but the later did survived at room temperature very long. It is thus conceivable that attaching orientation as revealed in the photomicrographs was not relevant to survival of *L. acidophilus*.

CONCLUSION

In this experiment, we reported the ability of banana, soybean and pearl barley powders to preserve *L. acidophilus* during cryopreservation and storage. Individual components may be attributable to the protective effects exerted by banana, soybean and pearl barley powders. Soybean powder, similar to nonfat dry milk powder, could preserve freeze-dried form of the bacteria during storage at 4 and 25°C. Because soybean contains proteins and oligosaccharides, it can also be used as a nutrient source for fermentation. The inoculum system containing soybean can potentially be used as a probiotic freeze-dried starter culture.

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