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Original Research Article

Protective effect of soybean powder as a cryoprotectant and encapsulating material on the survival of *Lactobacillus fermentum* SK54 during freeze drying, storage and exposure to simulated gastrointestinal conditions

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ABSTRACT

The aim of the current study was to focus on the impact of soybean powder as a cryoprotectant and microencapsulation on the survival of *Lactobacillus fermentum* SK54 from freeze-drying, storing and exposing to simulated gastrointestinal tract conditions. Seven cryoprotectants e.g., soybean powder, skim milk, galactooligosaccharide (GOS), inulin, sucrose, lactose and glucose were tested for their ability to protect *L. fermentum* SK54 during freeze-drying. Results revealed soybean powder, skim milk and GOS could highly protect *L. fermentum* SK54 with the survival rates 87.06-88.52% as the cryoprotectants. The optimal concentrations of soybean powder, skim milk and GOS were demonstrated to be 13% (w/v). The freeze-dried cells with soybean powder had the best survival rate from storing at 4 °C and 25 °C for 5 months. Microencapsulation of *L. fermentum* SK54 cells by the extrusion technique was adopted for this study. The encapsulated beads with soybean powder significantly improved the survival of the bacteria in the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). The survival rate of encapsulated cells with soybean powder stored at 4 °C and 25 °C for 5 months was markedly improved when compared with encapsulated cells without soybean powder. Soybean powder and soybean powder incorporated in encapsulated beads were the suitable cryoprotectants for *L. fermentum* SK54 in freeze-drying and storage processes. The results in this study indicate that soybean powder is the most suitable cryoprotectants when used alone and incorporated into encapsulated beads prepared by extrusion technique had the potential to protect probiotic cells during the manufacturing, storing processes and exposing to SGF and SIF.

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INTRODUCTION

Some selected strains of *Lactoacillus* spp. are widely used as probiotics in the food, dairy and pharmaceutical industries. For industrial applications, probiotics may be preserved and distributed in the form of liquid suspension, spray-dried, frozen or freeze-dried (lyophilization) forms. The preservation and distribution techniques are required to guarantee stable cultures in terms of viability, cell metabolism and functional activity (De Giulio et al., 2005). Freeze-drying is one of the most commonly used techniques for preservation and the long-term storage of microorganisms. However, during the freeze-drying process, bacterial cells are exposed to additional stressful conditions such as water crystallization, low temperatures, low water activities and high osmotic pressure that can each produce structural and physical damage to probiotic cells such as physical changes to membrane lipids and denaturation of proteins (Carvalho et al., 2004; Rault et al., 2010). To prevent or reduce these adverse effects, many research studies have used cryoprotectants such as polyols, polysaccharides, disaccharides, amino acids and proteins to maintain the viability of bacterial cells during freeze-drying, subsequent storage and to protect them as they move through the acidic gastric environment (Hubálek 2003; Li et al., 2011). However, selection of a most suitable cryoprotective agent depends on many factors such as the species and strain of the microorganisms to be protected, the concentrations of cryoprotectants themselves, the composition of the freeze-drying medium and storage conditions (Carvalho et al., 2004; Otero et al., 2007). To improve the survival of probiotic against adverse environmental conditions, the microencapsulation is now one of the most commonly used techniques (Chan et al., 2009; Zou et al., 2011). Microencapsulation is a technique in which cells are entrapped within a matrix during the formation of the hydrocolloid spheres. The commonly used natural polymer-based materials for microencapsulation are pectin, alginate, chitosan, cellulose, xanthan gums and **κ**-carageenan.

The aim of this study was to evaluate the survival of *L. fermentum* SK54 using different cryoprotectants, the effect of selected cryoprotectants incorporated into pectin beads during freeze-drying and subsequent storage in different conditions. In addition, the viability of free cells and cells encapsulated in pectin beads under simulated conditions of the gastrointestinal tract have been investigated.

MATERIALS AND METHODS

Chemicals and reagents

Pectin (low-methoxyl) was a generous gift from Brenntag (Thailand) Co. Ltd. Other chemicals were obtained from different sources as indicated in parenthesis i.e., deMann Rogosa and Sharpe (MRS) medium (Difco, USA), skim milk (Difco, USA), soybean powder (Doi Kham Co. Ltd, Thailand), sucrose (Mitr Phol Sugar Co. Ltd, Thailand), lactose (Ajax Finechem, Australia), glucose (Ajax Finechem, Australia), inulin (Beneo-orafit, Belgium) and galactooligosaccharide (GOS) (Friesland Foods Domo, The Netherlands). system (Millipore, Bedford, USA). All other chemicals and solvents in this study were of analytical grade.

Bacterial strain and culturing conditions

Lactobacillus fermentum SK54 with high probiotic potential was isolated from healthy baby feces (Kaewiad et al., 2014). It was re-suspended in MRS broth and incubated at 37 °C for 24 h under anaerobic condition using anaerobic jars with the GasPak® system. The culture broth was centrifugated at 2,000 g for 10 min at 4 °C and the cell pellet was collected for further studies.

Freeze drying probiotic with different cryoprotectants

Soybean powder, skim milk, GOS, inulin, sucrose, lactose and glucose were used as cryoprotectants. Each of them was prepared as a 10% (w/v) solution. The bacterial cell pellet was suspended in each solution to obtain a cell concentration of about 10^{10} CFU/mL before freeze-drying. For the freeze-drying condition, this strain was freezed at -40 ˚C for 2 h followed by 18 h of primary drying at -40 ˚C, 8 h of secondary drying at -10 ˚C and the final step to 25 ˚C using a freeze-dryer (model FD-300 Airvac Engineering Pty Ltd., Dandenong, Australia). The viable cells before and after freeze-drying were enumerated by the drop plating technique. The cryoprotectants with high activity were then checked at other concentrations of 3, 8, 13 and 18% (w/v) solutions to determine their optimum effectiveness.

The bacterial cells suspension without cryoprotectant was freeze dried and used as a control. The survival rate was expressed as a percentage of the relative viability by the equation:

$$\text{Survival rate (\%)} = (Y / Y_0) \times 100.$$

Where Y is the number of viable cells after freeze-drying (Log CFU/mL), and Y_0 is the number of viable cells before freeze-drying (Log CFU/mL).

Long term storage of freeze-dried cells

The cryoprotectants at their optimum concentrations were then selected for studying survival stability. The freeze-dried samples were kept in gelatin capsules at 4 °C and 25 °C and the cell viability was determined regularly during a period of 5 months.

Cell microencapsulation procedures

The extrusion technique was used for preparation of the encapsulated cells. Pectin was used as the supporting matrices. From previous experiments, 13% (w/v) of soybean powder, skim milk and GOS had shown the best cryoprotectant activity. They were selected for testing in this experiment. *L. fermentum* SK54 (about 10^{10} CFU/mL) and each of the cryoprotectants were incorporated into 4% (w/v) pectin solution. Skim milk was not used in this study because it formed lumps when incorporated into those polymer solutions. To prepare the encapsulated cells, the above mixture was extruded drop wise through a 0.80 mm diameter needle connected to a 10 mL sterile syringe into 0.5 M sterile calcium chloride solution. The encapsulated cells in the form of beads were obtained and agitated continuously for 30 min. After hardening, the beads were washed twice with sterile distilled water, recovered by filtration through Whatman filter paper No. 4 and stored at 4 °C in 0.1% (w/v) peptone water before freeze-drying. For the freeze-drying process, fresh beads were removed from the peptone water and subjected to freeze-drying process as previously described.

Size analysis and morphology of beads

The diameters of 20 freeze-dried beads were measured using an optical microscope at a magnification of 10X. For the morphology study, both intact and cross-sections of freeze-dried beads were coated with gold to increase the conductivity and their mechanical stability under vacuum and examined with a scanning electron microscope (JSM-5800LV, JEOL, Japan). The outer surface and internal structure of the freeze-dried beads were observed.

Survival assay and enumeration of microencapsulated cells

The beads (0.1 g) were suspended in 20 mL of sodium phosphate buffer (NaH_2PO_4, after adjusting the pH to 7.4 with 1 M NaOH). The cells were released from the beads by stirring the suspension with magnetic stirrer at 400 rpm for 5 min. Serial dilutions were prepared and viable cells were counted. The encapsulation efficiency of the formulation for the bacterial cells was calculated as:

$$\text{Encapsulation efficiency (\%)} = (N / N_0) \times 100.$$

Where N is the number of cells released from the beads, and N_0 is the number of free cells added to the polymer solutions during encapsulation process.

Survival of microencapsulated cells exposed to simulated gastric fluid and simulated intestinal fluid

Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as described by Gbassi et al., (2009) with some modifications. For the study of survival in SGF and SIF, 0.1 g of beads or 1 mL of free cell suspension of *L. fermentum* SK54 were mixed in 10 mL of SGF and incubated at 37 °C with constant shaking at 50 rpm. After 180 min in SGF, the samples were removed from the SGF solution into 10 mL SIF and continuously incubated at 37°C. The survival of bacterial cells in SGF and SIF was measured at 30, 60, 120, 180 min (SGF), 210, 240, 300, 360 and 420 min (SIF) of incubation time.

Long term storage of freeze dried microencapsulated cells

The freeze-dried beads were kept in gelatin capsules and stored at 4 °C and 25 °C for 5 months. At monthly intervals, the viability of *L. fermentum* SK54 cells was enumerated.

Statistical analysis

All experiments were analyzed in triplicate. The results were expressed as means ± standard errors of the means (SD). Comparison of mean values was performed by ANOVA for multiple comparisons with posthoc Tukey test using PSPP analysis open source software.

RESULTS

Effect of cryoprotectants on the survival of the cells during freeze drying and storing

L. fermentum SK54 cells were freeze-dried using different cryoprotectants to test for their cryoprotectant activities during processing and storag. Soybean powder, skim milk and GOS showed similar preservation activity and significantly better than inulin, sucrose, lactose and glucose during freeze-drying. Thirteen percent (w/v) soybean powder, skim milk and GOS gave the highest survival rate of *L. fermentum* SK54 with 89.69%, 89.24% and 89.17%, respectively. The bacterial cells without cryoprotectants gave only 75.78% survival rate. The freeze-dried cells in these cryoprotectants were filled into gelatin capsule and kept at 4 °C and 25 °C for 5 months.

In Figure 1(A), after storage at 4 °C for 5 months, the survival rate of *L. fermentum* SK54 in soybean powder, skim milk and GOS was 92.99%, 92.33% and 86.35%, respectively. Figure 1(B), the survival rate of cells by all cryoprotectants decreased after 5 months at 25 °C. The survival rate of *L. fermentum* SK54 in soybean powder, skim milk and GOS was 81.40%, 79.94% and 73.61%, respectively. The survival of freeze-dried cells without cryoprotectants could not be detected after 3 and 2 months of storage at 4 °C and 25 °C, respectively.

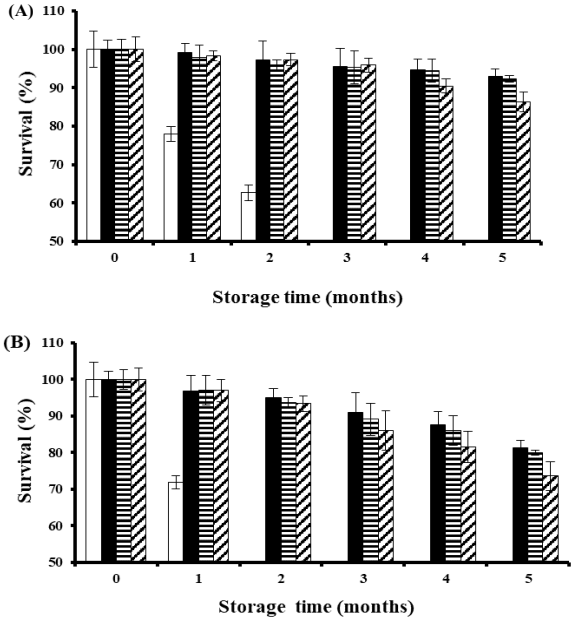

Figure 1. Survival rate of freeze-dried *L. fermentum* SK54 in the presence of different cryoprotectants with their optimal concentrations: without cryoprotectant (control group, white column), 13% (w/v) soybean powder (black column), 13% (w/v) skim milk (horizontal striped column), 13% galactooligosaccharide (diagonal striped column) at 4 °C (A) and 25 °C (B) for 5 months. Error bars represent SD.

The effect of cryoprotectants and microencapsulation on the survival of cells during freeze drying process

According to the data shown in Table 1, the entrapment efficiency of *L. fermentum* SK54 into pectin beads with and without cryoprotectants (soybean powder and GOS) before freeze drying varied between 89.24-92.76%. The bead containing soybean powder gave the survival rate of the cell significantly higher than the beads containing GOS. The pectin beads containing soybean powder gave the survival rate 89.63-91.71%. Pectin beads without cryoprotectant gave a low survival rate 69.01-70.28% (Table. 1).

Morphology of the encapsulated beads

The fresh beads of *L. fermentum* SK54 using pectin as the encapsulation polymer were spherical in shape. The diameters of freeze-dried pectin beads with cryoprotectants were 1.40-1.64 mm. The beads without cryoprotectant were 1.17-1.25 mm in diameter

(Table. 1). The morphologies of the beads containing soybean powder under SEM are shown in Figure 2. The surface of the dried beads was wrinkled due to the loss of water. Cross sections of the beads showed an internal porous structure. The close up of the outer and inner surface of the beads revealed the presence of densely packed encapsulated bacterial cells.

Survival of microencapsulated cells exposed to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

The effect of SGF and SIF on the viability of *L. fermentum* SK54 in pectin beads is shown in Figure 3. After 180 min in SGF, the beads containing soybean powder and GOS showed the survival rate 88.67-89.67%. After 240 min in SIF, the beads containing soybean powder and GOS showed the survival rate 84.92% and 65.43%, respectively. The pectin beads without cryoprotectants, the survival rate decreased to 65.57% after 180 min in SGF and none survived after 60 min in the SIF.

Effect of microencapsulation and cryoprotectants on the survival of the cells during storage

The survival rate of *L. fermentum* SK54 in any forms of encapsulated beads stored at 4 °C gradually decreased every month as shown in Figure 4(A). After 5 months, the pectin beads containing soybean powder gave the survival rate 82.89%. In the case of the beads without cryoprotectants, no viable cells could be detected after 3 months of storage. At 25 °C storage temperature as shown in Figure 4(B), the pectin beads containing soybean powder gave the survival rate 68.23%. No viable cells from the beads without cryoprotectant could be detected after 2 months of storage. Overall, 13% (w/v) of soybean powder was added into the polymer solution to improve the survival rate of the encapsulated cells during storage at all temperature. At 4 °C storage temperature showed the higher survival rate of *L. fermentum* SK54 than it kept at 25 °C storage temperature.

DISCUSSION

Freeze-drying has long been used for the preservation and storage of probiotic cells for food and pharmaceutical applications. Maintaining their survival and functional characteristics during freeze-drying and storage are the crucially importance to improve the production processes. However, the freeze-drying technique brings about adverse effects such as damage to cell membranes, protein denaturation and water crystallization all leading to a decrease of the viability of the probiotic cells (Ampatzoglou et al., 2010). To reduce such undesirable side effects, different cryoprotectants have been used to increase the survival of *L. fermentum* SK54 during the freeze-drying process and storage conditions. Soybean powder has provided the highest cell viabilities for *L. fermentum* SK54, followed by skim milk and GOS after both freeze-drying and storage.

According to Hubálek (2003), cryoprotectants can be categorized into three groups based on their ability to pass through the cell wall and membrane, which affects their protective mechanism. The first group includes permeable compounds that can enter both the cell wall and the cytoplasmic membrane, such as dimethyl sulfoxide and glycerol. The second group consists of semi-permeable compounds that can penetrate the cell wall but not the membrane, including mono- and disaccharides, amino acids, oligosaccharides, and low molecular weight polymers. The third group comprises non-permeable compounds that cannot penetrate the cell wall or interact directly with the membrane, such as high molecular weight polymers, polyethylene glycol-6000, proteins, and polysaccharides.

Soybean powder and skim milk are complex substances and also can provide high protective activity to *L. fermentum* SK54. The components of soybean powder are proteins, oils, soluble carbohydrates (e.g. sucrose, oligosaccharides), insoluble carbohydrates (fiber) and minerals. The larger molecules present in skim milk and soybean powder such as protein and insoluble carbohydrates could not penetrate the cell wall and are adsorbed on the outer cell surface forming a viscous layer. They protect the cells by causing a partial efflux of water from the cell, inhibiting the growth of ice crystals, and maintaining the amorphous structure of ice in the close proximity to the cell (Hubálek, 2003). Sugars in soybean and skim milk can penetrate the cell wall but not cytoplasmic membrane and they enhance the protective effects during freeze-drying and storage by the proposed mechanisms mentioned above. Amino acids in skim milk provide good protection on the bacteria during freeze-drying as a result of the reaction between the amino groups of the cryoprotectant compound and the carboxyl groups of the proteins within the microorganism, hence stabilizing the protein structure (Abadias et al., 2001). Minerals, especially calcium in soybean and skim milk can increase the survival rate after freezing or freeze-drying (King and Su 1994). In an overview, each component of soybean powder or skim milk has a different cryoprotective activity and exerts a concerted preservative effect lead to increase survival of the cells. GOS are mostly sugar-like compounds (oligosaccharides and polyhydroxylated compounds) comprised of between two and ten monomers. The prebiotic properties of GOS have been elucidated and include resistance to digestion by pancreatic and brush border enzymes (Saulnier et al., 2009). Polyhydroxylated compounds have been used as preservatives for LAB during freeze-drying or vacuum drying (Sendra et al., 2008; Guergoletto et al., 2010).

Table 1. Encapsulation efficiency, freeze-dried beads size and survival rate of encapsulated *L. fermentum* SK54 after freeze drying

Samples*	Before freeze drying (Log CFU/g)	After freeze drying (Log CFU/g)	% Survival	Beads size (mm)
SK54+P	9.99 ± 0.07	7.00 ± 0.10	70.28 ± 2.27	1.25 ± 0.27
SK54+P+SB	10.14 ± 0.18	9.30 ± 0.20	91.71 ± 2.20	1.71 ± 0.27
SK54+P+GOS	9.94 ± 0.03	8.35 ± 0.28	82.19 ± 3.21	1.51 ± 0.32

* SK54: *L. fermentum* SK54, P: pectin, SB: soybean powder and GOS: galactooligosaccharide

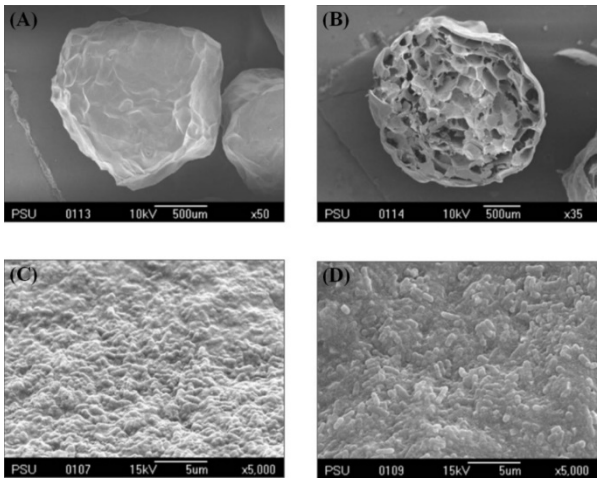

Figure 2. Morphology of pectin beads containing *L. fermentum* SK54 and soybean powder: outer structure (A); inner structure (B) and appearance of the encapsulated bacterial cells on the outer (C) and inner (D) surface of the beads.

In order to exert beneficial health effects, ingested *Lactobacillus* spp. must survive during their transit through highly acidic conditions of the stomach and the enzymes and bile salts in the duodenum in order to reach their target sites in the small intestine and colon in sufficiently large quantities to facilitate colonisation (Cui et al., 2000). Microencapsulation is one of the techniques used to protect probiotic cells from these harsh environments (Kailasapathy, 2006; Burgain et al., 2011). The tolerance of the free cells and encapsulated beads to the simulated conditions of the stomach and intestine was determined. In the encapsulated beads, the survival rate in SGF was better than that of free cells because of gelation of the pectin to form specific and strong interactions between the gelling ions and the blocks of guluronic and galacturonic acid residues, respectively (Braccini and Pérez 2001). The addition of soybean powder to the pectin matrix did better protection of encapsulated cells than GOS beads in simulated gastrointestinal tract conditions (P>0.05). These results resemble those of an earlier study by, Iyer and Kailasapathy (2005) who showed that when the microencapsulation of a probiotic using alginate with Hi-Maize starch was prepared by an extrusion technique they provided greater protection against gastric fluid than using alginate beads. Chan et al. (2011) revealed that filler (fiber or protein) could improve sphericity, flowability and mechanical strength and reduce porosity of the calcium alginate beads. Soybean powder play a role as a protective barrier of the cells against SGF and they also have a buffering capacity, therefore they potentially protect the cells from adverse acidic conditions (Trachoo et al., 2008).

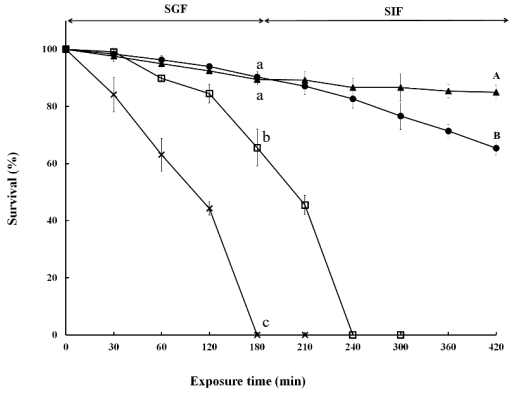

Figure 3. Survival rate of *L. fermentum* SK54 in pectin beads exposed to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF): Free cell (x), pectin bead without cryoprotectant (□), pectin bead with soybean powder (▲) and pectin bead with GOS (●). Different lowercase and uppercase letters indicate significant differences (P>0.05) at the same time.

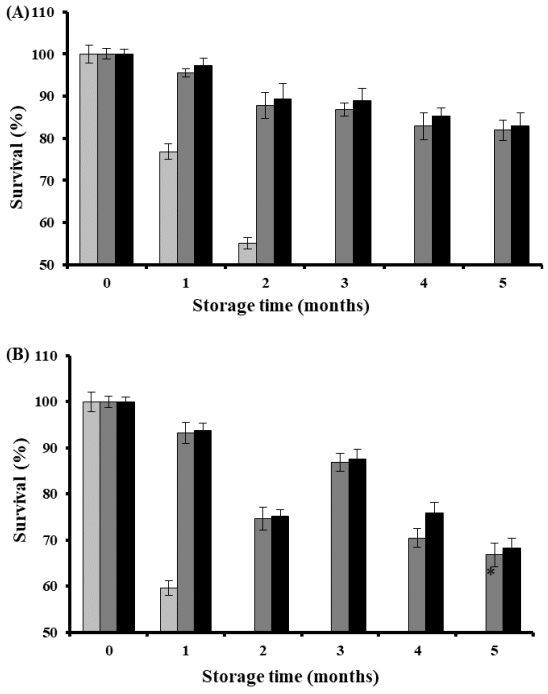

Figure 4. Survival rate of encapsulated *L. fermentum* SK54 in pectin beads containing galactooligosaccharide (GOS) or soybean powder: pectin + *L. fermentum* SK54 (light grey column), pectin + soybean powder (grey column), pectin + GOS (black column) after storage at 4 °C (A) and 25 °C (B) for 5 months. Error bars represent SD.

CONCLUSIONS

In conclusion, among the seven tested cryoprotectants, soybean powder, skim milk and GOS all produced a more effective protection for *L. fermentum* SK54 compared with lactose and glucose during the freeze-drying and storing. Pectin beads containing soybean powder enhanced the cell viability of *L. fermentum* SK54 during the freeze-drying process and improved the cells survival in SGF and SIF. The effect of soybean powder as cryoprotectant and encapsulating material for *L. fermentum* SK54 offers an effective means of delivery of viable cells to the intestine and maintains their survival during freeze-drying process, storage and the adverse gastrointestinal conditions for using in pharmaceutical and food products.

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REFERENCES

Abadias, M., Teixido, N., Usall, J., Benabarre, A. and Vinas, I. 2001. Viability, efficacy and storage of freeze dried biocontrol agent *Candida sake* using different protective and rehydration media. Journal of Food Protection, 64, 856–861.

Ampatzoglou, A., Schurr, B., Deepika, G., Baipong, S. and Charalampopoulos, D. 2010. Influence of fermentation on the acid tolerance and freeze drying survival of *Lactobacillus rhamnosus* GG. Biochemical Engineering Journal, 53, 65–70.

Braccini, I. and Pérez, S. 2001. Molecular basis of Ca^{2+} induced gelation in alginates and pectins: the egg-box model revisited. Biomacromolecules, 2, 1089–1096.

Burgain, J., Gaiani, C., Linder, M. and Scher, J. 2011. Encapsulation of probiotic living cells: from laboratory scale to industrial applications. Journal of Food Engineering, 104, 467–483.

Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X. and Gibbs, P. 2004. Relevant for the preparation of freeze dried lactic acid bacteria. International Dairy Journal, 14, 835–847.

Chan, E.S., Lee, B.B., Pogaku, R. and Poncelet, D. 2009. Prediction models for shape and size of ca-alginate macrobeads produced through extrusion-dripping method. Journal of Colloid and Interface Science, 388, 63–72.

Chan, E.S., Wong, S.L., Lee, P.P., Lee, J.S., Ti, T.B., Zhang, Z., Poncelet, D., Ravindra, P., Phan, S.H. and Yim, Z.H. 2011. Effects of starch filler on the physical properties of lyophilized calcium-alginate beads and the viability of encapsulated cells. Carbohydrate Polymers, 83(1), 225-232.

Cui, J.H., Goh, J.S., Kim, P.H., Choi, S.H. and Lee, B.J. 2000. Survival and stability of *Bifidobacteria* loaded in alginate poly-*l*-lysine microparticles. International Journal of Pharmaceutics, 210, 51–59.

De Giulio, B., Orlando, P., Barba, G., Coppola, R., Rosa, M.D., Sada, A., Prisco, P.P.D. and Nazzaro, F. 2005. Use of alginate and cryo-protective sugars to improve the viability of lactic acid bacteria after freezing and freeze drying. World Journal of Microbiology and Biotechnology, 21, 739–746.

Gbassi, G.K., Vandamme, T., Ennahar, S. and Marchioni, E. 2009. Microencapsulation of *Lactobacillus plantarum* spp. in an alginate matrix coated with whey proteins. International Journal of Food Microbiology, 129, 103–105.

Guergoletto, K. B., Magnani, M., Martin, J. S., Andrade, C. G. T. de J. and Garcia, S. 2010. Survival of *Lactobacillus casei* (LC-1) adhered to prebiotic vegetal fibers. Innovative Food Science and Emerging Technologies, 11(2), 415–421.

Hubálek, Z. 2003. Protectants used in the cryopreservation of microorganisms. Cryobiology, 46, 205–229.

Iyer, C. and Kailasapathy, K. 2005. Effect of co-encapsulation of probiotics with prebiotics on increasing the viability of encapsulated bacteria in simulated gastrointestinal conditions and in yoghurt. Journal of Food Science, 70, 18–23.

Kaewiad, K., Kaewnopparat, S. and Kaewnopparat, N. 2014. *In vitro* comparison of probiotic properties of *Lactobacillus fermentum* SK54 Isolated from new born baby with *Lactobacillus rhamnosus* GG ATCC 53103. Advanced Materials Research, 1060, 215–218.

Kailasapathy, K. 2006. Survival of free and encapsulated probiotic bacteria and their effect on the sensory properties of yoghurt. LWT - Food Science and Technology, 39, 1221–1227.

King, V.A.E. and Su, J.T. 1994. Dehydration of *Lactobacillus acidophilus*. Process Biochemistry, 28, 47–52.

Li, B., Tian, F., Liu, X., Zhao, J., Zhan, H. and Chen, W. 2011. Effects of cryoprotectants on viability of *Lactobacillus reuteri* CICC 6226. Applied Microbiology and Biotechnology, 92, 609–616.

Otero, M.C., Espeche, M.C. and Nader-Macías, M.E. 2007. Optimization of the freeze-drying media and survival throughout storage of freeze dried *Lactobacillus gasseri* and *Lactobacillus delbrueckii* subsp. *delblueckii* for veterinarian probiotic applications. Process Biochemistry, 42, 1406–1411.

Rault, A., Bouix, M. and Béal, C. 2010. Cryotolerance of *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 is influenced by the physiological state during fermentation. International Dairy Journal, 20, 792–799.

Saulnier, M.D., Spinler, K.J., Gibson, R.G. and Versalovic, J. 2009. Mechanisms of probiosis and prebiosis: considerations for enhanced functional foods. Current Opinion in Biotechnology, 20, 135–141.

Sendra, E., Fayos, P., Lario, Y., Fernandez-Lopez, J., Sayas-Barbera, E. and Perez-Alvarez, A. 2008. Incorporation of citrus fibers in fermented milk containing probiotic bacteria. Food Microbiology, 25, 13–21.

Trachoo, N., Wechakama, P., Moongngarm, A. and Suttajit, M. 2008. Stability of freeze dried *Lactobacillus acidophilus* in banana, soybean powder and pearl barley powders. International Journal of Biological Sciences, 8, 119–124.

Zou, Q., Zhao, J., Liu, X., Tian, F., Zhang, H.P., Zhang, H. and Chen, W. 2011. Microencapsulation of *Bifidobacterium bifidum* F-35 in reinforced alginate microspheres prepared by emulsification/internal gelation. International Journal of Food Science and Technology, 46, 1672–1678.