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Impact of encapsulation techniques on the viability of *Bifidobacterium longum* and *Streptococcus thermophilus*

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Abstract The effect of encapsulation techniques with extrusion and emulsion on the survival of probiotic bacteria *Bifidobacterium longum* TISTR 2195 and *Streptococcus thermophilus* TISTR 2289 was investigated. Sodium alginate and carrageenan were used as encapsulating agents and the encapsulated beads were coated with an outer layer of skim milk. The viability of the encapsulated probiotic cells was determined under a simulated gastric condition at pH 2.0 and a freeze-drying condition. Results showed that the cell viability of the encapsulated *S. thermophilus* and *B. longum* with sodium alginate–skim milk using extrusion technique was higher than that of cells encapsulated with carrageenan-skim milk using emulsion technique. The survival rates of alginate–skim milk encapsulated *S. thermophilus* in acidic and freeze-drying conditions were 78.65% and 82.97%, respectively. The carrageenan–skim milk encapsulated probiotic exhibited the lowest proportion of viable cells. These findings indicated that the extrusion encapsulation of probiotics with sodium alginate–skim milk is an effective technique to improve the survival of probiotic bacteria under simulated gastric and freeze-drying conditions.

Keywords: *Bifidobacterium longum*, Encapsulation, Probiotic, Survival rate, *Streptococcus thermophilus*

Introduction

Probiotic bacteria are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO, 2001). Over the past two decades, the many health benefits exhibited by probiotics have been reported for example, the lowering of serum cholesterol, enhancement of immune function, improvement in lactose intolerance,

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reduction of the risk of diarrhea, treatment of many intestinal disorders such as inflammatory bowel diseases and many allergic responses, and so on (Marteau and Boutron-Ruault, 2002; Shah *et al*., 2016). Currently, probiotic bacteria have been added to many food products—such as dairy products (yogurt, milk) and non-dairy products (juices, mayonnaise sauce) to exert healthy impacts on consumers. Probiotic bacteria should be capable of surviving in the intestinal digestive tract at a minimum level of 6 log CFU/ml (Fritzen-Freire *et al*., 2012).

Encapsulation, a process to immobilize cells within supporting material, is being adopted to protect probiotic cells from harsh conditions during food processing, storage and during their passage through the digestive tract (Chuprom, 2010). Extrusion and emulsion methods are widely being used for the microencapsulation of probiotics. Extrusion is a physical method using a simple and gentle operation to entrap cells. Probiotic cells are added to hydrocolloidal supporting material and extruded through a syringe. The cell suspension is dripped into calcium chloride solution to form gel beads. This method avoids the use of corrosive solvents, resulting in high cell viability and lower cell damage (Krasaekoopt *et al*., 2003; Burgain *et al*., 2011; Martín *et al*., 2015). Emulsion is a chemical method involving a discontinuous phase (cell–polymer suspension) and a continuous phase (vegetable oil). Cells in hydrocolloid suspension are added to vegetable oil and homogenized to form a water in oil emulsion. Calcium chloride (a solidifying agent) is added to the emulsion to generate gel beads, the size of which varies depending on the agitation speed (Krasaekoopt *et al*., 2003; Burgain *et al*., 2011; Martín *et al*., 2015).

Previous studies have described various supporting materials—such as carrageenan (Ding and Shah, 2009; Shi *et al*., 2013), xanthan gum (Ding and Shah, 2009), alginate (Ding and Shah, 2009; Pan *et al*., 2013), chitosan–alginate (Gandomi *et al*., 2016) and alginate incorporated with resistant starch (Fahimdanesh *et al*., 2012; Bigdelian and Razavi, 2014) that can improve probiotic cell protection during exposure to adverse and storage conditions. Although some research has investigated the effect of supporting materials on the survivability of probiotic cells, few studies have compared the efficacy of encapsulation techniques on cell viability. The objective of this study was to compare the effect of encapsulation techniques (extrusion, emulsion) and supporting materials on the survival of probiotic *B. longum* TISTR 2195 and *S. thermophilus* TISTR 2289 in both simulated gastric conditions and the freeze-drying process.

Materials and methods

Materials

Probiotic bacteria *B. longum* TISTR 2195 and *S. thermophilus* TISTR 2289 were purchased from the Biodiversity Research Center, Thailand Institute of Scientific and Technological Research. The used encapsulating agents were commercial grade carrageenan and sodium alginate. Skim milk powder was obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India.

Preparation of inocula

B. longum TISTR 2195 and *S. thermophilus* TISTR 2289 were cultured in De Man, Rogosa, Sharpe (MRS) broth (HiMedia Laboratories Pvt. Ltd, Mumbai, India) containing 0.05% (w/v) of L-Cysteine hydrochloride, and MRS broth, respectively. Both strains were grown anaerobically at 37℃ for 48 h. Cell cultures were centrifuged at 8,500 rpm for 20 min at 4℃. The cell pellet was washed twice with 0.85% (w/v) sterilized saline solution. The washed cells were resuspended in 10 ml sterilized saline solution and counted cell numbers through plating on MRS agar incubated at 37℃ for 48 h.

Probiotic encapsulation by extrusion technique

Probiotics *B. longum* TISTR 2195 and *S. thermophilus* TISTR 2289 were encapsulated by extrusion technique modified from Chandramoulia *et al*. (2004). The bacterial culture (approximately 7–8 log CFU/ml) was mixed in either 2% (w/v) sodium alginate or 2% (w/v) carrageenan. The suspensions were dropped using a sterile disposable syringe with a 22G needle and immersed into sterile 0.1 M calcium chloride for 30 min. The beads were then filtered through filter paper (Whatman #4) and washed twice with 0.1% (w/v) peptone solution containing 0.85% (w/v) NaCl. Encapsulated *B. longum* and encapsulated *S. thermophilus* beads were coated with 10% (w/v) skim milk and stored at 4℃ before testing.

Probiotic encapsulation by emulsion technique

The emulsion technique of *B. longum* and *S. thermophilus* encapsulation was adapted from Sultana *et al*. (2000). Ten milliliters of probiotic cells suspension were mixed with 40 ml of either of 2% (w/v) sodium alginate or 2% (w/v) carrageenan. Sixty milliliters of vegetable oil were transferred into the mixture and stirred by homogenizer at 1,000 rpm for 30 min until the mixture became homogenous. The mixtures were then gelled by adding 100 mL of 0.1

M calcium chloride rapidly and left for 30 min until the gels separated at the bottom of the solution. The oil layer was discarded, and the gel beads were collected through centrifugation at 7,500 rpm for 15 min. The beads were washed twice with 0.1% (w/v) peptone solution containing 0.85% (w/v) NaCl and 0.05 M $CaCl_2$ and then coated with 10% (w/v) skim milk.

Viability of free and encapsulated probiotic bacteria under simulated gastric condition

One hundred milligrams of encapsulated probiotic beads were immersed in 0.9 ml phosphate buffer pH 2.0 containing 0.3% (w/v) pepsin (Sigma) and incubated at 37℃ for 3 hours. The beads were then filtered through filter paper (Whatman #4) and washed twice with 0.85% (w/v) NaCl solution. The beads (0.1g) were broken down by vortex mixer at room temperature for 15 min. The viable probiotic cells were counted through plating on MRS agar and incubated anaerobically at 37℃ for 48 h, reported as log CFU/g. For free probiotics, 0.1 g of cell suspension was exposed into 0.9 ml phosphate buffer pH 2.0 containing 0.3% (w/v) pepsin (Sigma). After 3 hours of incubation at 37℃, the viability of the free probiotic cells was measured. The survival rate was calculated using the equation (1).

$$\text{Survival rate (\%)} = (N/N_0) \times 100 \quad (1)$$

where N is the number of viable cells (log CFU/g) after exposure to a simulated gastric condition and N_0 is the number of initial viable cells (log CFU/g).

Survivability of free cell, encapsulated probiotic B. longum and encapsulated S. thermophilus under freeze-drying condition

The encapsulated *B. longum*, encapsulated *S. thermophilus* and free probiotic cells were frozen at −18℃ overnight and dried by using a freeze dryer (ScanVac Coolsafe 110-4, Demmark) at −105℃ for 24 h. The viable freeze-dried probiotic cells were then counted on MRS agar after incubation under anaerobic condition at 37℃ for 48 h, reported as log CFU /g. The survival rate was calculated using the equation (2).

$$\text{Survival rate (\%)} = (N/N_0) \times 100 \quad (2)$$

where N is the number of viable cells (log CFU/g) after exposure to a freeze-drying condition and N_0 is the number of initial viable cells (log CFU/g).

Statistical analysis

The data were obtained from triplicate trials in a completely randomized design. Analysis of variance was computed by SPSS statistic software. These data were compared by the Duncan′s multiple range test.

Results

Encapsulation of B. longum TISTR 2195 *and S. thermophilus* TISTR 2289

Encapsulation is a technique used to protect probiotic cells from harmful conditions such as temperature, pH, and so on, and to enhance the survival of probiotics in the gastrointestinal tract. In this study, *B. longum* TISTR 2195 and *S. thermophilus* TISTR 2289 were entrapped with sodium alginate and carrageenan using extrusion and emulsion techniques. The cell suspensions of *B. longum* TISTR 2195 and *S. thermophilus* TISTR 2289 used for encapsulation were ranged of 8.66–8.72 log CFU/ml and 7.89–8.94 log CFU/ml, respectively. The number of encapsulated probiotic cells in different supporting materials is shown in Table 1. The highest survival rate of *B. longum* TISTR 2195 (96.07%) was observed after encapsulation with sodium alginate coated with skim milk (alginate–skim milk) using the extrusion method. The numbers of viable alginate–skim milk encapsulated *B. longum* (8.32 log CFU/g) cells were significantly higher than those of the carrageenan–skim milk encapsulated cells ($p < 0.05$). For encapsulation of *S. thermophilus* TISTR 2289 using the extrusion method, the survivability of alginate–skim milk encapsulated and carrageenan–skim milk encapsulated *S. thermophilus* was similarly high (99.11% and 99.33%, respectively). In addition, both strains encapsulated using the extrusion technique had more viable cells than those using the emulsion technique.

Table 1. Survival of encapsulated probiotic in sodium alginate coated with skim milk and carrageenan coated with skim milk

Supporting material	**Encapsulation technique**	***B. longum***		***S. thermophilus***	
		Log CFU/g	% survival	Log CFU/g	% survival
Alginate–skim milk	Extrusion	$8.32^{a1/}$ ±0.02	96.07	7.82±0.12	99.11
Alginate–skim milk	Emulsion	8.05^{bc}±0.02	92.32	7.80±0.13	98.85
Carrageenan–skim milk	Extrusion	8.08^{b} ±0.05	92.88	8.88±0.09	99.33
Carrageenan–skim milk	Emulsion	8.00^{c} ±0.00	92.06	8.59±0.06	92.37

All data are represented as mean ± SD (n = 3)
[1]/: Values with different letters in the same column are significantly different ($p < 0.05$)

Viability of free and encapsulated probiotics under simulated gastric condition

Probiotic bacteria confer health benefits on the host when they survive in food products or during gastrointestinal transition at a concentration of at least 6 log CFU per g or ml. We determined the survivability of free and encapsulated probiotic cells under simulated gastric condition to evaluate the protective effect of encapsulation techniques with various encapsulants. Figure 1 and Figure 2 illustrate the viability of free and encapsulated probiotic cells. The survival of free cells *B. longum* and *S. thermophilus* after incubation at pH 2.0 for 3 hours was reduced to 7 log CFU/g and 5 log CFU/g, respectively. In contrast, the encapsulated cells demonstrated greater viability than the free cells under the same conditions.

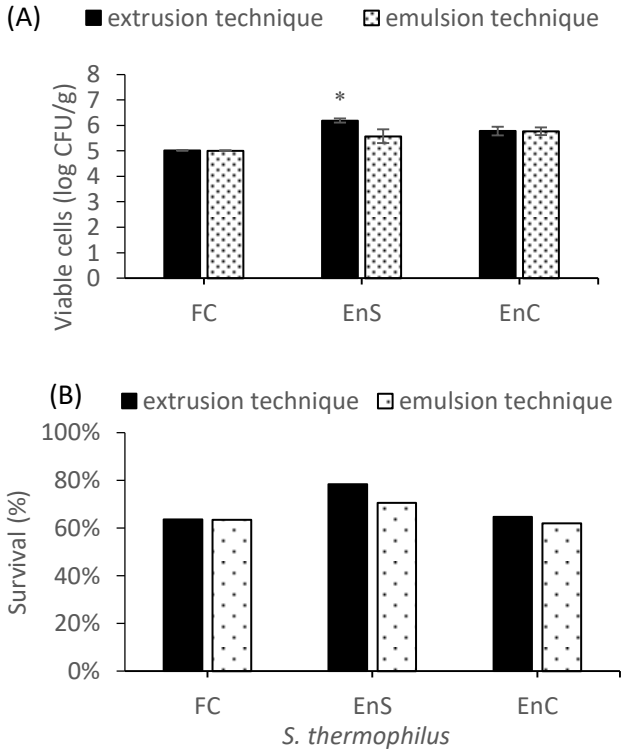

Figure 1. Viability of free cells (FC) and encapsulated *S. thermophilus* TISTR 2289 after exposure to a simulated gastric condition for 3 hours. The data is presented as mean ± SD (n = 3). EnS: encapsulated cells with sodium alginate coated with skim milk; EnC: encapsulated cells with carrageenan coated with skim milk. * represents values that differ significantly ($p < 0.05$)

The number of viable cells using extrusion encapsulated bacteria was higher than those of bacteria encapsulated using the emulsion technique. *S. thermophilus* encapsulated with alginate–skim milk displayed a significantly higher number of viable cells than those encapsulated with carrageenan–skim milk ($p < 0.05$) (Figure 1A). The survival rate of alginate–skim milk encapsulated bacterial was 78.65% whereas the survival rate of carrageenan–skim milk encapsulated bacteria was only 64.65%. In addition, we observed a high survival rate of alginate–skim milk encapsulated bacteria using the emulsion technique (Figure 1B). Although the extrusion encapsulation technique exhibited a greater protective effect on the viability of *S. thermophilus*, there was no significant difference observed between the survival of encapsulated *B. longum* using the extrusion method and the emulsion method (Figure 2). Interestingly, the resistance of encapsulated *B. longum* to simulated gastric juice was greater than the encapsulated *S. thermophilus*.

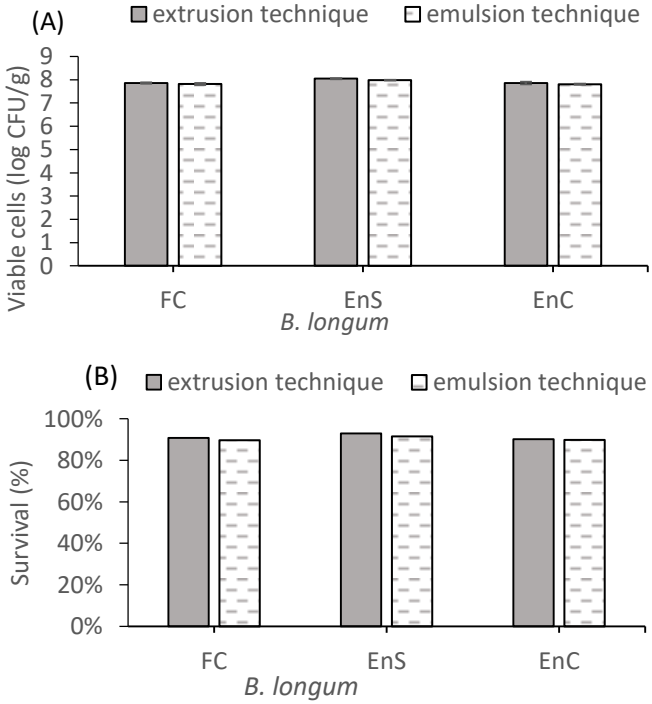

Figure 2. Viability of free cells (FC) and encapsulated *B. longum* TISTR 2195 after exposure to a simulated gastric condition for 3 h. The data is shown as mean ± SD (n = 3). EnS: encapsulated cells with sodium alginate coated with skim milk; EnC: encapsulated cells with carrageenan coated with skim milk

Survival of free and encapsulated probiotic bacteria after freeze-drying condition

To extend storage life of encapsulated probiotics and be able to incorporate them into food products, freeze dry processing was carried out. As show in Figure 3, alginate–skim milk encapsulated *S. thermophilus* TISTR 2289 using the extrusion technique showed the highest cell survival at about 82.97%, while the viability of probiotics encapsulated using the emulsion technique was 73.64%. The survival rate of free and encapsulated *B. longum* during freeze-drying is demonstrated in Figure 4. *B. longum* encapsulated with alginate–skim milk using the extrusion method had the highest viable cell numbers of 7.01 log CFU/g (Figure 4A) and its survival rate was the highest at 80.94% as compared with the other strain, coating, and encapsulation method (Figure 4B). The lowest survival rate (74.68%) of encapsulated *B. longum* was observed in the emulsion system with carrageenan–skim milk. However, encapsulation of probiotic cells in both techniques afforded better protection than the non-encapsulated cells.

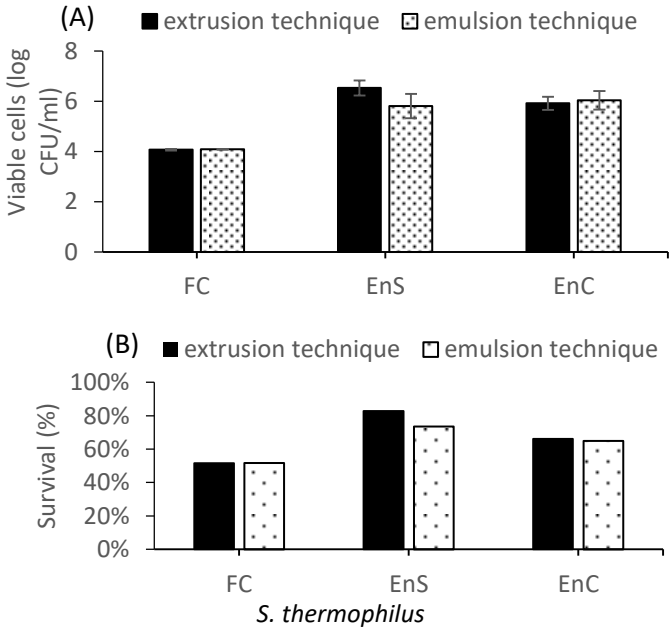

Figure 3. Survival (%) of free cells (FC) and encapsulated *S. thermophilus* TISTR 2289 after exposure to freeze-drying condition. The data is presented as mean ± SD (n = 3). EnS: encapsulated cells with sodium alginate coated with skim milk; EnC: encapsulated cells with carrageenan coated with skim milk

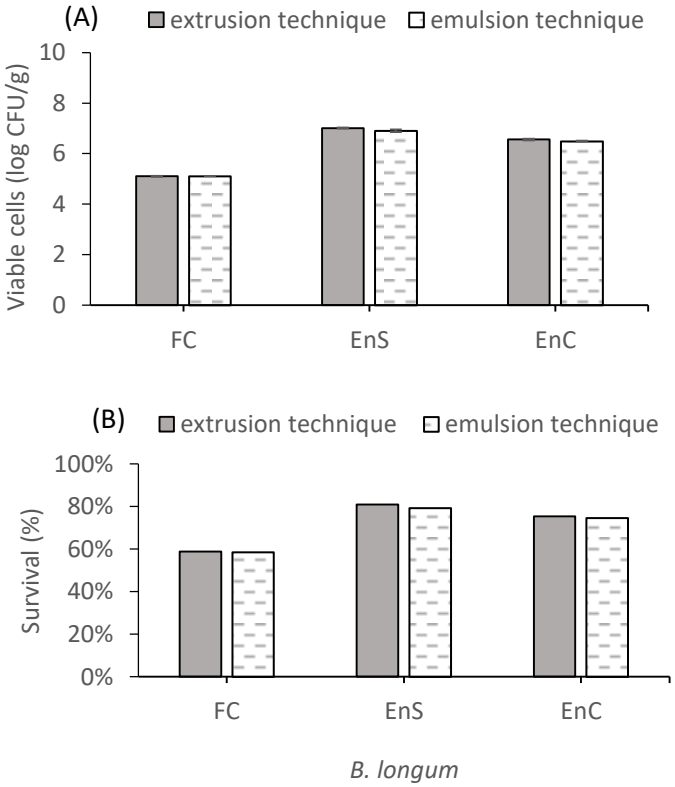

Figure 4. Survival (%) of free cells (FC) and encapsulated *B. longum* TISTR 2195 after exposure to freeze-drying condition. The data is shown as mean ± SD (n = 3). EnS: encapsulated cells with sodium alginate coated with skim milk; EnC: encapsulated cells with carrageenan coated with skim milk

Discussion

The present study was designed to determine the effect of encapsulation techniques and encapsulants on the survivability of probiotic cells under simulated gastric condition and freeze-drying condition. The results of this study show that encapsulation with sodium alginate coated with skim milk using the extrusion technique significantly increased the viability of *S. thermophilus* compared to the free cells and cells encapsulated with carrageenan–skim milk after exposure a to simulated gastric conditions and a freeze-drying condition. This may be related to alginate, a linear heteropolysaccharide extracted from various types of seaweed, comprising D-mannuronic and L-guluronic acid (Burgain *et al.*, 2011) which can form an irreversible gel with the addition of calcium chloride. Sodium ions from the

polymer of guluronic acids exchange with divalent cations (Ca^{2+}), resulting in a cross-linked polymer, which can entrap the cells in a structure (Martń *et al*., 2015). This extremely strong gel cannot be homogenized at high-speed stirring (Manev *et al*., 2013). The extrusion technique uses a gentle operation, leading to spherical gel bead formation, which causes low levels of injury to probiotic cells (Chuprom, 2010).

Additionally, coating gel beads with skim milk could protect cells and improve the survival of encapsulated probiotics under a simulated gastric condition and freeze-drying because of skim milk′s buffering capability and the decreased porosity of the encapsulated beads. These results are consistent with other studies illustrating the improved viability of *Lactobacillus plantarum* and *L. bulgaricus* using alginate–skim milk encapsulation during exposure to a simulated gastrointestinal condition and freeze-drying (Pan *et al*., 2012; Wang *et al*., 2016). Shi *et al*. (2013) reported an improvement in the survival of *L. bulgaricus* in simulated gastric fluid pH 2.5 and 2 when encapsulated in carrageenan–locust bean gum coated with milk.

In summary, the research finding indicated that encapsulation by the extrusion technique is a more effective method of protecting probiotic cells in a simulated gastric condition and freeze-drying than that achieved by the emulsion technique. Further, sodium alginate–skim milk can potentially be used as supporting material for both encapsulation techniques. It could maintain the stability of viable cells more effectively than carrageenan under adverse conditions. Further research should be done to elucidate the stability and viability of encapsulated probiotics in many types of food products and during storage conditions.

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