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Activity of Encapsulated *Lactobacillus bulgaricus* in Alginate-whey Protein Microspheres

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ABSTRACT

In this work, alginate-whey protein was used as wall materials for encapsulating Lactobacillus delbrueckii subsp. bulgaricus *(*L. bulgaricus*). The characteristics of encapsulated and free* L. bulgaricus *showed that the free* L. bulgaricus *lost viability after 1 min exposure to simulated gastric fluid (SGF) at pH 2.0 and 2.5. However, the viability of encapsulated* L. bulgaricus *did not decrease in SGF at pH 2.5 for 2 h incubation. The viable numbers of encapsulated* L. bulgaricus *decreased less than 1.0 log unit for 2 h incubation in SGF at pH 2.0. For bile stability, only 1.2 log units and 2.0 log units viability of the encapsulated* L. bulgaricus *was lost in 1 and 2% bile for 1 h exposure, respectively, compared with no survival of free* L. bulgaricus *under the same conditions. Encapsulated* L. bulgaricus *was completely released from the microspheres in simulated intestinal fluid (SIF, pH 6.8) in 3 h. The viability of the encapsulated* L. bulgaricus *retained more 8.0 log CFU/g after stored at 4°C for four weeks. However, for free* L. bulgaricus*, only around 3.0 log CFU/mL was found at the same storage conditions. Results showed that the encapsulation could improve the stability of* L. bulgaricus.

Key words: Encapsulation, Alginate, Whey protein, Probiotics, Stability

INTRODUCTION

Several studies have shown that the probiotics play an important role in human health; they can prevent diarrhea, balance the intestinal microflora, stimulate of the immune system, have anticarcinogenic properties and lactose intolerance (Shah 2007). FAO has recommended that the least viability of probiotics should be at 10^7 CFU/g of product at the time of consumption. The biggest problem in probiotics application is its sensitivity to environment conditions (Carvalho et al. 2004). Hence, protecting the viability of probiotics is one of the premises of its application.

Encapsulation is one of most effective methods that improve the viability and stability of probiotic bacteria (Gbassi et al. 2009; Heidebach et al. 2010; Dolly et al. 2011). Alginate is by far the most widely used wall material for encapsulation. However, encapsulation of probiotic bacteria in alginate microspheres was not able to protect the organisms effectively from high acidic environment (Sultana et al. 2000; Krasaekoopt et al. 2003; Dong et al. 2013; Pan et al. 2013; Shi et al. 2013 a, b). Whey protein or denatured whey protein is another extensively used as wall material for the encapsulation of probiotics. Denatured whey protein can be obtained through heating whey protein solution (Anandharamakrishnan et al. 2007; Tang et al. 2013). The application of polysaccharide-protein matrix for probiotics encapsulation is a relatively new strategy and can be seen as a promising alternative approach developed for probiotic

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encapsulation. In this study, extrusion technique was employed for the encapsulation of *L. bulgaricus* with wall material combinations of denatured whey protein and sodium alginate. Characteristics of encapsulated *L. bulgaricus* were investigated and compared with those of free *L. bulgaricus*.

MATERIAL AND METHODS

Cell culture Preparation

Lactobacillus bulgaricus was provided by Hangzhou Wahaha Group Co Ltd. (ZhejiangChina) as a 25% glycerol stock in MRS broth (Difco, Michigan, USA) at -80°C. All the glass-wares used in this study were sterilized at 121°C for 15 min. The culture was activated in MRS broth at 37°C and propagated two times prior to use in the experimental trials. The fresh culture was prepared by adding 1% inoculum to MRS broth and incubated at 37°C for overnight. The cells were harvested by centrifugation at 3,000 g at 4°C for 10 min. After the removal of supernatant, the cells were washed with sterile 0.85% (w/v) sodium chloride solution.

Encapsulation of *L. bulgaricus*

Whey protein powder was mixed with sterile distilled water. The solution was stirred gently using a magnetic stirrer to dissolve the whey protein prior to heat treatment. In order to denature the whey protein, the solution was kept at 80°C for 30 min in a water bath (Tang et al. 2013). Alginate-whey protein microspheres were made using an Inotech Encapsulator IER-50 (Inotech Biosystems Intl. Inc.) by extruding a mixture containing *L. bulgaricus* suspended in sodium alginate solution and de-natured whey protein into 0.1 M $CaCl_2$ solution while gently stirring with a magnetic bar. The microspheres were prepared using a 450 µm nozzle. The resulting microspheres were allowed to harden in the $CaCl_2$ solution for 30 min, rinsed with distilled water, filtered, and sealed in sterilized conical tubes for storage at 4°C.

Survival assay and numeration of encapsulated and free *L. bulgaricus*

pH stability: Microspheres or free *L. bulgaricus* were added to test tubes containing SGF (0.2%, w/v, NaCl at pH 2.5 or pH 2.0) and incubated at 37°C for 0, 10, 30, 60, 90 and 120 min. At pre-determined time, wet microspheres were removed from the SGF, washed with saline solution, dispersed and broken down in SIF (50 mm KH_2PO_4) pH 6.8. The survival of encapsulated or free *L. bulgaricus* was immediately assayed (Pan et al. 2013; Shi et al. 2013 a, b).

Bile stability: Microspheres or free *L. bulgaricus* suspension were added to test tubes containing simulated bile (1 or 2%, w/v) porcine bile extract (Sigma-Aldrich) and incubated at 37°C for 1 and 2 h. After pre-determined time, the beads were washed with saline solution. Microspheres were dissolved in SIF (pH 6.8). The viability of encapsulated or free *L. bulgaricus* was immediately assayed as above.

Release profile: Microsphere samples were added to SIF solution and incubated at 37°C with shaking at 100 rpm/min. At predetermined time points, 100 µL of this solution was taken out and immediately assayed for the activity. The same volume of the fresh medium was added to replace the volume of the withdrawn samples. The cumulative amount of released *L. bulgaricus* was plotted against the time (Pan et al. 2013; Shi et al. 2013 a, b).

Storage stability: Microspheres or free *L. bulgaricus* were stored at 4°C. At pre-determined time points, samples were taken out for activity measurement. Total viable counts of *L. bulgaricus* were determined by a pour plate method using MRS agar after serial 10-fold dilutions in saline solution. Plates were incubated at 37°C for 48 h (Pan et al. 2013; Shi et al. 2013 a, b).

Statistical analysis

All the experiments were repeated at least three times and the results were presented in mean value ± standard deviation (SD). Statistical analysis was performed using Original 8.0 for Windows. Student's t test was used to compare the significance of differences. The differences were considered significant at the level of $P < 0.05$.

RESULTS AND DISCUSSIONS

Preparation of encapsulated *L. bulgaricus* microspheres

One of necessary pre-requisite for encapsulation method is high encapsulation yield. The initial number of viable *L. bulgaricus* in aqueous suspension used to prepare the microspheres was about 10.01 log CFU/mL. After encapsulation, the

number of viable cells recovered in pH 6.8 SIF was 9.98 log CFU/g. The encapsulation yield of *L. bulgaricus* in alginate-whey protein microspheres was close to 100%. It indicated that this encapsulation process led to a high encapsulation yield. High encapsulation efficiency of 96% was also reported for the encapsulation of probiotic yeasts in alginate-whey protein microspheres (Hébrard et al. 2010). Therefore, it appeared that alginate-whey protein matrices had a good compatibility with functional biological substances, including *L. bulgaricus*.

The size of alginate-based microspheres can be affected by various factors such as nozzle size, polymer concentration, hardening time in calcium chloride, initial cell concentration, etc (Chandramouli et al. 2004; Dong et al. 2013; Shi et al. 2013 a, b). In this work, the, nozzle size was 450 μm. Alginate-whey protein microspheres with diameter around 800 μm and a spherical shape were obtained (results not shown). In the case of encapsulation for food application, the size of the microspheres is an important factor. Large microspheres can adversely affect the texture of food after supplementation by causing coarseness; small microspheres may not give sufficient protection. Alginate-based microspheres often with between 1.0 to 3.0 mm are obtained, which are too large for many food applications (Sheu and Marshall 1993; Hansen et al. 2002; Muthukumarasamy et al. 2006; Dong et al. 2013; Shi et al. 2013 a, b). In the present study, alginate-whey protein microspheres were below 1.0 mm. It might not produce enough detrimental effect on the texture of food. Smaller microspheres could be obtained when smaller nozzle size was used.

Stability of encapsulated *L. bulgaricus* in SGF

One of major problems in efficacy of probiotic food is low survival of the cells in the gastric pH. Encapsulated and free *L. bulgaricus* were tested for the survival under simulated gastric pH of 2.0 and 2.5 (Fig. 1). It was found that the encapsulation could improve the pH tolerance of *L. bulgaricus*. The numbers of encapsulated cells did not decrease in SGF pH at 2.5 for 2 h incubation. The viable count of the encapsulated *L. bulgaricus* decreased less than 1.0 log unit for 2 h incubation in pH 2.0 SGF. However, free cells of *L. bulgaricus* were extremely sensitive to acidic environments and were not detectable after 1 min exposure to SGF at pH 2.5 and 2.0 (results not shown). These results agreed with several earlier reports. Chandramouli et al. (2004) found that the viable numbers of *L. acidophilus* encapsulated in alginate microspheres was significant increased in SGF at pH 2.0. Encapsulated *L. acidophilus* in alginate microspheres survived better after sequential incubation in simulated gastric and intestinal juices (Krasaekoopt et al. 2004). Higher survival was also reported when the encapsulated lactobacilli in alginate microspheres were incubated in SGF (Lee et al. 2004).

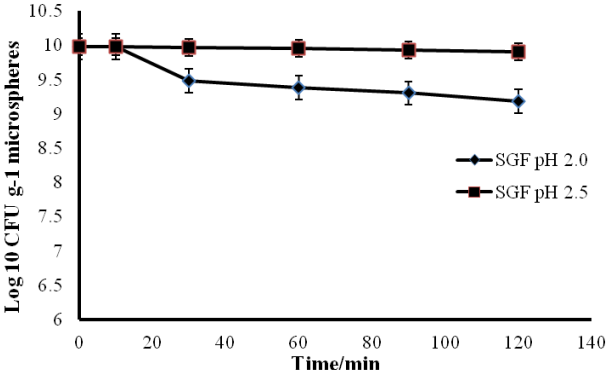

Figure 1 - Survival of encapsulated *Lactobacillus bulgaricus* in pH 2.0 SGF and pH 2.5 SGF.

Stability of encapsulated *L. bulgaricus* in bile salt stability

The results of free and encapsulated *L. bulgaricus* subjected to different concentrations of bile salts (1 and 2%) are shown in Table 1. It indicated a similar trend for viability of encapsulated and free *L. bulgaricus* in bile salt. From the initial count of 10.01 log CFU/mL, no survival was found in 1 and 2% bile salt after 1 h exposure. The decrease rate was lower for the encapsulated *L. bulgaricus*.

Table 1 illustrated around 2.0 log and 3.0 log reduction of *L. bulgaricus* after 2 h incubation in 1 and 2% bile salt concentrations, respectively. The numbers declined more as the bile concentration and incubation time increased. The sensitivity of many probiotics strains to bile concentrations encountered in the human gastro-intestinal tract has been reported by several authors (Chung et al. 1999; Amor et al. 2002; Picot & Lacroix 2004). Clark and Martin (1994) observed 5.0 log reductions in viable cell counts of *B. adolescentis* in 2% bile solution after 12 h incubation at 37°C. Amor et al. (2002) found that the viability of *B. lactis* and *B. adolescentis* was more susceptible to the damaging effects of bile salts. In the present study, higher survival rate of the cells in alginate-

whey protein microspheres after bile solution treatment was pre-requisite for providing the host with a beneficial health effect.

Table 1 - Survival of free and encapsulated *L. bulgaricus* after treatment in simulated bile concentrations of 1 and 2% for 1 h and 2 h (Log $_{10}$ CFU/g microspheres).

Condition/time	Bile concentration (%)		
	0	1	2
Free *L. bulgaricus* (1 h)	10.01±0.13	0	0
Encapsulated *L. bulgaricus* (1 h)	9.98±0.12	8.78±0.03	7.98±0.08
Free *L. bulgaricus* (2 h)	10.01±0.08	0	0
Encapsulated *L. bulgaricus* (2 h)	9.96±0.14	7.69±0.07	7.03±0.15

Values shown are means ±standard deviations (n =3). Means in the table differ significantly ($P < 0.05$).

Release of encapsulated *L. bulgaricus* in SIF

The release of the cells from microcapsules in colon is essential for the growth and colonization of probiotics. Figure 2 illustrates the release of cells of the encapsulated *L. bulgaricus* in pH 6.8 SIF. When encapsulated *L. bulgaricus* was placed into SIF at pH 6.8, the microspheres began to swell and eventually disintegrated. For alginate-whey protein microspheres, the cells of *L. bulgaricus* released within 3 h and 70% of cell had already been released within 1 h. Gunsaekaran et al. (2007) encapsulated caffeine using whey protein hydrogel and observed similar release profiles, in which a burst release of caffeine happened within 1 h at pH 7.5. At pH 6.8, whey proteins are negatively charged, which may interact with the carboxyl group from the alginate molecules. This electrostatic repulsion also favors the dissociation of the gel network. It is well known that alginate gel formed through calcium ions is very rigid and only swells slowly at neutral pH. Mandal et al. (2006) found that the release of cells progressed with increased incubation. A progressive release of viable cells from whey protein-based microcapsules in SIF was also reported by Picot and Lacroix (2004).

Storage stability of encapsulated *L. bulgaricus*

The storage stability of free and encapsulated *L. bulgaricus* during four weeks at 4°C is shown in Figure 3. The viability of the encapsulated *L. bulgaricus* was higher compared to free *L. bulgaricus*. The viability of free *L. bulgaricus* was from 10.01 log CFU/mL to 9.93 log CFU/mL after one week, and around 3.0 log CFU/mL after four weeks. However, the number of encapsulated *L. bulgaricus* cells only decreased from 9.97 log CFU/mL to 8.37 after four weeks. The viability of encapsulated *L. bulgaricus* cells was fully preserved in one week. Therefore, encapsulation of *L. bulgaricus* in alginate-whey protein microspheres could improve the storage stability of free *L. bulgaricus* cells. Several studies that encapsulated bacteria in alginate-based microspheres reported better survival ability than non-encapsulated bacteria during the storage period (Truelstrup Hansen et al. 2002; Krasaekoopt et al. 2003; Pan et al. 2013; Shi et al. 2013 a, b).

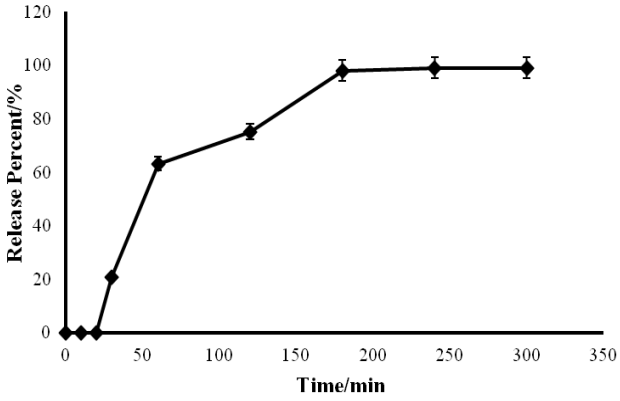

Figure 2 - Release profile of encapsulated *Lactobacillus bulgaricus* in pH 6.8 SIF.

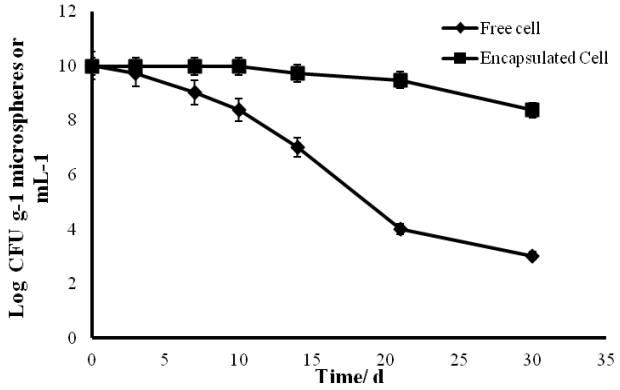

Figure 3 - Storage stability of free and encapsulated *Lactobacillus bulgaricus* at 4°C.

CONCLUSIONS

Alginate-whey protein microspheres encapsulating model *L. bulgaricus* were produced by extrusion method. Encapsulated microspheres had better survival ability after bile salt concentrations, and at low pH, long storage time as compared to free cells. Encapsulated *L. bulgaricus* was released from the microspheres within a reasonable time so as to exert their beneficial effects. These findings demonstrated alginate-whey protein microspheres were effective wall carriers for probiotics encapsulation.

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REFERENCES

Amor KB, Breeuwer P, Rombouts P, Verbaarschot FM, Akkermans ADL, et al. Multiparametric flow cytometry and cell sorting for the assessment of viable, injured and dead bifidobacterium cells during bile salt stress. *Appl Environ Microbiol*. 2002; 68: 5209-5216.

Anandharamakrishnan C, Rielly CD, Stapley AGF. Effects of process variables on the denaturation of whey proteins during spray-drying. *Drying Technol.* 2007; 25: 799-807.

Carvalho AS, Silva J, Ho P, Teixeira P, Malcata FX, Gibbs P. Relevant factors for the preparation of freeze-dried lactic acid bacteria. *Int Dairy J*. 2004; 14: 835-847.

Chandramouli V, Kailaspathy K, Peiris P, Jones M. An improved method of microencapsulation and its evaluation to protect *Lactobacillus* spp. in simulated gastric conditions. *J Microbiol Meth*. 2004; 56: 27-35.

Chung HS, Kim YB, Chun SL, Ji GE. Screening and selection of acid and bile resistant bifidobacteria. *Int J Food Microbiol.* 1999; 47: 25-32.

Clark PA, Martin JH. Selection of Bifidobacteria for use as dietary adjuncts in cultured dairy foods: III-Tolerance to simulated bile concentrations of human small intestines. *Cultured Dairy Products J*. 1994; 29: 20-21.

Dolly P, Anishaparvin A, Joseph GS, Anandharamakrishnan C. Microencapsulation of *Lactobacillus plantarum* (mtcc 5422) by spray-freeze-drying method and evaluation of survival in simulated gastrointestinal conditions. *J Microencapsul* 2011; 28: 568-574.

Dong QY, Chen MY, Xin Y, Qin XY, Chen Z, Shi LE, et al. Alginate-based and protein-based materials for probiotics encapsulation: A review. *Int J Food Sci Technol.* 2013; 48: 1339-1351.

Gbassi GK, Vandamme T, Ennahar S, Marchioni E. Microencapsulation of *Lactobacillus plantarum* spp. in an alginate matrix coated with whey proteins. *Int J Food Microbiol.* 2009; 129: 103-105.

Gunasekaran S, Ko S, Xiao L. Use of whey proteins for encapsulation and controlled delivery applications. *J Food Eng*. 2007; 83: 31-40.

Hansen LT, Allan-Wojtas PM, Jin YL, Paulson AT. Survival of Ca-alginate microencapsulated *Bifidoacterium* spp. in milk and simulated gastrointestinal conditions. *Food Microbiol*. 2002; 19:35-45.

Hébrard G, Hoffart V, Beyssac E, Cardot JM, Alric M, Subirade M. Coated whey protein/alginate microparticles as oral controlled delivery systems for probiotic yeast. *J Microencapsul.* 2010; 27: 292-302.

Heidebach T, Forst P, Kulozik U. Influence of casein-based microencapsulation on freeze-drying and storage of probiotic cells. *J Food Eng*. 2010; 98:309–316.

Krasaekoopt W, Bhandari B, Deeth H. Evaluation of encapsulation techniques of probiotics for yoghurt. *Int Dairy J*. 2003; 13: 3-13.

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

Lee JS, Cha DS, Park HJ. Survival of freeze-dried *Lactobacillus bulgaricus* KFRI 3673 in chitosan-coated calcium alginate microparticles. *J Agr Food Chem*. 2004; 52: 7300-7305.

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

Muthukumarasamy P, Allan-Wojtas P, Holley RA. Stability of *Lactobacillus reuteri* in different types of microcapsules. *J Food Sci*. 2006; 71: M20-M24.

Pan LX, Fang XJ, Yu Z, Xin Y, Liu XY, Shi LE, et al.. Encapsulation in alginate-skim microspheres improves viability of *Lactobacillus bulgaricus* in stimulated gastro-intestinal conditions. *Int J Food Sci Nutr.* 2013; 64: 380-384.

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

Shah NP. Functional cultures and health benefits. *Int Dairy J*. 2007; 17: 1262-1277.

Sheu TY, Marshall RT. Microentrapment of lactobacilli in calcium alginate gels. *J Food Sci*. 1993; 58: 557-561.

Shi LE, Li ZH, Li DT, Xu M, Chen HY, Zhang ZL, et al. Encapsulation of probiotics *Lactobacillus bulgaricus* in alginate-milk microspheres and evaluation of survival in simulated gastrointestinal conditions. *J Food Eng.* 2013a; 117: 99-104.

Shi LE, Zhang ZL, Song YQ, Zhou ML, Yu WM, Tang ZX. Encapsulation of *Lactobacillus bulgaricus* in carragenan-locust bean gum coated milk microspheres with double layer structure, *LWT-Food Sci Technol.* 2013b; 54: 147-151.

Sultana K, Godward G, Reynolds N, Arumugaswamy R, Peiris P, Kailasapathy K. Encapsulation of probiotic bacteria with alginate–starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt. *Int J Food Microbiol*. 2000; 62: 47-55.

Tang ZX, Huang XQ, Baxi S, Chambres JR, Sabour PM, Wang Q. Whey protein improves survival and release characteristics of bacteriophage Felix O1 encapsulated in alginate microspheres. *Food Res Int.* 2013; 52: 460-466.

Hansen LT, Allan-Wojtas PM, Jin YL, Paulson AT. Survival of Ca-alginate miecoencapsulated *Bifidobacterium* spp. in milk and simulated gastrointestinal conditions. *Food Microbiol*. 2002; 62: 47-55.

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