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Survival of Freeze-Dried *Lactobacillus bulgaricus* KFRI 673 in Chitosan-Coated Calcium Alginate Microparticles

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The aim of this study was to investigate the effect of alginate microparticles coated with three kinds of chitosans of different molecular weights on the survival of *Lactobacillus bulgaricus* KFRI 673 in simulated gastric (SGJ) and intestinal juices (SIJ) and on their stability during storage at 4 and 22 °C. *L. bulgaricus* KFRI 673 loaded in alginate microparticles was prepared by spraying the mixture of sodium alginate and cell culture into the calcium chloride solution using an air-atomizing device. When *L. bulgaricus* KFRI 673 was exposed to SGJ of pH 2.0 for 60 min, none of the microorganism survived. Contrary to this result, microbiological analysis indicated that microencapsulation in alginate microparticles improved the survival of acid-sensitive *L. bulgaricus* KFRI 673 in SGJ and that high molecular weight chitosan coating resulted in the highest survival in SGJ. To study storage stability of free and microencapsulated cells, in vitro studies were conducted at 4 and 22 °C during a 4 week period. Both free and microencapsulated cells showed similar stabilities during 4 weeks of storage at 4 °C. However, the stability of *Lactobacillus* at 22 °C was appreciably improved when loaded in high molecular weight chitosan-coated alginate microparticles. In conclusion, microencapsulation of lactic acid bacteria with alginate and chitosan coating offers an effective way of delivering viable bacterial cells to the colon and maintaining their survival during refrigerated storage.

KEYWORDS: ***Lactobacillus bulgaricus*; alginate; chitosan coating; microparticles; storage stability**

INTRODUCTION

Probiotics are defined as live microbial food ingredients that have a beneficial effect on human health by increasing humoral immunity and improving the balance of intestinal microflora (*1*−*4*). Lactic acid bacteria used as probiotics are commonly incorporated into foods to provide a wide variety of health benefits (*5*). For these bacteria to exert positive health effects, they have to reach their site of action alive and establish themselves in certain numbers (*5*). However, a major barrier to the survival of ingested microorganisms is the acidic environment (pH 2.0) of the stomach (*6*).

Microencapsulation techniques have been successfully used to enhance dairy fermentation for the production of concentrated lactic acid bacteria (*7*−*10*) and to improve the survival of microorganisms in dairy products (*11*, *12*), mayonnaise (*13*), and gastric juice (*4*, *6*, *12*, *14*−*16*). Among the encapsulation devices, microencapsulation in calcium alginate microparticles has been widely used for the immobilization of lactic acid bacteria owing to its ease of handling, nontoxic nature, and low cost (*17*−*19*).

Sodium alginate is an anionic linear polysaccharide containing 1,4-linked D-mannuronic acid and L-guluronic acid residues (*20*, *21*). Sodium alginate is gelled when it comes in contact with calcium ions in solution by cross-linking between the carboxylate anions of alginate guluronate units and the calcium ions (*22*). However, alginate microparticles (calcium alginate) are chemically unstable when chelators such as phosphate and citrate and nongelling cations such as sodium or magnesium ions are present (*23*). To increase the stability of alginate microparticles and to minimize the loss of encapsulated material, the microparticles are coated with polycationic polymers of poly-L-lysine (*14*, *24*), polyvinylamine (*25*), and chitosan (*16*, *17*, *26*, *27*).

Chitosan, a polycationic polysaccharide derived from the natural polymer chitin, forms polyelectrolyte complexes with alginate, a polyanionic polymer (*28*, *29*). The addition of cationic chitosan, resulting in chitosan-coated alginate microparticles, further strengthens the alginate gel structure of microparticles (*27*).

In this study, alginate microparticles loaded with *Lactobacillus bulgaricus* KFRI 673 were coated using chitosan of different molecular weights (low, medium, and high) by an air-atomizing device. The air atomization technique is a solvent-free technique, as it is not harmful to live organisms, and

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Table 1. Viscometric Average Molecular Weight and Deacetylation Degree of Chitosan

chitosan	DOD[a] (%)	M_v[b]
A	85.00	3.852×10^3
B	88.37	1.824×10^4
C	92.08	1.709×10^5

[a] Degree of deacetylation; determined by viscometry as described by Hwang et al. (*35*). [b] Viscometric average molecular weight; determined by ^{1}H NMR as described by Hwang et al. (*35*).

produces tiny microparticles with no adverse effects on the textural and sensory quality of the carrier food (*30*, *31*).

The objective of this study was to investigate the effect of chitosan-coated alginate microparticles on the survival of *L. bulgaricus* KFRI 673 in simulated gastric and intestinal juices as well as on their stability during 4 weeks of storage at 4 and 22 °C.

MATERIALS AND METHODS

Materials. Sodium alginate of medium viscosity, xanthan gum, and phosphate-buffered saline tablet of pH 7.4 were purchased from Sigma Chemicals (St. Louis, MO). Calcium chloride, Tween 20 (polyoxyethylene sorbitan monolaurate), and KCl−HCl buffer solution of pH 2.0 were purchased from Yakuri Pure Chemical Co. (Kyoto, Japan). Glycerol was purchased from Junsei (Tokyo, Japan), and lactic acid was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Skim milk solution and MRS broth were purchased from Difco (Detroit, MI).

Chitosan A was supplied by Sowha Chemical Co. (Tokyo, Japan) and chitosans B and C were supplied by Biotech Co., Ltd. (Mokpo, Korea). The average molecular weight and the degree of deacetylation of the three chitosan samples are given in **Table 1**.

Microorganism and Media. *L. bulgaricus* KFRI 673, purchased from Korea Food Research Institute, were transferred twice in MRS broth at 37 °C. Culture cells were harvested after 28 h (late log phase) by centrifugation (7519*g*, 10 min at 4 °C), washed, and resuspended in 1% peptone water to a final concentration of $\sim 10^9$ colony-forming units (cfu)/mL.

Preparation of Chitosan-Coated Alginate Microparticles Loaded with *L. bulgaricus* KFRI 673. The microencapsulating procedure, a two-stage procedure outlined by Gåserød et al. (*32*), was modified to make it more suitable for bacterial cells. The alginate mixture was prepared by adding 2% (w/v) sodium alginate, 5.5% (w/v) MRS broth, 5% (v/v) glycerol, 0.26% xanthan gum, 0.1% Tween 20, and 20% (v/v) cell suspension into distilled water and then mixed together. The mixture was infused into an air-atomizing device (Spray Systems Co., Incheon, Korea) and sprayed into a pan (30 cm × 30 cm × 8 cm, stainless steel) containing 1000 mL of 0.5 M calcium chloride solution under gentle stirring with a magnetic bar. This air-atomizing device was operated at an air pressure of 0.6 kgf/cm^2 and a liquid pressure of 0.4 kgf/cm^2. The divalent calcium ions cross-linked the droplets of sodium alginate to form alginate microparticles. The microparticles formed were allowed to harden in $CaCl_2$ solution for 15 min and filtered through two layers of unsterile filter paper (catalog no. 1004 110, Whatman, Maidstone, U.K.). The filtered alginate microparticles were rinsed twice with distilled water and then transferred to a solution of 0.8% (w/v) chitosan in a 1% (v/v) lactic acid solution. The microparticles were stirred gently with a magnetic bar for 15 min to evenly coat the surface of the alginate microparticles.

The resulting chitosan-coated alginate microparticles were again separated by paper filtration and rinsed twice with distilled water. They were frozen at −72 °C for 6 h. The final chitosan-coated alginate microparticles were obtained after freeze-drying using a vacuum freeze-dryer (SFDSM06, Samwon Freezing Engineering Co., Busan, Korea) at −75 °C under a pressure of 5.33 Pa for 18 h.

Morphology Analysis. Microparticles were examined by scanning electron microscope (SEM) for size and surface morphologies (JSM-5310LV, JEOL Ltd., Tokyo, Japan) at 25 kV. The microparticles were mounted on metal grids using double-sided tape and coated with gold under vacuum.

Survival of *L. bulgaricus* KFRI 673 in Simulated Gastric and Intestinal Juices. The survival of *L. bulgaricus* KFRI 673 was studied under different pH conditions simulating the gastric environment. Conical tubes containing 20 mL of simulated gastric juice (SGJ) without pepsin, KCl−HCl buffer solution (pH 2.0), and simulated intestinal juice (SIJ) without pancreatin, phosphate-buffered saline solution (pH 7.4), were preincubated at 37 °C for 10 min and incubated with 1.0 mL aliquots of the cell suspension, respectively. At intervals of 0, 30, 90, and 180 min, 1.0 mL aliquots were removed from conical tubes and assayed for *L. bulgaricus* KFRI 673 by serially diluting 1.0 mL aliquots of the test material in 1% peptone water and pour-plating 1 mL on MRS agar. The plates were incubated at 37 °C for 48 h.

The colony forming units of microorganisms were counted to assess survival. Average survival of *L. bulgaricus* KFRI 673 was determined from three separately diluted and plated samples. The experiments were replicated three times. Therefore, the results are presented in mean survival rate ± standard deviation.

Release of Microencapsulated *L. bulgaricus* KFRI 673 in Simulated Intestinal Juice. Microparticle samples (1 g) were added to conical tubes containing 20 mL of SIJ of pH 7.4 without pancreatin and incubated at 37 °C. At time intervals of 0, 30, 90, and 180 min, 1.0 mL aliquots were removed and assayed for *L. bulgaricus* KFRI 673 by plating suitable dilutions in triplicate as described previously.

Survival of Microencapsulated *L. bulgaricus* KFRI 673 after Sequential Incubation in Simulated Gastric and Intestinal Juices. Microparticle samples (1 g) were added to conical tubes containing 20 mL of SGJ of pH 2.0 without pepsin and incubated at 37 °C for 0, 30, 90, and 180 min. After incubation, the microparticles were removed by sieve and were subsequently placed in 20 mL of SIJ of pH 7.4 without pancreatin. Conical tubes were then incubated at 37 °C for 180 min. After incubation in the SIJ of pH 7.4, 1.0 mL aliquots were removed and assayed for *L. bulgaricus* KFRI 673 by plating suitable dilutions in triplicate as described previously.

Stability of Free and Microencapsulated *L. bulgaricus* KFRI 673 during Storage at 4 and 22 °C. Cell suspension (1 mL) was added to 9 mL of 10% (w/v) skim milk solution. Microparticle samples (1 g) and 1 mL of cell suspension samples were stored for 4 weeks at 4 and 22 °C. Microparticle samples (1 g) and 1 mL of cell suspension samples were collected at intervals of 1 week, and then the survival of 1 mL of cell suspension samples and 1 g of microparticle samples after 180 min of incubation in the SIJ (pH 7.4) was assayed for *L. bulgaricus* KFRI 673 by plating suitable dilutions in triplicate as described previously.

RESULTS AND DISCUSSION

Morphological Analysis. The air-atomizing technique produced free-flowing microparticles in sizes ranging mainly between 40 and 80 μm after freeze-drying. **Figure 1** shows the shapes and surface morphologies of alginate and chitosan-coated alginate microparticles. Alginate microparticles (**Figure 1a**) were generally spherical with a wrinkled surface and a collapsed center. The wrinkled surface was probably due to the loss of water content during the freeze-drying process (*30*). Skjåk-Bræk et al. (*33*) reported that alginate microparticles usually had a heterogeneous structure with a dense surface layer and a loose core due to the heterogeneous gelation mechanism, which resulted in the collapsed center during the drying process.

Incorporation of low molecular weight chitosan (chitosan A) did not cause any changes in the shape and size but modified the surface of alginate microparticles (**Figure 1b**). Alginate microparticles coated with chitosan A were more spherical and smoother than the higher molecular weight chitosan-coated alginate microparticles, which showed partial collapse in the center (**Figure 1c**,**d**). The occurrence of partial collapse in higher molecular weight chitosan-coated alginate microparticles may have resulted from binding of the high molecular weight

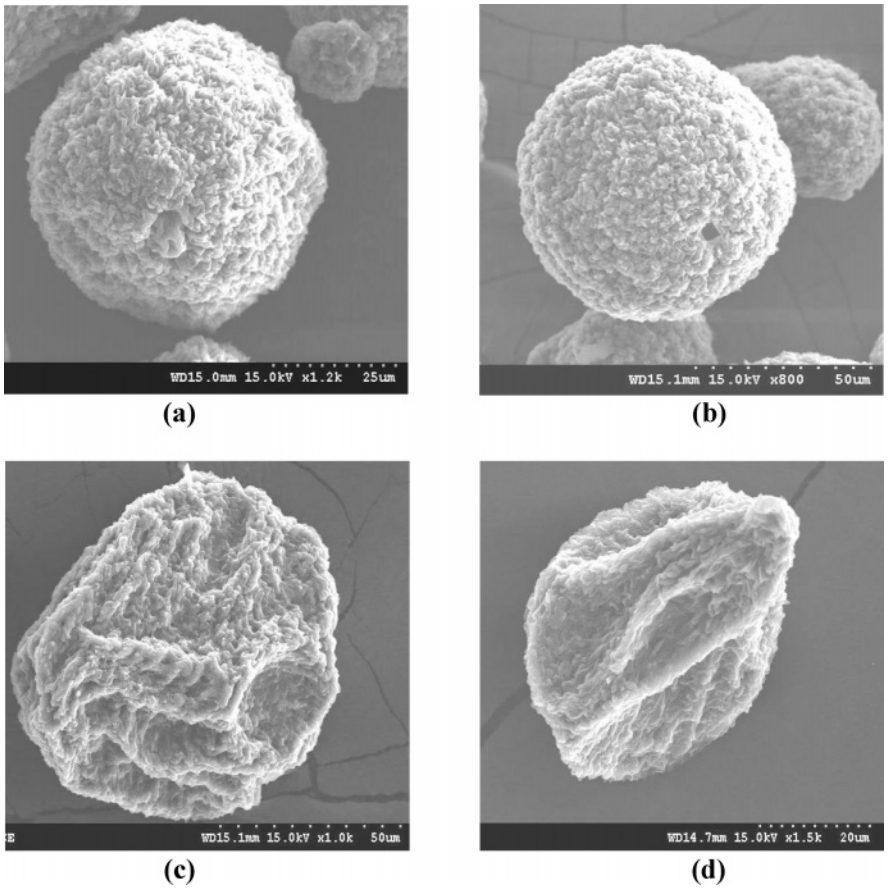

Figure 1. SEM micrographs: (**a**) calcium alginate; (**b**) chitosan A-coated calcium alginatel; (**c**) chitosan B-coated calcium alginate; (**d**) chitosan C-coated calcium alginate.

chitosan onto only the surface randomly with limited binding to the alginate gel network. The low molecular chitosan, on the other hand, diffuses rapidly into the microparticles and uniformly distributed through the whole capsule due to its low viscosity (*23*, *32*).

Survival of *L. bulgaricus* KFRI 673 in Simulated Gastric and Intestinal Juices. When *L. bulgaricus* KFRI 673 was exposed to SGJ of pH 2.0 without pepsin, none of the microorganisms survived after 60 min (**Figure 2**). However, the bacterial population was fully maintained in SIJ (pH 7.4) without pancreatin. This is in good agreement with the similar study done by Rao et al. (*6*), who reported that no *Bifidobacterium pseudolongum* survived in the simulated gastric environment of pH 1.33 for 60 min, but at pH 6.06 and 7.13 survival was fully maintained. Our results suggested that *L. bulgaricus* KFRI 673 was sensitive to the acidic environment and that ingestion of unprotected lactic acid bacteria would result in reduced viability. Therefore, *L. bulgaricus* KFRI 673 can be microencapsulated to ensure greater survival in the gastric environment.

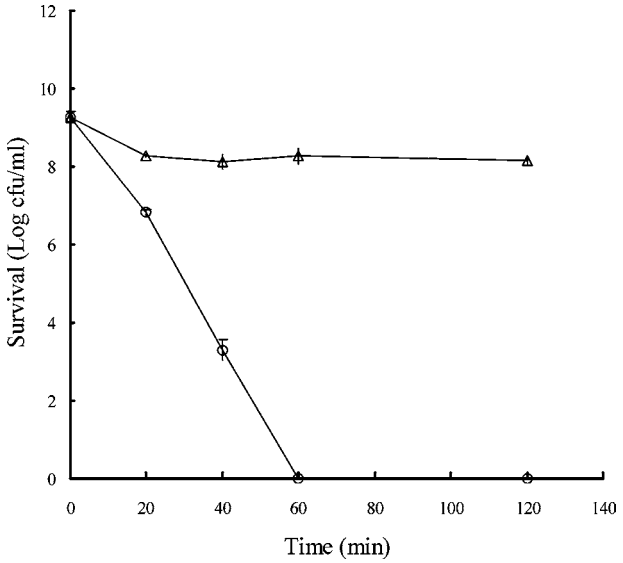

Figure 2. Survival of *L. bulgaricus* KFRI 673 in simulated gastric juice of pH 2.0 (○) and intestinal juice of pH 7.4 (△) at 37 °C (mean ± SD, $n = 3$).

Release of Microencapsulated *L. bulgaricus* KFRI 673 in Simulated Intestinal Juice. When encapsulated *L. bulgaricus* KFRI 673 was placed directly into SIJ of pH 7.4 without pancreatin, the release of viable cells was similar among all four samples as shown in **Figure 3**. There was a complete dissolution of microparticles within 30 min, which allowed the release of encapsulated *L. bulgaricus* KFRI 673. Although microparticles completely dissolved in SIJ of pH 7.4, the recovery of *L. bulgaricus* KFRI 673 showed a decrease of ~2 log cycles. Initial cell numbers of *L. bulgaricus* KFRI 673 dropped from 1.6×10^9 to 7.0×10^7 cfu/mL in alginate microparticles. The viability of *L. bulgaricus* KFRI 673 in chitosan A- and chitosan B-coated alginate microparticles decreased from 1.57×10^9 to 3×10^7 cfu/mL and from 1.9×10^9 to 2.0×10^7 cfu/mL, respectively. Numbers of *L. bulgaricus* KFRI 673 in chitosan C-coated alginate microparticles also declined from 9.33×10^8 to 9.67×10^6 cfu/mL. Truelstrup Hansen et al. (*4*) reported that encapsulated *Bifidobacteria lactis* Bb-12 best resulted in 100% recovery for subsequent enumera-

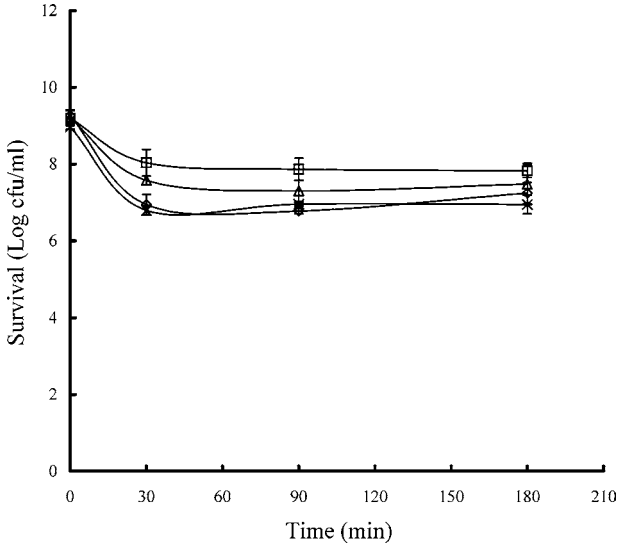

Figure 3. Release of microencapsulated *L. bulgaricus* KFRI 673 in simulated intestinal juice of pH 7.4 at 37 °C: (□) calcium alginate; (△) chitosan A-coated calcium alginate; (◇) chitosan B-coated calcium alginate; (×) chitosan C-coated calcium alginate (mean ± SD, $n = 3$).

tion by homogenizing alginate microparticles in peptone saline using a tissue homogenizer (Polytron) for 30 s. However, the recovery of *Bifidobacteria* in phosphate saline was 34% of that obtained in peptone saline.

Survival of Microencapsulated *L. bulgaricus* KFRI 673 after Sequential Incubation in Simulated Gastric and Intestinal Juices. For lactic acid bacteria to exert positive health effects, they have to colonize on the colon in large quantities (*12*). As explained above, free *L. bulgaricus* KFRI 673 was very feeble in the low-pH environment. **Table 2** shows the survival of *L. bulgaricus* KFRI 673 loaded in alginate and chitosan-coated alginate microparticles as a function of time after sequential incubation in SGJ of pH 2.0 without pepsin followed by SIJ of pH 7.4 without pancreatin. The amount of bacteria in alginate microparticles without chitosan coating gradually declined with incubation time by 6 log cycles after 180 min. It appeared that gastric juice entered the less protected microparticles through the surface pinholes, resulting in reduced bacterial survival (*6*). In chitosan A-coated alginate microparticles, the number of *L. bulgaricus* KFRI 673 decreased to ~5 log cycles after 180 min of sequential incubation. However, the viability of *L. bulgaricus* KFRI 673 was reduced by 4 log cycles in alginate microparticles coated with chitosan B and by 3 log cycles with higher molecular weight chitosan C.

When *L. bulgaricus* KFRI 673 was exposed to SIJ, the bacterial population was fully maintained. However, the free cell did not retain any viability when exposed to SGJ for 60 min (**Figure 2**). To reduce this loss, microencapsulation using alginate was investigated. Encapsulated *L. bulgaricus* KFRI 673 survived the simulated gastric environment in large numbers even if exposed to SGJ for 180 min **(Table 2)**. This result suggested that the microencapsulation technique could protect *Lactobacillus* from the gastric environment, and this is in good agreement with the results of Cui et al. (*14*), who indicated that the survival of *Bifidobacterium bifidum* was highly enhanced in low-pH conditions when immobilized with alginate or poly-L-lysine treatment.

Chitosan was applied as a membrane coating material to increase the mechanical strength and stability of alginate microparticles in the gastric environment (*27*). In our study, high molecular weight chitosan coating resulted in the highest survival of bacteria in SGJ. It was desirable for microparticles to be spherical because a nonspherical form, such as microparticles with tails that would easily break off, caused microbes to escape (*9*). The crushed shape also caused microbes to escape through the surface of the microparticles. Variations in the molecular weight and nonuniform shape of the chitosan-coated microparticles may have affected the survival of *L. bulgaricus* KFRI 673 in SGJ. Considering its greater protecting ability, alginate microencapsualtion with higher molecular weight chitosan coating can be a good candidate for improving the survival of bacteria if the shape of the microparticles is more spherical. The selection of more acid-resistant species (*3*) and resistant starch as prebiotic additives with alginate during encapsulation (*12*) enhanced the survival of the probiotic bacteria in acidic conditions. The survival of probiotic bacteria can also be affected by physiological factors such as time of ingestion, type of food, transit time, and gastrointestinal conditions (*14*).

Stability of Free and Microencapsulated *L. bulgaricus* KFRI 673 during Storage at 4 and 22 °C. Microencapsulation of various bacterial cultures including probiotics has been a common practice for expanding their shelf life (*34*). **Figure 4** shows the stability of free and encapsulated *L. bulgaricus* KFRI 673 during 4 weeks of storage in the refrigerator at 4 and 22 °C. The viability of free and microencapsulated cells showed similar stability for 4 weeks of storage at 4 °C (**Figure 4a**); numbers of free *L. bulgaricus* KFRI 673 decreased from 1.57×10^9 to 5.13×10^8 cfu/mL in 10% skim milk solution, and the survival of *L. bulgaricus* KFRI 673 loaded in alginate microparticles decreased from 7.00×10^7 to 9.67×10^6 cfu/mL. The survival of *L. bulgaricus* KFRI 673 decreased from 3.00×10^7 to 6.67×10^6 cfu/mL in alginate microparticles coated with chitosan A and from 2.00×10^7 to 7.33×10^6 cfu/mL in alginate microparticles coated with chitosan B, respectively. The viability of *L. bulgaricus* KFRI 673 in alginate microparticles coated with chitosan C decreased from 9.67×10^6 to 3.67×10^6 cfu/mL. However, upon storage at 22 °C (**Figure 4b**), initial numbers of free *L. bulgaricus* KFRI 673 decreased from 1.57×10^9 to 2.67×10^6 cfu/mL, and the survival of *L. bulgaricus* KFRI 673 loaded in alginate micro-

Table 2. Survival of Microencapsulated *L. bulgaricus* KFRI 673 after Sequential Incubation in Simulated Gastric Juice of pH 2.0 and Intestinal Juice of pH 7.4 at 37 °C

	viability[a] (log cfu/mL)			
sample	0 min	30 min	90 min	180 min
uncoated calcium alginate	7.82 ± 0.17	4.47 ± 0.07	4.08 ± 0.47	1.96 ± 0.24
chitosan A[b]-coated calcium alginate	7.48 ± 0.54	4.88 ± 0.12	4.45 ± 0.13	2.75 ± 0.10
chitosan B[b]-coated calcium alginate	7.24 ± 0.28	4.66 ± 0.22	4.62 ± 0.10	3.03 ± 0.50
chitosan C[b]-coated calcium alginate	6.94 ± 0.24	4.93 ± 0.20	5.13 ± 0.11	3.18 ± 0.22

[a] Mean ± SD, $n = 3$. [b] Physical characteristics of chitosans A–C are given in **Table 1**.

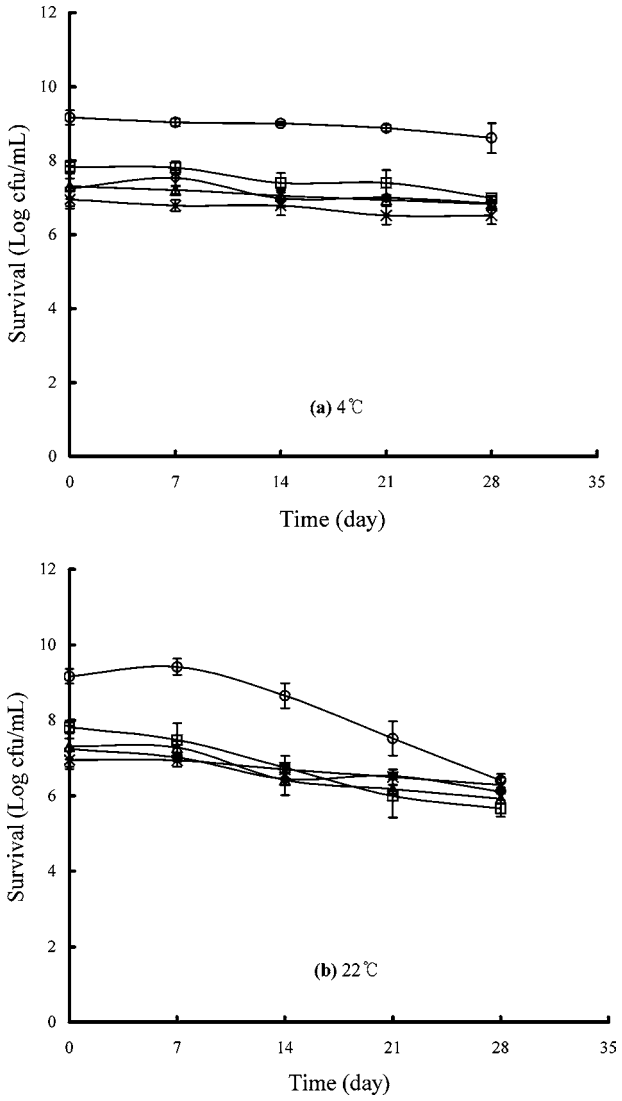

Figure 4. Stability of free and microencapsulated *L. bulgaricus* KFRI 673 during 4 weeks of storage at (**a**) 4 °C and (**b**) 22 °C: (○) free cell culture; (□) calcium alginate; (△) chitosan A-coated calcium alginate; (◇) chitosan B-coated calcium alginate; (×) chitosan C-coated calcium alginate (mean ± SD, $n = 3$).

particles decreased from 7.00×10^7 to 5.00×10^5 cfu/mL. The survival of *L. bulgaricus* KFRI 673 decreased from 3.00×10^7 to 8.67×10^5 cfu/mL in alginate microparticles coated with chitosan A and from 2.00×10^7 to 1.27×10^6 cfu/mL in alginate microparticles coated with chitosan B, respectively. The alginate microparticles coated with chitosan C, on the other hand, maintained the initial level of survival, from 9.67×10^6 to 2.07×10^6 cfu/mL, throughout the storage period at 22 °C as well as 4 °C.

Both free and microencapsulated cells showed similar stabilities during 4 weeks of storage at 4 °C. However, the stability of *L. bulgaricus* KFRI 673 loaded in alginate microparticles coated with high molecular weight chitosan C was appreciably improved at 22 °C. This was probably due to the protection of thicker membranes with higher molecular weight chitosan (*27*). Several studies showed that the survival of microencapsulated bacteria was improved in alginate microparticles over that of nonencapsulated bacteria during the storage period (*3*, *4*, *12*, *16*). Koo et al. (*16*) reported that *L. bulgaricus* YIT 9018 loaded in chitosan-coated alginate microparticles showed higher storage stability than free cell culture.

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