J. Microbiol. Biotechnol. (1999), **9**(2), 132–139

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Improvement of *Bifidobacterium longum* Stability Using Cell-Entrapment Technique

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Received: August 12, 1998

Abstract A cell-entrapment technique using compressed air was applied to *Bifidobacterium longum* KCTC 3128 for the improvement of bifidobacteria viability. The main cell-entrapment matrix used was alginate, and viability improvement of the *B. longum* entrapped in alginate lattices was monitored along with the effects of other additional biopolymers. A prerequisite for acquiring consistent results was the uniformity of bead size and cell distribution which was achieved by using compressed air and mixing the cell suspension with sterilized alginate powder, respectively. The viability losses of the *B. longum* entrapped in alginate beads in the presence of three different substances logarithmically increased in relation to the reaction time, and proportionately decreased with an increased alginate concentration and bead diameter. The strongest improvement in *B. longum* viability was exhibited with a bead containing 3% alginate and 0.15% xanthan gum.

Key words: Cell-entrapment technique, *Bifidobacterium longum*, alginate, viability loss

Bifidobacteria constitute a major part of the intestinal microflora of humans and animals [24]. The beneficial effects of bifidobacteria are well recognized, therefore, many products containing bifidobacteria, including fermented milk, yogurt, and health foods, are now produced for probiotic purposes [14]. In order to create the desired therapeutic effects, there must be a sufficient amount of bifidobacteria present in the food product. Accordingly, it is suggested that a minimum of 10^5cfu/g of viable bifidobacteria cells should be existent in a product at the time of consumption [14]. However, the number of viable bifidobacteria cells have been found to decrease in the presence of lactic acid bacteria (LAB), possibly due to inhibitory substances produced by *Lactobacillus* spp. and *Streptococcus* spp [19]. The main inhibitory substances produced by LAB are acids and hydrogen peroxide [10]. The pH of yogurt can decrease to 3.7 or below during storage in cool conditions, which may be detrimental to bifidobacteria. Similarly, the hydrogen peroxide produced by yogurt organisms, especially *Lactobacillus bulgaricus*, can also affect the viability of bifidobacteria. These bactericidal effects are synergistic in the presence of hydrogen peroxide under acidic conditions [22]. In the case of powdered dietary adjuncts, the number of viable bifidobacteria cells decreases during storage. When freeze dried or fermented products containing bifidobacteria are introduced to the alimentary canal (gastrointestinal tract), a large number of bifidobacteria cells are killed by the free hydrochloric acid present in the gastric juices [7, 9]. Despite withstanding the gastric transit, the surviving bifidobacteria will lose most of their viability during transit in the small intestine compartment due to bile salts secreted from the gall bladder into the duodenum [16]. Ibrahim and Bezkorovainy [15] reported that bifidobacteria showed a growth limitation in the presence of sodium glycocholate, however, they could recover their original character in TPY broth without bile salt. Grill *et al.* [11] reported that bifidobacteria deconjugated bile salts through conjugated bile salt hydrolase (BSH), which possibly also contributes to the composition of gut flora. Moreover, this activity accentuates the capacity of bile salts to inhibit certain microorganisms. In order to increase viability, lactic acid bacteria were microencapsulated within cross-linked chitosan membranes formed by emulsificaion/interfacial polymerization [12]. Rao *et al.* [21] reported that the survival of microencapsulated *B. pseudolongum* using cellulose acetate phthalate (CAP) was improved in simulated gastric and intestinal juices. In addition to these materials, several biopolymers such as gellan gum, -carrageenan/locust bean gum, and alginate are commonly used for immobilizing lactic acid bacteria [1, 4, 8, 20]. When immobilizing many kinds of cells, alginate is typically used as the ionic polysaccharide, in

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conjunction with calcium ions as the crosslinker, due to its gentle and simple immobilizing conditions. Substances that affect the survival of bifidobacteria, including organic acids, gastric acid, and bile salts, can permeate the gel immobilizing bifidobacteria [23].

The aim of this study was to establish the optimal conditions of bead preparation using alginate as a matrix for entrapping bifidobacteria, determine the effects of simulated gastric juices, bile salts, and hydrogen peroxide on the survival of *B. longum* entrapped in gel beads, and, finally, improve the stability of bifidobacteria using this optimal cell-entrapment technique.

MATERIALS AND METHODS

Bacterial Strain and Its Cultivation

B. longum KCTC 3128 was obtained from KCTC (The Korea Collection for Type Cultures, Genetic Resource Center, Taejon). This strain was routinely prepared for inoculum by incubation in an anaerobic system (Forma Scientific Inc., U.S.A.) filled with mixed gases consisting of N_2(75%), H_2(10%), and CO_2(5%) for 20 h at 37°C. It was then subcultured (20 h at 37°C) in TPY broth after inoculation in a trypticase-proteose peptone-yeast extract (TPY) broth containing 0.5% glucose as the sole carbohydrate source. The *B. longum* for entrapment was incubated for 20 h at 37°C in a 2.5-l fermenter containing 1,000 ml of TPY broth to which a fresh inoculum at 2% (v/v) had been added.

Bacterial Cell Entrapment

B. longum was harvested in the late exponential phase (for 20 h at 37°C) by centrifugation for 10 min at 3,000 rpm. Through centrifugation in a sterile disposable bag (Seward Co., U.K.), a cell suspension was prepared by mixing 250 ml of a 0.85% (w/v) NaCl saline solution with the wet cells obtained from 500 ml of the bifidobacterial culture. This cell suspension was then individually mixed with 5 g, 7.5 g, and 10 g of autoclaved alginate powder (Sigma. Co.) using the laboratory blender stomacher 400 (Seward Co., U.K.) to produce final alginate concentrations of 2, 3, and 4% (w/v). These mixtures were next transferred to the bead forming device (Fig. 1), and dropped through a 24G blunt-ended needle into a sterile 0.1 M $CaCl_2$ solution using compressed air (2 kg/cm^2) filtered by a sequence of 5, 1, 0.22 air filters set in the air compressor. The beads were hardened in this solution for 1 h. The size of the beads formed was about 3 mm in diameter. The mixture containing 3% alginate produced beads with 1.06, 1.70, and 3.17 mm diameters when using approximately 3, 2.3, and 2 kg/cm^2 of compressed air pressure, respectively. The diameters of the calcium alginate beads were measured with an eyepiece micrometer on an optical microscope at a magnification of 100×, their means and standard deviations also calculated. At least 100 randomly selected beads were measured for each sample, and then they were washed with sterile saline prior to use in order to remove any excess calcium ions and untrapped cells.

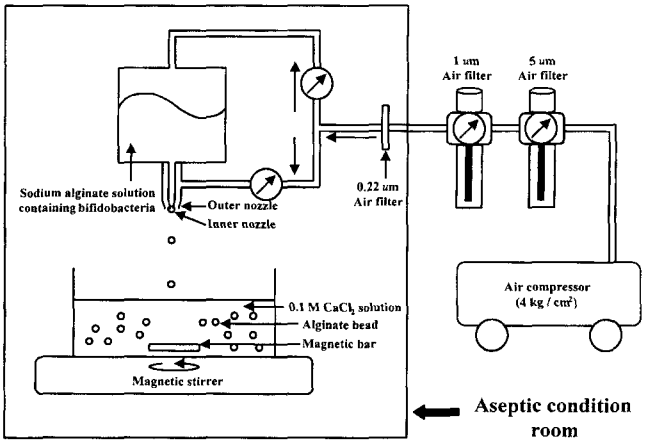

Fig. 1. The schematic diagram of a beads forming device.

Solubilization of Gel Beads

Potassium phosphate monobasic (Shinyo Co., Japan), sodium citrate (Shinyo Co., Japan), sodium phosphate dibasic (Shinyo Co., Japan), and sodium phosphate monobasic (Hayashi Ltd., Japan) were all tested for the dissolution of the alginate lattice. Ten beads containing 4% alginate and with a mean diameter of 3.13 mm were added to 30 ml of 0.01 M, 0.1 M, and 1 M of these solutions and mixed in the stomacher 400. The resulting solubilizing activities were then investigated.

Viable Cell Counts of *B. longum* Entrapped in Beads

Ten alginate beads were dissolved in 30 ml of a sterile solution of 0.1 M sodium citrate by mixing in the stomacher 400 for 10 min. This dissolved solution was then used for the viable cell count. In order to determine the number of living cells, 0.1 ml of the decimal dilutions in physiological saline were plated on TPY agar gel and incubated in an anaerobic system for 48 h at 37°C. Thereafter, the colonies grown in this medium were counted.

Structure of Alginate Lattice of Beads

The SEM specimens were prepared by drying the beads with entrapped *B. longum* and coating them with platinum for conductivity. These specimens were then examined using a Hitachi S-700 (Japan) scanning electron microscope at 10 kV (×3,000).

Selection of Entrapping Material by Addition of Biopolymers

The various entrapping materials used in this study are listed in Table 1. They were all sterilized in powder and

Table 1. List of entrapping materials used in this study.

Entrapping materials	Source
Soluble starch	Junsei Co., Japan
Whey powder	Sigma Co., USA
Insoluble starch	Oriental Chemical Ind., Korea
Xanthan gum	Jiwon Chemical Co., Korea
Agar	Showa Chemical Inc., Japan
Locust bean gum	Jiwon Chemical Co., Korea
Guar gum	Jiwon Chemical Co., Korea
Gum arabic	Jiwon Chemical Co., Korea
Pectin	Jiwon Chemical Co., Korea
Gelatin	Jiwon. Chemical Co., Korea

mixed with the cell suspension to obtain a final concentration of 0.3% (w/v). The resultant concentrations of xanthan gum and alginate were 0.15% (w/v) and 3% (w/v), respectively. These mixtures were then used to make 3-mm diameter beads.

Acid, Bile Salts, and Hydrogen Peroxide Tolerances of Entrapped *B. longum*

The acid tolerance of the *B. longum* entrapped in the beads was determined by measuring the viability of bacterium after exposure to simulated gastric juices mainly consisting of HCl. Simulated gastric juices without pepsin [0.08 M HCl containing 0.2% (w/v) NaCl, pH 1.33] were prepared as described by Rao *et al.* [21]. An experiment was conducted in which 100 beads were exposed to 10 ml of simulated gastric juices for 3 h at 37°C. During this exposure, 10 beads were harvested at various intervals, washed with a physiological saline solution, and then dissolved in a 0.1 M sodium citrate solution. A bile salt solution (pH 7.34) containing 0.6% Oxgall (Difco Co.) was used to determine bile salt tolerance of *B. longum* entrapped in alginate beads. Accordingly, 100 beads were exposed to 10 ml of the 0.6% bile salt solution for 6 h at 37°C. Ten exposed beads were harvested at intervals of 1 h, washed with a physiological saline solution, and then dissolved in a 0.1 M sodium citrate solution. The hydrogen peroxide tolerance of the *B. longum* entrapped in the beads was determined by measuring of the viability of the bacterium after exposure to hydrogen peroxide solution. A hydrogen peroxide solution was prepared that contained 1,000 ppm of H_2O_2. Accordingly, 100 beads were exposed to 10 ml of the hydrogen peroxide solution for 3 h at 37°C. Ten exposed beads were harvested at intervals of 1 h, washed with a physiological saline solution, and then dissolved in a 0.1 M sodium citrate solution. The total living cell numbers were determined using the plate count method. For untrapped cells, the wet cells obtained from the centrifugation of 10 ml of the bifidobacterial culture were all individually resuspended in 10 ml of simulated gastric juices, a bile salt solution, and a hydrogen peroxide solution. The exposure to simulated gastric juices, bile salt solution, and hydrogen peroxide solution was continued for 3 h, 6 h, and 3 h, respectively at 37°C. One ml of the resuspended solution was harvested each hour and the viable cell numbers were determined.

Stability of Entrapped *B. longum* in an Acidified Milk

One liter of fresh milk was pasteurized for 30 min at 100°C. The pasteurized milk was adjusted to pH 4.5 by mixing with acetic acid and lactic acid filtered using a 0.22 μm membrane filter in the mole proportion of 3 to 2. Thirty ml of a rennet solution [1% (w/v) rennet containing 6% (w/v) NaCl] filtered by 0.22 μm membrane filter was then added to the adjusted milk. Beads entrapping *B. longum* were then stored in the acidified milk at 4°C, and the bifidobacteria viable cell number was determined after 2, 5, 7, and 10 days.

Results and Discussion

Preparation of Beads

B. longum was mixed with different alginate concentrations and beads were prepared using a compressed air device as illustrated in Fig. 1. Different sizes of 3% alginate beads, measuring 1.06, 1.70, and 3.17 mm in diameter, were obtained through a 24G needle using about 3.0, 2.3, and 2.0 kg/cm^2 of compressed air pressure, respectively. As shown in Fig. 2, the sizes were distributed within narrow ranges. Therefore, similar bead types had almost identical sizes. Several researchers, including Audet and Lacroix [2], studied bead formation using a two-phase method applying phase separation between oil and water. The fractionation of these beads by size required the use of several sieves with different pore sizes [8, 18]. Variations in bead size create difficulties in the correct determination of the survival

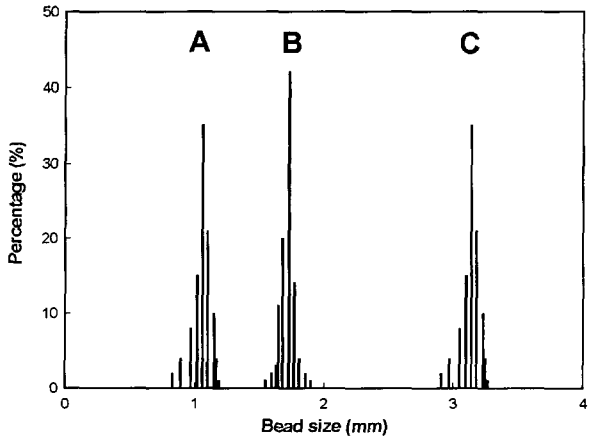

Fig. 2. The size distribution of three kinds of 3% alginate beads entrapping *B. longum*.
A: 1.06 mm diameter beads; B: 1.70 mm diameter beads; C: 3.17 mm diameter beads.

changes of *B. longum* entrapped in beads in the presence of acid, bile salts, or hydrogen peroxide. Accordingly, the two-phase method uses a peristaltic pump equipped with a syringe to produce beads with almost the same size [2]. Although the harvested beads are almost the same size, their size differences are, however, greater than those of the beads produced by the device used in this study. Furthermore, the two-phase method is not suitable for large-scale bead production, its ability to regulate bead diameter is limited according to the change between the nozzle size of the syringe and the ejection power of the peristaltic pump, plus it has difficulties in forming beads from high viscosity solutions. Therefore, in terms of bead size distribution, the method employed in this study is superior to those reported on earlier. The preparation of the alginate/cell mixture for bead preparation is usually performed by the addition of wet cells to a previously sterilized sodium alginate solution under aseptic conditions [2, 17]. A homogeneous mixture, therefore, cannot be obtained because the density of the sodium alginate solution is higher than that of the cell suspension. Consequently, a uniform cell number for each bead produced using this solution cannot be expected. This problem can be solved by the addition of sterilized sodium alginate powder to the previously mixed cell suspension. As a result, there is a uniform distribution of cells in the alginate/cell mixture plus a nearly identical cell number can be entrapped in each bead.

Solubilization of Beads

Phosphate and citrate dissolve alginate beads by removing the calcium ion from a calcium-alginate lattice. Solutions of these salts were prepared in concentrations of 0.01 M, 0.1 M, and 1 M. Table 2 indicates the results of the solubility of 4% alginate beads in 30 ml of each concentration. 4% alginate beads were found to dissolve within 10 min in all the concentrations of the sodium citrate solution. The solubility of beads in a sodium phosphate dibasic solution was observed to be slightly lower than in a sodium citrate solution, and significantly higher than in either a potassium phosphate monobasic solution or a sodium phosphate monobasic solution. Based on these results, a sodium citrate solution was selected to dissolve the alginate beads. A cell suspension of *B. longum* (3.20×10^8 cfu/ml) was mixed with 0.01, 0.1, and 1 M concentrations of a sodium citrate solution and incubated for 10 min before the viable cells were counted. The survival percentages of the bacterium in the 0.01 M, 0.1 M, and 1 M concentrations of a sodium citrate solution were about 98%, 96%, and 79%, respectively (Table 3). The 0.1 M concentration of a sodium citrate solution was finally selected for dissolving the alginate beads due to its rapid dissolving time and the minimal changes of viable cell numbers observed during the dissolution of the beads.

Table 2. Solubility of 4% alginate beads of 3.13 mm mean diameter containing *B. longum* by phosphate and citrate salts solutions.

Kinds of solutions	Concentration (M)	pH	Time required for complete dissolution (min)
Sodium citrate	0.01	8.20	10
	0.1	8.21	4
	1	8.26	4
Sodium phosphate dibasic	0.01	8.98	16
	0.1	9.08	8
	1[a]	-	-
Sodium phosphate monobasic	0.01	5.00	> 30[b]
	0.1	4.55	> 30
	1	4.08	14
Potassium phosphate monobasic	0.01	4.99	> 30
	0.1	4.52	> 30
	1	4.19	15

[a]Not tested because of the supersaturated solution.
[b]Over 30 min.

Table 3. Survival of *B. longum* in sodium citrate solution of different concentrations.

Sodium citrate concentration (M)	Viable cell counts ($\times10^8$)		Survival (%)
	Initial	After 10 min	
0.01	3.20	3.15	98
0.1	3.20	3.08	96
1	3.20	2.52	79

Effects of the Concentration of the Entrapping Material and Bead Size on the Survival of the Entrapped *B. longum*

Beads with a diameter of about 3 mm were prepared separately using 2%, 3%, and 4% (w/v) alginate under 2 kg/cm^2 of compressed air pressure. In addition, 3% alginate beads with 1.06±0.05 and 1.70±0.09 mm diameters were prepared under 3.0 and 2.3 kg/cm^2 of compressed air pressure, respectively. These beads were then independently exposed to simulated gastric juices for 3 h. Figure 3 shows the results of the viable cell numbers of entrapped *B. longum* after exposure to simulated gastric juices. The viable cell numbers decreased exponentially. In the case of 2% alginate beads with a 3.14±0.23 mm diameter, the viable cell numbers of *B. longum* were 1.71×10^7 cfu/bead before exposure and 2.60×10^4 cfu/bead after exposure. In the case of 3% alginate beads with a 3.17±0.22 mm diameter, the viable cell numbers of *B. longum* decreased from 2.88×10^7 cfu/bead to 1.26×10^5 cfu/bead after 3 h. In the case of 4% alginate beads with a 3.13±0.15 mm diameter, the viable cell numbers of *B. longum* were 2.71×10^7 cfu/bead before exposure and 4.94×10^5 cfu/bead after 3 h.

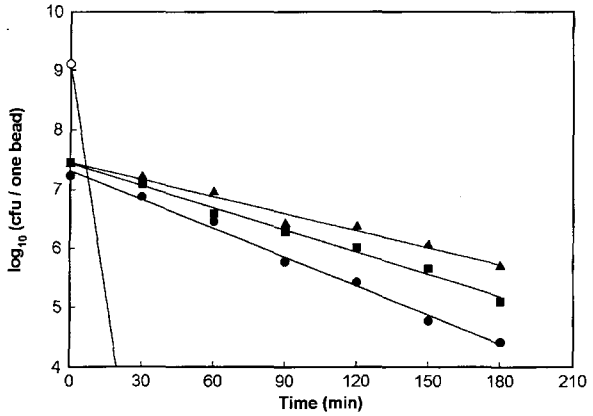

Fig. 3. Survival of *B. longum* entrapped in alginate beads with different alginate concentrations after the exposure to simulated gastric juices.
●: 2% (w/v) alginate bead; ■: 3% (w/v) alginate bead; ▲: 4% (w/v) alginate bead; ○: untrapped *B. longum*.

Therefore, the survival of *B. longum* entrapped in 2, 3, and 4% alginate beads was 0.2, 0.4, and 1.8%, respectively. The viable cell numbers of untrapped *B. longum* were 1.27×10^9 cfu/ml before exposure and below 10^3 cfu/ml within 30 min. These results indicate that the higher the concentration of alginate in the beads, the lower the death rate of the *B. longum* entrapped in the beads.

Beads of different sizes were prepared using a 3% alginate mixture in order to test the effect of bead size on the survival characteristics of entrapped *B. longum* in the presence of simulated gastric juices (Fig. 4). The viable cell numbers of *B. longum* in beads with a 1.06 mm diameter were 7.82×10^5 cfu/bead initially, however, none were detected after exposure to simulated gastric juices for 3 h. When 3% alginate beads with a 1.70 mm diameter were used, the viable cell numbers of *B. longum* were

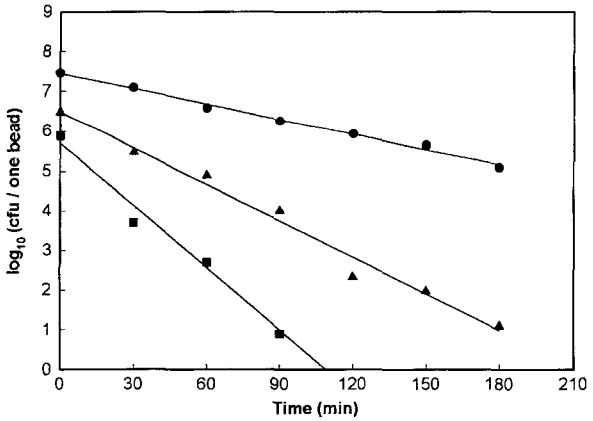

Fig. 4. Survival of *B. longum* in 3% alginate beads with the various bead diameters after the exposure to simulated gastric juices.
■: 1.06 mm diameter bead; ▲: 1.70 mm diameter bead; ●: 3.17 mm diameter bead.

3.15×10^6 cfu/bead initially and 1.25×10 cfu/bead after 3 h. As expected, a lower death rate of entrapped *B. longum* was experienced with a larger bead. Betty and Stephanopoulos [3] reported that ethanol rather than glucose would rapidly penetrate the alginate lattice since the molecular weight of ethanol was smaller than that of glucose. They also claimed that the slower diffusion of these substances into beads containing higher concentrations of alginate was due to the lower number and depth of pores rather than pore size. Tanaka *et al.* [23] reported that the diffusion of a substance with a high molecular weight into alginate beads was limited by an increasing alginate concentration in a bead. In addition, they observed that glucose was freely diffused into 2% alginate gel beads with a 3.3±0.2 mm diameter and its concentration between the inner beads and solution was equalized within 30 min. Chen *et al.* [6] reported that the diffusion coefficients of Cu^{2+} and other small species into calcium alginate beads are close to that of water. It is speculated that the proton (H^+) of hydrochloric acid, a major component of simulated gastric juices, can freely diffuse into alginate beads within 3 h because its molecular weight is significantly smaller than that of Cu^{2+}. However, the improvement of entrapped *B. longum* in the presence of simulated gastric juices with an increased alginate concentration or bead size cannot be satisfactorily explained. A decrease in the diffusion rate of protons into the inner bead due to a decrease in the number or depth of the pores and an increase in the bead size is insufficient justification. Accordingly, Krisch and Szajani [17] reported that the resistance of bacteria against solutes such as ethanol and acetic acid is related to their physiological change. Hilge-Rotmann and Rehm [13] reported that cells entrapped in alginate beads contained more saturated fatty acid than untrapped cells. Castillo-Agudo [5] suggested that the more ethanol tolerant *Saccharomyces* strains showed a clear reduction in fatty acid unsaturation compared with the ethanol-sensitive strains. We conclude that the physical characteristics of the calcium-alginate lattice and the physiological changes in the cells are both involved in the increased survival of bifidobacteria entrapped in alginate beads in the presence of simulated gastric juices.

Structure of the Alginate Lattice of Beads

The structure of the alginate lattice of beads was investigated using a scanning electron microscope (SEM, Hitachi S-700, Japan) after cutting dried alginate beads with a razor. It was observed that the rod-shaped *B. longum* entrapped in 3% alginate beads was evenly distributed in the alginate lattice (Fig. 5). A large number of pores were also observed in the alginate lattice. Therefore, substances influencing the survival of bifidobacteria such as acids, bile salts, and hydrogen peroxide are able to penetrate the beads through these pores in the alginate lattice. To improve the survival of entrapped *B. longum* against harmful substances, it is

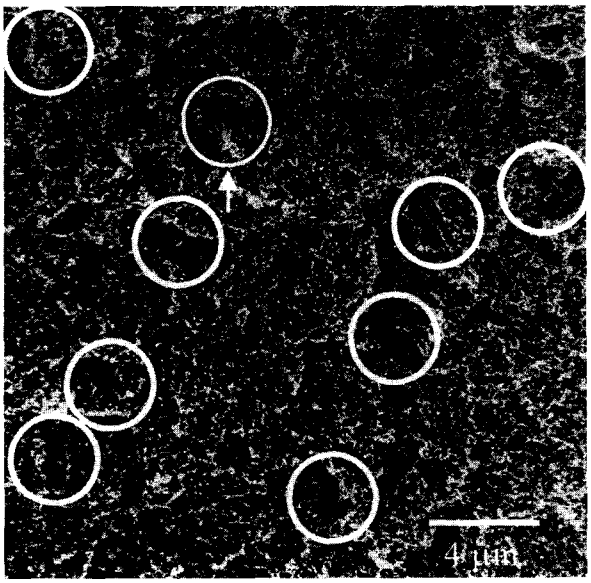

Fig. 5. SEM photograph of *B. longum* entrapped in alginate bead.

important to either reduce the number and size of the pores in the alginate lattice or devise other methods to decrease the diffusion of these harmful substances into the interior of the beads.

Acid Tolerance of *B. longum* Entrapped in Beads Mixed with Protective Materials

Beads containing various biopolymers were prepared in order to investigate the survival of *B. longum* entrapped in beads in the presence of acid in relation to a variation in the entrapping materials. Table 4 indicates that the survival of *B. longum* entrapped in calcium alginate beads containing biopolymers in the presence of simulated gastric juices varied according to the biopolymer added. The viable cell numbers of *B. longum* entrapped in beads containing only 3% alginate decreased from 2.88×10^7 cfu/bead to 1.26×10^5 cfu/bead after exposure to simulated gastric juices for 3 h (0.4% of survival percentage). Whereas, the survival of bifidobacteria entrapped in beads prepared with 0.3% soluble starch, 0.3% whey, or 0.15% xanthan gum to 3% alginate in the presence of simulated gastric juices was higher than that of bifidobacteria entrapped in beads containing only 3% alginate. Xanthan gum was used at a concentration of 0.15% to obtain a similar bead density compared to other additives. From among the beads with added biopolymers, the *B. longum* entrapped in beads mixed with 3% alginate and 0.15% xanthan gum exhibited the highest survival against simulated gastric juices. The viable cell numbers decreased from 2.5×10^7 cfu/bead to 3.71×10^6 cfu/bead after 3 h (14.8% survival rate). Thus, the survival rate of bifidobacteria with the addition of 0.15% xanthan gum was 37 times higher than the control. In the case of beads prepared with additions of either 0.3% insoluble starch, 0.3% agar, 0.3% locust bean gum, 0.3% guar gum, 0.3% pectin, or 0.3% gelatin to 3% alginate, the survival of *B. longum* entrapped in beads in the presence of simulated gastric juices was consistently lower than that of bacterial cells entrapped in beads containing only 3% alginate. In conclusion, we determined that the optimal entrapping material for improving the survival of *B. longum* was 3% alginate mixed with 0.15% xanthan gum.

Bile Salt Tolerance of Entrapped *B. longum*

Beads containing *B. longum* were exposed to a solution containing 0.6% ox gall (Difco Co.) which has a similar composition to human bile. Figure 6 shows that the viable cell numbers of *B. longum* entrapped in beads with 3% alginate and an addition of 0.15% xanthan gum decreased from 2.50×10^7 cfu/bead to 1.02×10^7 cfu/bead after exposure to a 0.6% ox gall solution for 6 h (40.8% survival rate). However, the viable cell numbers of *B. longum* entrapped

Table 4. Survival of *B. longum* in 3% alginate beads after the exposure to simulated gastric juices with various additives.

Types of beads	Addition (%)	Diameter of beads (mm)[a]	Viable cell counts ($\log_{10}$cfu/one bead)	
			for Control	after 3 h
Alginate	N[b]	3.170.22	7.459	5.101
Alginate+Soluble starch	0.3	3.180.13	7.342	5.594
Alginate+Whey	0.3	3.150.28	7.121	5.493
Alginate+Insoluble starch	0.3	3.110.31	7.436	5.021
Alginate+Xanthan gum	0.15	3.140.15	7.398	6.569
Alginate+Agar	0.3	3.100.21	6.753	3.780
Alginate+Locust bean gum	0.3	3.190.23	7.513	4.566
Alginate+Guar gum	0.3	3.180.24	6.810	4.063
Alginate+Gum arabic	0.3	3.090.21	6.495	<3
Alginate+Pectin	0.3	3.120.25	7.360	3.297
Alginate+Gelatin	0.3	3.140.18	7.198	<3

[a]Each value represents mean diameter based on 100 beads.
[b]No additive.

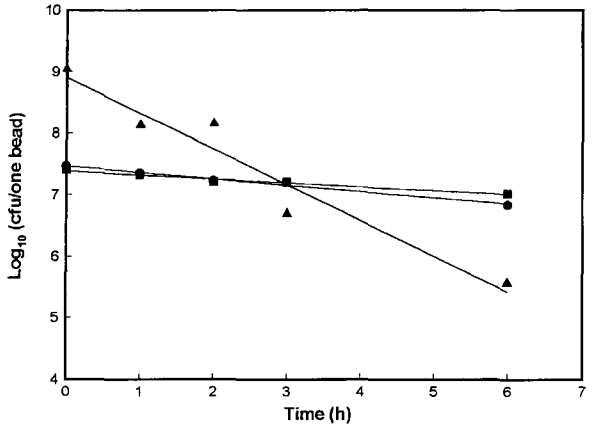

Fig. 6. Survival of *B. longum* in 3% alginate beads with or without 0.15% (w/v) xanthan gum and untrapped *B. longum* after the exposure to bile salts solution.
●: 3% alginate bead; ■: 3% alginate mixed with 0.15% xanthan gum bead; ▲: untrapped *B. longum*.

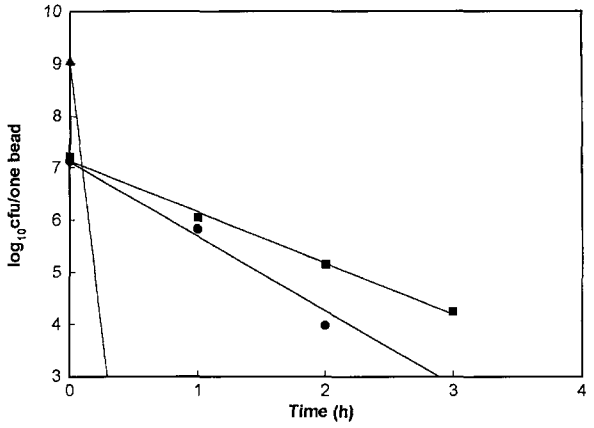

Fig. 7. Survival of *B. longum* in 3% alginate beads with or without 0.15% (w/v) xanthan gum and untrapped *B. longum* after the exposure to hydrogen peroxide solution.
●: 3% alginate bead; ■: 3% alginate mixed with 0.15% xanthan gum bead; ▲: untrapped *B. longum*.

in beads with only 3% alginate decreased from 2.87×10^7 cfu/bead to 6.65×10^6 cfu/bead after 6 h (23.2% survival rate), and the viable cell numbers of untrapped *B. longum* decreased from 1.09×10^9 cfu/ml to 3.63×10^5 cfu/ml after 6 h (0.03% survival rate). Consequently, the survival of *B. longum* was improved by 18% with the addition of 0.15% xanthan gum. These results indicate that the entrapment of *B. longum* in 3% alginate beads mixed with 0.15% xanthan gum is very effective in improving the survival of the bacterium against bile salts.

Hydrogen Peroxide Tolerance of Entrapped *B. longum*

The hydrogen peroxide tolerance of entrapped *B. longum* was tested using a 1,000 ppm hydrogen peroxide solution. The viable cell numbers of *B. longum* entrapped in beads with 3% alginate decreased from 1.32×10^7 cfu/bead to 9.95×10^2 cfu/bead after exposure to this diluted hydrogen peroxide solution for 3 h (0.007% survival rate). The viable cell numbers of the bacterium entrapped in beads with a mixture of 3% alginate and 0.15% xanthan gum after 3 h were 0.1% of the initial viable cell numbers. The addition of 0.15% xanthan gum increased the survival rate of *B. longum* 15.5 times over. The viable cell numbers of untrapped *B. longum* decreased from 1.06×10^9 cfu/ml to 10^3 cfu/ml after exposure to a 1,000 ppm hydrogen peroxide solution for 1 h, and none were detected after 2 h (Fig. 7). These results show that a 1,000 ppm hydrogen peroxide solution was more detrimental to the survival of bifidobacteria than simulated gastric juices or a 0.6% bile salt solution. Accordingly, hydrogen peroxide is the major concern during bead preparation and storage, whereas, gastric juices are the major constraint to maintaining the viability of *B. longum* entrapped in alginate beads in the gastrointestinal tract.

Stability of Entrapped *B. longum* in Acidified Milk

The survival of *B. longum* entrapped in beads was investigated after these beads were stored for 10 days in acidified milk with a pH of around 4.5. Figure 8 shows that the viable cell numbers of untrapped *B. longum* decreased from 2.23×10^9 cfu/ml to 4.48×10^8 cfu/ml after storage. It was also observed that the viable cell numbers of *B. longum* entrapped in 3% alginate beads, both with and without 0.15% xanthan gum, hardly changed during the storage. Thus, the *B. longum* entrapped in beads had a better survival rate in the acidified milk environment than the untrapped bacterial cells. In conclusion, entrapment in beads containing 3% alginate and 0.15% xanthan gum significantly improves the survival of the bacterium during the storage and distribution of acidified milk products, and facilitates the delivery of a higher number of viable cells to

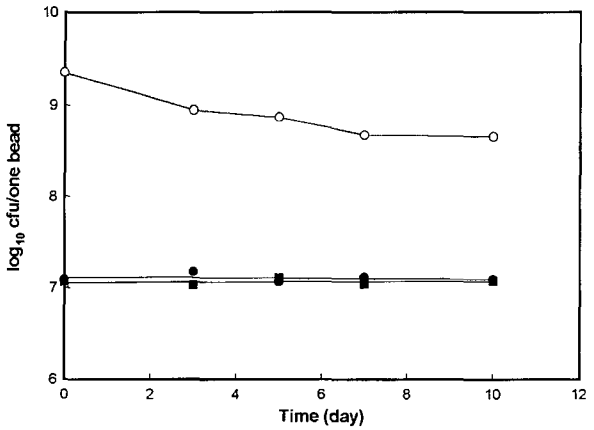

Fig. 8. Survival of *B. longum* in 3% alginate beads with or without 0.15% xanthan gum and untrapped *B. longum* during storage in fermented milk for 10 days.
●: 3% alginate bead; ■: 3% alginate mixed with 0.15% xanthan gum bead; ○: untrapped *B. longum*.

the large intestine through the stomach and the small intestine.

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