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Survival of *Bifidobacterium breve* in Acidic Solutions and Yogurt, Following Immobilization in Calcium Alginate Beads

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Abstract Sodium alginate was used to immobilize *Bifidobacterium breve* ATCC 15700 cells. The ability of the Ca-alginate beads to protect the *B. breve* ATCC 15700 was evaluated under different conditions including alginate concentration, bead size, pH, hydrogen peroxide, and storage period. The survival of the *B. breve* ATCC 15700 was estimated in pasteurized yogurt, containing either the immobilized or free cells, throughout the storage period. The survival cells in bead after exposure to acidic solution (pH 3.0) increased with increase of both the alginate gel concentration and bead size. Also, immobilized cells in alginate bead were more resistant than the free cells to hydrogen peroxide, storage period, and the environment inside yogurt. When retreated beads with skim milk and nonretreated beads were tested in acidified pH 3.0 TPY media including acetic and lactic acid, the number of viable cells in the retreated bead was approximately 10-fold higher than that of nonretreated beads. This suggests that the skim milk operated as a material decreasing the diffusion of acid and hydrogen peroxide into alginate gels. From this research, it was found that yogurt itself supported immobilized cells with an improved protection from the extreme acidity in yogurt.

Key words: *Bifidobacterium breve*, immobilization, Ca-alginate, yogurt, survivability

Bifidobacteria are a part of the normal intestinal microflora of humans and animals, and play an important beneficial role in the host's health [1, 17]. When they present in sufficient numbers, bifidobacteria maintain a favorable balance between the population of beneficial and potentially harmful microorganisms in the gastrointestinal tract [7]. However, factors such as gastrointestinal disorders, stress, antibacterial agents, and aging can disturb the balance of intestinal microflora, significantly decrease the number of beneficial bifidobacteria, and result in gastroenteritis with symptoms such as abdominal cramps, fever, diarrhea, vomiting, and other chronic gastrointestinal diseases [1, 12, 23]. Attempts have been made to achieve a balanced intestinal microflora by inclusion of bifidobacteria into food [15, 24]. Yogurt is commonly used as a vehicle to deliver bifidobacteria [18]. In order to create the desired health promoting effects, there must be a sufficient number of bifidobacteria present in food products. Accordingly, it is suggested that a minimum of 10^5 CFU/g of viable bifidobacteria cells should exist in a product at the time of consumption [13]. However, the numbers 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. [21]. Major inhibitory substances produced by LAB are acids and hydrogen peroxide [8]. The pH level of yogurt can decrease to 3.7 or below during storage in cool conditions, which may be detrimental to the survival of bifidobacteria. Similarly, hydrogen peroxide produced by yogurt organisms, especially *Lactobacillus bulgaricus*, can also affect the viability of bifidobacteria [25]. These bactericidal effects are synergistic in the presence of hydrogen peroxide under acidic conditions [14, 29]. Therefore, a high number of viable bifidobacteria in yogurt may not always be maintained because of the acidity of yogurt and the low acid tolerance of bifidobacteria [16]. Furthermore, delivery of viable bifidobacteria to the large intestine has been limited because of the extreme acidity found in the human stomach [3]. Thus, the success of bifidobacteria-containing food products depends on the viability of bifidobacteria in the product during its shelf life as well as on the resistance of the bacteria to the conditions existing in the upper gastrointestinal tract. It was reported that the immobilization of bifidobacteria could be used as a method to ensure greater survival rate

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under acidic, gastric, and intestinal conditions [26]. In order to increase survivability, lactic acid bacteria were microencapsulated within crosslinked chitosan membranes formed by emulsification/interfacial polymerization [9]. Rao *et al.* [26] 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, κ-carrageenan/locust bean gum, and alginate are commonly used for immobilizing lactic acid bacteria [4, 2, 22]. When immobilizing different types of cells, alginate is typically used as the ionic polysaccharide, in conjunction with calcium ions as the crosslinker, due to its gentle and simple immobilizing conditions. However, substances that affect the survival of bifidobacteria, including organic acids, gastric acid, and bile salts, can permeate the gel immobilizing bifidobacteria [26].

In this study, calcium alginate was used to immobilize *B. breve* ATCC 15700 cells, the ability of the Ca-alginate beads to protect the *B. breve* was evaluated under different conditions including alginate concentration, bead size, pH, hydrogen peroxide, and storage period, and the survival of the *B. breve* in yogurt containing either the immobilized or free cells throughout the storage period was estimated.

B. breve ATCC 15700 purchased from the American Type Culture Collection (ATCC), Rockville, MD, U.S.A., was used in this study. This strain was routinely prepared for an inoculum by incubating in an anaerobic system (Forma Scientific Inc., U.S.A.) filled with mixed gases consisting of N_2 (75%), H_2 (10%), CO_2 (5%) for 24 h at 37°C. It was subsequently subcultured (20 h at 37°C) in trypticase-proteose peptone-yeast extract (TPY) broth containing 0.5% glucose as a sole carbohydrate source. *B. breve* ATCC 15700 for entrapment was incubated for 20 h at 37°C in a 2.5 l fermenter containing 1,000 ml of TPY broth where a fresh inoculum at 2% (v/v) was added.

Sodium alginate (Jiwon Tech., Co., Korea) was used to immobilize *B. breve* ATCC 15700 cells. Polymer powder was dispersed in physiological saline (0.85% NaCl) by gentle stirring. This solution was continuously stirred to achieve complete hydration of the polymer. The solution was autoclaved for 15 min at 121°C. Bifidobacterial cells from an overnight (20 h) culture were immobilized by mixing one part of the culture concentrate with four parts of sodium alginate solution. Beads were manufactured by using either the two-phase or extrusion technique methods. In the case of the two-phase method, cells were entrapped by mixing one part of the culture concentrate with four parts of sodium alginate (selected concentration). One part of the mixture was then added dropwise to five parts of soybean oil (250 ml in a 800 ml beaker) which was stirred at 200 rpm by a magnetic stirrer. Within 10 min, a uniformly turbid emulsion was obtained with no evidence of a free aqueous phase. Calcium chloride (0.05 M) was added quickly but gently (20 ml/sec) down the side of the beaker until the water/oil emulsion was broken. Calcium-alginate beads were formed within 10 min. These beads were collected by using a sieve (S4020, Sigma, U.S.A.) and washed with sterile saline water. In the case of the extrusion technique method, beads were manufactured by dropping polymer solution into 0.05 M $CaCl_2$ solution through a syringe needle (21G) under gentle stirring. The formed beads were hardened in $CaCl_2$ solution for 60 min, separated from the solution with a sterile stainless steel sieve (S4020, Sigma, U.S.A.), and washed with sterile saline solution.

The beads of different sizes were prepared by adjusting the stirring speed and needle gauge. Diameters of the calcium alginate beads were measured with an eyepiece micrometer on an optical microscope at a magnification of 100×. At least 100 randomly selected beads were measured for each sample. The total viable numbers of untrapped *B. breve* ATCC 15700 expressed as CFU were determined by the plate count method using TPY agar. To count the viable cell numbers in beads, 100 beads were washed with a sterile saline solution and dissolved for 5 min in 30 ml of sterile 1% sodium citrate solution with the aid of a Stomacher 400 (Seward Co., U.K.). The cultivation of *B. breve* ATCC 15700 on a TPY agar plate was carried out anaerobically under N_2 (75%), H_2 (10%), CO_2 (5%) at 37°C for 48 h.

Beads with a diameter of about 3.30 mm were prepared separately by using 1%, 2%, 3%, and 4% (w/v) alginate. These beads were then independently exposed to 10 ml of the acidified TPY broth which adjusted to pH 3.0 by mixing with acetic acid and lactic acid in the mole proportion of 3 to 2 for 5 h. Figure 1 shows the results of the viable cell

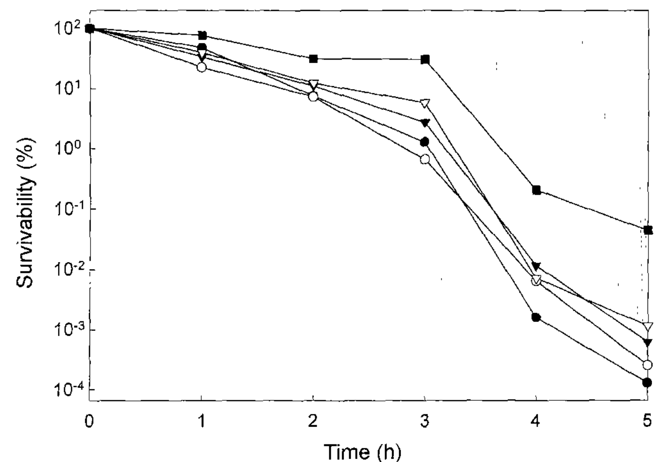

Fig. 1. Survival of *Bifidobacterium breve* ATCC 15700 in 3.30 mm diameter of beads in relation to the change of Ca-alginate concentration after being exposed to the acidified TPY broth.

●, free cell; ○, 1% alginate bead; ▼, 2% alginate bead; ▽, 3% alginate bead; ■, 4% alginate bead.

numbers of immobilized *B. breve* ATCC 15700 after being exposed to the acidified TPY broth. The viable cell numbers decreased with the time that the immobilized *B. breve* ATCC 15700 was exposed to the acidic solution. In the case of 1% alginate beads, the viable cell numbers of *B. breve* ATCC 15700 were 3.61×10^6 CFU/bead before exposure and 9.33 CFU/bead after exposure. In the cases of 2%, 3%, and 4% alginate beads, the viable cell numbers of *B. breve* ATCC 15700 decreased from 6.42×10^6 CFU/bead, 4.83×10^6 CFU/bead, and 3.14×10^6 CFU/bead to 4.02×10 CFU/bead, 5.60×10 CFU/bead, and 1.36×10^3 CFU/bead after 5 h, respectively. The best survivability of *B. breve* ATCC 15700 was obtained with beads made of 4% sodium alginate. These results indicate that the concentration of alginate had a significant effect on cell survivability; the higher the concentration of alginate, the lower the death rate of *B. breve* ATCC 15700 immobilized in the beads. The viable cell numbers of nonimmobilized *B. breve* ATCC 15700 were 1.31×10^8 CFU/ml before exposure and below 1.63×10^2 CFU/ml within 5 h. In the acidic conditions, therefore, survivability of immobilized *B. breve* ATCC 15700 was higher than that of the free cells. Beads of different sizes were prepared by using a 4% alginate mixture to test the effects of beads size on the survival characteristics of immobilized *B. breve* ATCC 15700 against the acidified TPY broth, whose pH was adjusted to 3.0 by mixing with acetic acid and lactic acid in the mole proportion of 3 to 2 for 5 h. Figure 2 shows that the maximal survivability was obtained with immobilized cells in beads with diameters of 3.70 mm. The number of leaked cells gradually decreased as the bead diameter became larger until it reached to 3.70 mm, and thereafter it did not increase significantly with a larger bead diameter.

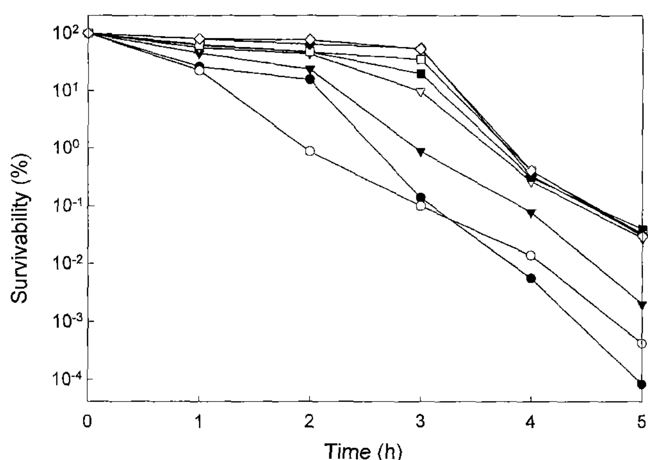

Fig. 2. Survival of *Bifidobacterium breve* ATCC 15700 in 4% Ca-alginate beads in relation to the change of bead size after being exposed to the acidified TPY broth.
●, free cell; ○, 0.30 mm bead; ▼, 2.98 mm bead; ▽, 3.30 mm bead; ■, 3.79 mm bead; □, 4.91 mm bead; ◆, 5.10 mm bead; ◇, 8.00 mm bead.

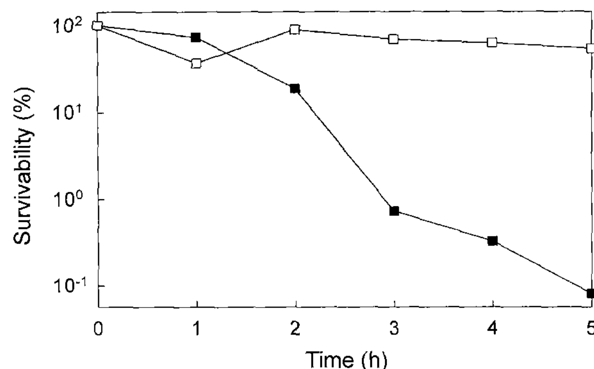

Fig. 3. Survival of entrapped cells after being exposed to 1,000 ppm of hydrogen peroxide for 5 h.
■, free cells; □, immobilized cells.

The hydrogen peroxide tolerance of immobilized *B. breve* ATCC 15700 was tested in modified TPY containing 1,000 ppm of hydrogen peroxide (Fig. 3). The viable cell numbers of *B. breve* ATCC 15700 immobilized in beads with 4% alginate decreased from 3.31×10^6 CFU/bead to 1.77×10^6 CFU/bead after being exposed to this diluted hydrogen peroxide solution for 5 h (53.5% survival), whereas free cells decreased from 1.67×10^8 CFU/ml to 1.35×10^5 CFU/ml (0.8×10^{-3}% survival). These results indicate that the immobilized cells were more resistant to hydrogen peroxide than the nonimmobilized cells.

The survivability of immobilized and free cells in 4°C (cold condition) for 2 weeks is shown in Fig. 4. The number of immobilized cells decreased slightly from 2.87×10^6 CFU/bead to 1.19×10^5 CFU/bead, whereas the number of nonimmobilized cells dropped markedly from 2.13×10^8 CFU/ml to 2.53×10^4 CFU/ml. It is clear that the immobilized cells show an enhanced survivability during storage at 4°C.

To estimate the survivability of the *B. breve* ATCC 15700 in yogurt containing either the immobilized or free

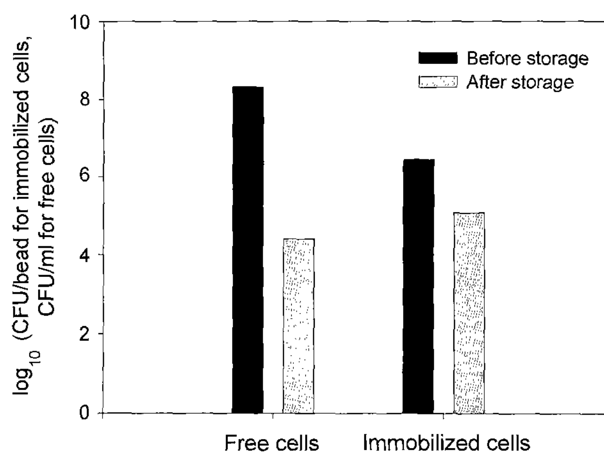

Fig. 4. Survival of immobilized cells and free cells stored at 4°C for 2 weeks.

cells throughout the storage period, acidified milks were manufactured on a small scale (1,000 ml) as follows: one liter of fresh milk purchased from a local supermarket was pasteurized at 85°C for 30 min. After cooling to 42°C, milk was inoculated [0.02% (w/v)] with a lactic starter culture (YC-180, Hansen's Lab., Denmark), and incubated at 42°C until the pH reached 4.4. Yogurt (250 ml) in 500 ml sterilized bottles was pasteurized at 85°C for 30 min to kill the lactic cultures. Yogurt samples were tested randomly by plating to ensure no viable lactic culture remained before inoculating with either immobilized or free bifidobacterial cells. For determining the survivability of immobilized and free cells, beads (about 5,000 each) and free cells (2% subcultured bifidobacterial cell) were each separately mixed with 250 ml of yogurt in a sterile bottle and stored at 4°C for up to 4 weeks. The survivability of bifidobacteral cells was determined at weekly intervals by sampling the entire contents of individual containers. Free cells (2% bifidobacterial culture) were subjected to the same treatment as beads. The survivability of immobilized and free cells of *B. breve* ATCC 15700 in pasteurized yogurt at 4°C is shown in Fig. 5. The viable count of immobilized cells dropped from 3.10×10^6 CFU/bead to 6.78×10^5 CFU/bead in 4 weeks, and that of free cells from 2.45×10^8 CFU/ml to 4.10×10^4 CFU/ml in the same time period. Moreover, the significant difference in viability between immobilized and free cells was observed at the end of the first week.

To estimate the acid tolerance of immobilized bifidobacterial cells in yogurt, immobilized beads were soaked in pasteurized 10% skim milk broth for 30 min. The retreated beads were then washed with 0.85% sterile saline solution and the acid tolerance of the immobilized cells in the beads was tested in acidified (pH 3.0) TPY media including acetic and lactic acids.

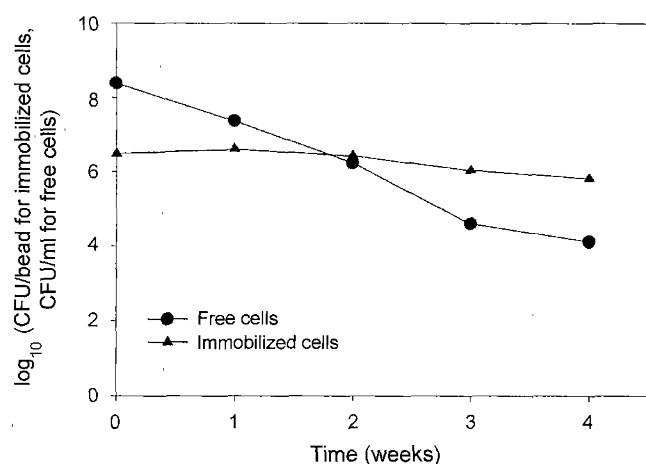

Fig. 5. Survival of immobilized cells and free cells of *Bifidobacterium breve* ATCC 15700 in pasteurized yogurt at 4°C for 4 weeks.

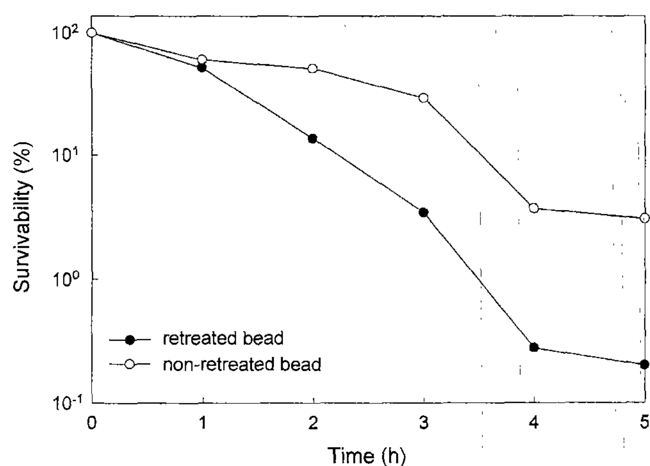

Fig. 6. Effect of retreatment on the survival of immobilized cells.

As shown in Fig. 6, the viable count of immobilized cells in nontreatment beads dropped from 3.08×10^6 CFU/bead to 6.22×10^3 CFU/bead in 5 h, while the viable count of immobilized cells in the retreated beads decreased only by 1.51 log cycles in the same period.

It is obvious that immobilization exerts a protective effect on *B. breve* ATCC 15700 in the acidic solution (pH 3.0). The viability of cells in alginate beads was dependent on the alginate concentration and bead size. It was reported that the slower diffusion rate of glucose and ethanol in more concentrated alginate gels is due to a decrease in the number and length of the pores rather than a decrease in the pore diameter [10]. Beads had difficulty in forming a spherical shape in solutions of low alginate concentration. In fact, this effect was in agreement with other investigations [19, 27, 31]. It has been shown that low-viscosity droplets are less able to retain their spherical shape against the drag forces upon being collided with the solution. On the other hand, high concentrations of sodium alginate (above 5%) cannot form small-sized droplets because of its physiological dough characteristics. Accordingly, cell immobilization will be limited to the possible range of gel concentration to form spherical beads. As for the bead size, the survival of cells in beads is higher with a larger size of bead, as shown in Fig. 3. Sheu *et al.* [28] indicated that larger bead diameters provided more protection for *Lactobacillus bulgaricus* in frozen desserts. However, very large-sized beads might cause coarseness of the texture of live microbial feed supplements, whereas small-sized beads did not provide sufficient protection of the bacteria. Thus, bifidobacteria should be immobilized in a limited range of bead sizes.

Tanaka *et al.* [30] reported that the diffusion of a high molecular weight substance into the alginate beads was limited by increasing the alginate concentration in beads, and glucose was freely diffused into 2% alginate gel beads of 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 molecular species in calcium alginate beads are close to that of water. It is thought that the proton (H^+) of organic acid might freely diffuse into the alginate beads within 5 h, because its molecular weight is significantly smaller than that of Cu^{2+}. Therefore, it is difficult to explain that the survival improvement of immobilized bifidobacteria against acidic solutions by the increase in alginate concentration or in the bead size was due to the decrease in diffusion rate of protons into the inner bead by both decreasing the number or depth of pores and by increasing bead size.

When stored at 4°C for 2 weeks, the survival of immobilized cells was better than that of free cells. This phenomenon was not due to the diffusion effect of substance into the alginate gel. Krisch and Szajani [20] reported that the resistance of bacteria against the solutes such as ethanol and acetic acid is due to their physiological change. Hilge-Rotmann and Rehm [11] reported that cells entrapped in alginate beads contained more saturated fatty acid than untrapped cells. In addition, Castillo-Agudo [5] suggested that the more ethanol tolerant *Saccharomyces* strains showed a clear reduction in the unsaturated fatty acid compared with the ethanol-sensitive strains. Therefore, we suggest that both the physical characteristics of the calcium-alginate lattice and the physiological change of cells might have increased the survivability of bifidobacteria immobilized in alginate beads against the environmental stresses.

Yogurt as a fermented product has a more complicated system than lactic acid, acetic acid, and hydrogen peroxide solutions. The starter cultures and antibacterial metabolic products such as lactic acid, hydrogen peroxide, and bacteriocin may affect the survivability of bifidobacteria in yogurt. The environment inside the beads probably protected the immobilized cells from those inhibitory factors by limiting their diffusion into the beads. It is obvious that immobilization exerted a protective effect on bifidobacterial cells in yogurt, as shown in Fig. 5. When placed into yogurt, the survival rate of immobilized cells was relatively higher than that of the immobilized cells in a simple system, such as the acidified TPY broth adjusted to pH 3.0 by mixing with acetic acid and lactic acid, and modified TPY containing 1,000 ppm of hydrogen peroxide. Although the titratable acidity or the total acid content in yogurt with pH around 4.4 was higher than that of the pH 3.0 TPY broth (data not shown), immobilized cells in yogurt were shown to be more resistant than those in the simple system. Therefore, this phenomenon indicates that some protection may be provided by yogurt itself. When retreated beads with skim milk and nonretreated beads were tested in acidified pH 3.0 TPY media including acetic and lactic acids, the survival rate of immobilized cells in nonretreated beads decreased by 0.002%, while the retreated beads decreased only by 0.031%. Therefore, the number of viable cells in the retreated beads was approximately 10-fold higher than that of the nonretreated beads. This suggests that the skim milk decreased the diffusion of acid and hydrogen peroxide into alginate gels. In conclusion, this research found that yogurt itself protected immobilized cells from the extreme acidity existing in yogurt.

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