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Survival of bifidobacteria in yogurt and simulated gastric juice following immobilization in gellan–xanthan beads

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Abstract

A novel acid-stable bead made of gellan gum and xanthan gum was used to immobilize Bifidobacteria. The beads (0.75% gellan and 1% xanthan gum) had an average diameter of 3 mm and did not shrink in 25% lactic acid solution, pH 1.5, or 20% acetic acid solution, pH 1.5, after storage at 4°C for 4 weeks. *Bifidobacterium infantis* ATCC 15697, the most acid-tolerant strain tested, was immobilized in gellan–xanthan beads and its survival in peptone water, pH 4, pasteurized yogurt, and simulated gastric juice was monitored. In peptone water, pH 4, the reduction in cell count of immobilized cells of *B. infantis* ATCC 15697 was not significantly different from that obtained with free cells during 6 weeks of storage at 4°C. However, counts of immobilized cells of *B. infantis* ATCC 15697 remained significantly higher than free cells ($P < 0.0001$) when both were exposed to simulated gastric juices at pH 2.5, 2.0 and 1.5. At pH 2.5, the viable count of free cells dropped from 1.23×10^9 CFU/ml to an undetectable level (< 10 CFU/ml) in 30 min, while the viable count of immobilized cells decreased by only 0.67 log cycle in the same time period. Immobilized cells also survived significantly better than free cells ($P < 0.05$) in pasteurized yogurt after refrigerated storage for 5 weeks.

Keywords: *Bifidobacterium*; Gellan–xanthan beads; Survival; Yogurt; Gastric juice

1. Introduction

Bifidobacteria are Gram-positive, non-motile, non-spore forming anaerobic rods with variable cellular morphology (Scardovi, 1986). They are part of the normal intestinal microflora of humans and animals and play an important beneficial role in the host's health (Hoover, 1993; Yaeshima, 1996; Holzapfel et al., 1998; Alander et al., 1999). When present in sufficient numbers, bifidobacteria maintain a favorable balance between the population of beneficial and potentially harmful microorganisms in the gastrointestinal tract (Gibson and Roberfroid, 1995). However, factors such as antibacterial drugs, stress, gastrointestinal disorders, and aging can disturb the balance of intestinal microflora, and significantly decrease the number of beneficial bifidobacteria, resulting in gastroenteritis with symptoms such as abdominal cramps, fever, vomiting, diarrhea, and

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other chronic gastrointestinal diseases (Mitsuoka, 1982; Gothefors, 1989; Hoover, 1993).

Attempts have been made to achieve a balanced intestinal microflora by inclusion of bifidobacteria into food (Ishibashi and Shimamura, 1993; Naidu et al., 1999). It has been recognized that selected strains for oral delivery must be of human origin, available in sufficient number in the food products at the time of consumption, and resistant to the gastric acidity in the upper gastrointestinal tract before reaching the colon (Gilliland, 1989; Holzapfel et al., 1998). Yogurt is commonly used as a vehicle to deliver bifidobacteria (Hughes and Hoover, 1991; Katz, 1999). However, 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 (Reuter, 1990; Iwana et al., 1993; Micanel et al., 1997). Furthermore, delivery of viable bifidobacteria to the large intestine, where they would be able to function, has been limited because of the extreme acidity found in the human stomach (Berrada et al., 1991; Clark and Martin, 1993; Mizota, 1996). 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 system existing in the upper gastrointestinal tract.

Immobilization of bifidobacteria to protect them against adverse effects of acid has been proposed (Rao et al., 1989; Modler et al., 1990; Hughes and Hoover, 1991). Rao et al. described a method to encapsulate freeze-dried *B. pseudolongum* using cellulose acetate phthalate (CAP) coated with beeswax, showing that encapsulated *B. pseudolongum* survived the simulated gastric environment in larger numbers than non-encapsulated cells. CAP is an enteric coating material used for control of drug release in the intestine. However, encapsulation of bifidobacteria in butter oil was ineffective in preventing acid injury to bacteria in both low acid yogurt (pH 5.85) and high acid yogurt (pH 4.47) (Modler and Villa-Garcia, 1993). Calcium alginate and k-carrageenan–Locust bean gum gel beads are the two most commonly used polymers for immobilizing viable cells during fermentation (Audet et al., 1988). However, alginate beads are not acid resistant, and it has been reported that the beads undergo shrinkage and decreased mechanical strength during lactic fermentation (Eikmeier and Rehm, 1987; Roy et al., 1987; Ellenton, 1998). k-Carrageenan–Locust bean gum gel beads have been used more often for lactic fermentation because they are less sensitive to acid than alginate. However, the formation of k-carrageenan–Locust bean gum beads requires potassium ions, which could damage the cells of *B. longum* during lactic fermentation (Paquin et al., 1990). Additionally, it is not recommended that potassium ions, which maintain the electrolyte equilibrium of body fluid, be included in the diet in large amounts. Gellan–xanthan beads are not only acid resistant but also are stabilized by calcium ions (Norton and Lacroix, 1990; Sanderson, 1990), which suggests that gellan–xanthan beads would be a good candidate to immobilize bifidobacterial cells and protect the cells from acid injury.

In this study, gellan gum, combined with xanthan gum, was used to immobilize bifidobacterial cells, and the ability of the gellan–xanthan beads to protect bifidobacteria was evaluated under different conditions including peptone water, pH 4, pasteurized yogurt, and simulated gastric juice.

2. Materials and methods

2.1. Bacterial strains and culture conditions

B. infantis ATCC 15697, *B. breve* ATCC 15700, *B. longum* ATCC 15707, *B. bifidum* ATCC15696, *B. adolescentis* ATCC 15703, and *B. bifidum* ATCC 29521, purchased from the American Type Culture Collection (ATCC), Rockville, MD, USA, were used in this study. Bifidobacteria were grown in MRS (BBL Microbiology System, Becton Dickinson, Rockville, MD, USA) medium supplemented with 0.05% L-cysteine HCl (Sigma, St. Louis, MO, USA) and 0.01% $CaCl_2.2H_2O$ (Fisher, Fairlawn, NJ, USA) at 37°C for 18–22 h anaerobically using the Gas Pak Plus System (BBL-71040 anaerobic system envelopes with palladium catalyst, Becton Dickinson, Cockeysville, MD, USA).

2.2. Immobilization technique

Gellan gum and xanthan gum (Sigma) were used in this study. Polymer powders were dispersed in deionized preheated water (80°C) by gentle stirring

and kept at 80°C for 1 h. The temperature was then raised to 90°C to achieve complete hydration of the polymers. The solutions were autoclaved for 15 min at 121°C.

Cells from overnight (18–22 h) bifidobacterial cultures (20 ml) were harvested by centrifugation (5000 rpm, 15 min), washed and resuspended in 5 ml of sterile water. The bacterial suspension was mixed with 15 ml of gum solution so as to obtain a gel with the same initial cell concentration as the free cell culture and the desired gum concentration. Beads were manufactured by dropping polymer solution into 0.1 M $CaCl_2$ solution through a syringe needle (21 G 1.5, Becton Dickinson, Franklin Lakes, NJ, USA) under gentle stirring. The formed beads were hardened in the $CaCl_2$ solution for 1 h and separated from the solution with a sterile stainless steel strainer. The beads were used immediately after preparation.

2.3. Bead size determination

The total volume of 100 beads was measured by displacement of water in a 10-ml graduated cylinder. The average bead volume and diameter was then calculated. Size determination was performed for three lots of manufactured beads.

2.4. Survival of bifidobacteria at different pH values

The survival of six bifidobacterial strains was evaluated in 0.1% peptone water at pH 4.0, 5.0, 6.0 and 7.0 adjusted with 25% lactic acid. After washing and resuspending, bifidobacterial cells in different pH peptone water were stored at 4°C and cell viability was assayed after 7 days. The experiments tested six strains at four pH levels with three replicates.

2.5. Viability of immobilized and free bifidobacteria in peptone water, pH 4

To evaluate the survival of immobilized cells under acidic conditions, 1 ml of fresh beads was added to test tubes containing 9 ml of peptone water, pH 4, and stored at 4°C for 6 weeks. The viable counts of immobilized bifidobacteria were determined at weekly intervals by sampling the contents of individual tubes. Free cell culture (1 ml) was subjected to the same conditions as beads to serve as a control. The experiment contained seven time points for both immobilized and free cells with three replicates.

2.6. Survival of immobilized and free bifidobacteria in yogurt

Yogurt was manufactured on a pilot plant scale as follows: pasteurized 2% partly skimmed milk (Beatrice Food. Toronto, Canada) purchased from a local supermarket, was supplemented with 4% nonfat dry milk (Carnation, Nestlé Food, Don Mills, Canada) and pasteurized at 85°C for 30 min. After cooling to 45°C, the milk was inoculated (1 unit/10 l) with a lactic starter culture (*Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus,* MY092, EZAL, Romain, France), and incubated at 45°C until the pH reached 4.6.

Yogurt (9 ml) in 50-ml plastic tubes was pasteurized at 85°C for 30 min to kill the lactic cultures. Yogurt samples were tested randomly by plating to make sure no viable lactic culture remained before inoculating with bifidobacterial cells. To determine the survival of immobilized cells, 1 ml of beads was mixed with 9 ml of yogurt in a sterile container and stored at 4°C for up to 8 weeks. The viability of bifidobacterial cells was determined at weekly intervals by sampling the entire contents of individual containers. Free cells (1 ml) were subjected to the same conditions as beads. The pH of the yogurt containing either the immobilized or free cells of bifidobacteria did not change throughout the storage period. Three different batches of yogurt were manufactured and employed in this study. For each batch of yogurt, the experiment contained nine time points for both immobilized and free cells with three replicates.

2.7. Survival of immobilized and free bifidobacteria in simulated gastric juice

To evaluate the survival of immobilized cells under gastric acidity, fresh beads (1 ml) were added to test tubes containing 9 ml of prewarmed (37°C) simulated gastric juice (0.08 M HCl containing 0.2% (w/v) NaCl) at pH 2.5, 2.0, and 1.5, and incubated at 37°C for 0, 15, 30, 60 and 120 min. The incubation

was terminated by placing the beads in 9 ml of 0.05 M sodium phosphate buffer, pH 7.0. Free cell culture (1 ml) was subjected to the same conditions as beads as a control. The experiment contained five time points for both immobilized and free cells with three replicates.

2.8. Cell enumeration

Viable cell samples (1 ml) were serially diluted in peptone water and 0.1 ml of the samples from the appropriate dilutions were spread plated onto supplemented agar. Viable cell counts, performed in duplicate, were determined after 48 h incubation at 37°C anaerobically.

To enumerate bifidobacteria in gel beads, 1 ml of beads (measured by displacement of water in a 10-ml graduated cylinder) was washed in sterile water, softened in 0.05 M sodium phosphate buffer, pH 7.0, for 10 min, and then ground completely with a porcelain mortar and pestle. Bifidobacterial cells released from beads were suspended in peptone water and serially diluted. Cell counts, performed in duplicate, were expressed in colony-forming units (CFU) per milliliter of gel.

2.9. Statistical analysis

The statistical analysis was conducted using the general linear model (GLM) procedure of SAS (SAS, 1990).

3. Results

3.1. Gel bead formation and optimization

Although gellan gum alone can form beads, the high setting temperature of the gellan gum at high concentration makes its use for entrapment of temperature sensitive bifidobacteria difficult (Camelin et al., 1993). In order to decrease the gel setting temperature, a low concentration of gellan gum was used in this study. However, the beads made of 0.5% gellan gum were soft and their shapes were irregular. By incorporating 0.5% xanthan gum, the bead quality was significantly improved, and spherical gellan–xanthan beads were obtained. Gellan gum at concentrations up to 0.75% did not gel in the absence of calcium ions at room temperature, even with the addition of xanthan gum (0.5–1%). This makes it easy to mix bacterial suspension with the gum solution before the beads were formed in $CaCl_2$ solution. Our results showed that 0.5–1% gellan gum combined with 0.5–1% xanthan gum were the best concentrations to make beads for immobilizing bifidobacterial cells based on the gel setting temperature and bead quality. Although no rheological properties of the gels were measured, gellan gum (0.75%), incorporating 1% xanthan gum, was considered to produce the best beads in terms of shape and stability.

3.2. Stability of gellan–xanthan beads in acidic solutions

To protect bifidobacteria from the acidity in yogurt and the human stomach, the beads must be acid-resistant. The stability of gellan–xanthan beads in acidic solutions was evaluated based on the change of bead diameter. The acid-stability tests were performed by soaking the beads for 4 weeks at 4°C in 25% lactic acid solution (pH 1.5) or 20% acetic acid solution (pH 1.5). Bead diameter remained the same in 25% lactic acid solution, and decreased marginally in 20% acetic acid solution (Table 1). Therefore, there was no significant change in the bead diameter during storage in the acidic solutions tested. Visual observation also showed that there was no damage to the beads during storage in lactic and acetic solutions.

3.3. Development of a method to enumerate bifidobacteria in gellan–xanthan beads

A method was developed to enumerate immobilized bifidobacteria in gellan–xanthan beads and its effectiveness was evaluated (Table 2). Dissolution of the beads was achieved by exposing them in 0.05 M sodium phosphate buffer, pH 7.0, for 10 min, and then grinding them using a sterile mortar and pestle gently and thoroughly. The results showed that the viable count of cells in 1 ml of beads were consistently lower than that of the free cells used to prepared the beads, however, the average difference (0.15 log cycles) was not significant.

Table 1
Size of gellan–xanthan beads after four week storage in 25% lactic acid solution (pH 1.5) and 20% acetic acid solution (pH 1.5) at 4°C[a]

Treatment	Trial	Before treatment		After treatment		$D_b - D_a$ (mm)
		V_b (mm^3)[b] /100 beads	D_b (mm)[c] /bead	V_a (mm^3) /100 beads	D_a (mm) /bead	
25% lactic acid	1	1400	2.99	1400	2.99	0
	2	1500	3.06	1520	3.07	−0.01
	3	1500	3.06	1480	3.05	0.01
	Average		3.04±0.03[d]		3.04±0.03	0
20% acetic acid	1	1550	3.09	1500	3.06	0.03
	2	1480	3.05	1480	3.05	0
	3	1500	3.06	1420	3.00	0.06
	Average		3.07±0.02		3.04±0.02	0.03

[a] Data are means of three replicates.
[b] V_b, V_a: volume of beads before and after treatment, which was measured by displacement of water by 100 beads using a 10-ml graduated cylinder.
[c] D_b, D_a: diameter of beads before and after treatment, which was calculated from the volume of the beads using the formula: $V = 1/6\,\pi D^3$.
[d] Mean±standard deviation.

Table 2
Effect of bead formation and dissolution steps on the viability of *B. infantis* ATCC 15697[a]

Trial	Immobilized cells[c] (log_{10} CFU/ml)	Free cells[b] (log_{10} CFU/ml)	Free cells − immobilized cells (log_{10} CFU/ml)
1	9.07	9.31	0.24
2	9.00	9.09	0.09
3	8.95	9.07	0.12
Average			0.15± 0.08[d]

[a] Data are means of three replications.
[b] Free cells were diluted appropriately and plated directly (in duplicate).
[c] Immobilized cells were enumerated by immobilizing free cells into gellan–xanthan beads, softened in 0.05 M sodium phosphate buffer, pH 7.0, for 30 min, and ground in a mortar with a pestle.
[d] Mean±standard deviation.

3.4. Effect of pH on the survival of bifidobacteria

Six strains of bifidobacteria were tested for survival at different pH in peptone water, and the viable number of each sample was determined after 7 days of refrigerated storage (Fig. 1). The results showed that the six strains were significantly different ($P < 0.0001$) in their response to the change of pH, with the greatest deviation occurring at pH 4. *B. infantis* ATCC 15697 had the highest viability among the six strains at pH 4 with a viable count of 1.11×10^8 cells/ml, which was only 0.9 log cycles less than the count at pH 7 after storage for 7 days. *B. breve* ATCC 15700 also survived well with a viable count of 1.8×10^7 cells/ml, that was 1.8 log cycles less than the count obtained at pH 7. The other four strains, *B. bifidum* ATCC 15696, *B. adolescentis* ATCC 15703, *B. longum* ATCC 15707, and *B. bifidum* ATCC 29521 survived poorly at pH 4, their viable count was 5.7, 4.8, 5.4 and 4.8 log cycles lower than that at pH 7, respectively.

3.5. Viability of immobilized and free cells in peptone water, pH 4

B. infantis ATCC 15697, which was the most acid-resistant strain among the other bifidobacterial

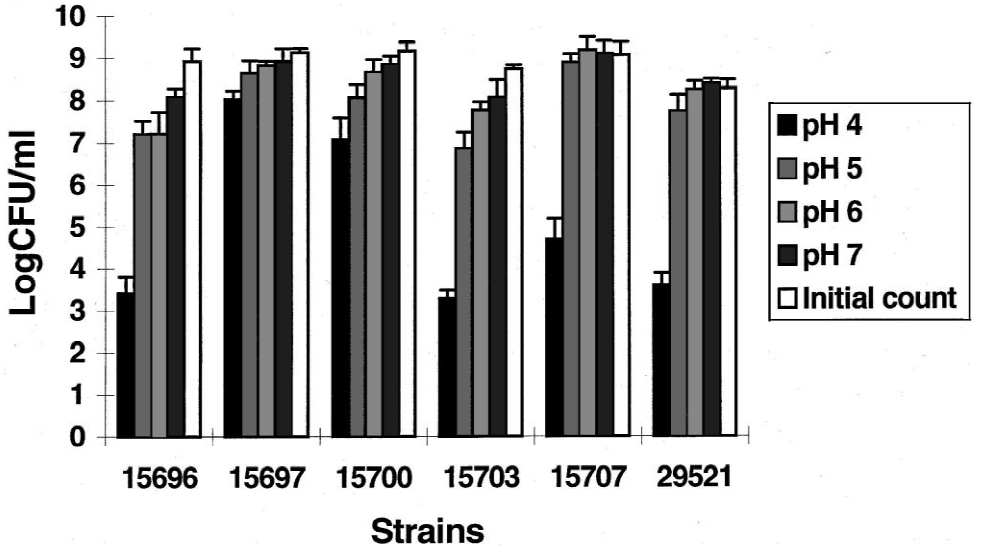

Fig. 1. Effect of pH on the survival of bifidobacteria. Bifodobacterial cells were incubated in MRS broth for 20 h at 37°C anaerobically prior to storage at 4°C in peptone water, at pH values of 4, 5, 6 and 7 for 7 days. Each value in the figure represents the mean from three replicates.

strains tested in this study, were immobilized in gellan–xanthan beads. Viability of immobilized and free cells of *B. infantis* ATCC 15697 in peptone water, pH 4 during storage at 4°C was determined (Fig. 2), this showed that there was no significant difference in the rate of decline of viable numbers between the immobilized and free cells. The viability of both immobilized and free cells of *B. infantis* ATCC 15697 dropped at a similar rate of about 0.4–1.4 log cycles at the end of the first 4-week period, and about 2 log cycles at the end of week 5. The viable counts of *B. infantis* ATCC 15697 decreased to an undetectable level in 6 weeks from the initial counts of 3.8×10^8 CFU/ml of immobilized cells and 1.3×10^9 CFU/ml of free cells.

3.6. Survival of immobilized and free cells in yogurt

The survival of immobilized and free cells of *B. infantis* ATCC 15697 in pasteurized yogurt at 4°C is shown in Fig. 2. The viable count of immobilized cells dropped from 1.2×10^9 CFU/ml to an undetectable level in 8 weeks, and that of free cells from 1.85×10^9 CFU/ml to an undetectable level in 7 weeks. Free cells and immobilized cells exhibited no significantly different losses in viability during the first 4 weeks of storage. But a significant difference

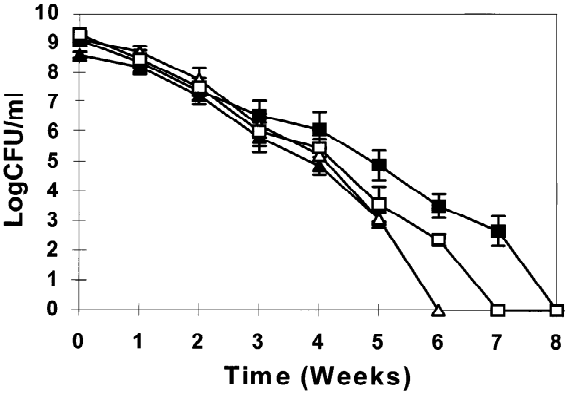

Fig. 2. Viability of immobilized and free cells of *B. infantis* ATCC 15697 in peptone water, pH 4 (▲;△) and in yogurt (pH 4.40±0.12) (■;□). Immobilized (solid symbol) and free cells (open symbol) were stored at 4°C in peptone water, pH 4, and in yogurt which had been previously pasteurized at 85°C for 30 min. Viable counts were determined at weekly intervals. Each point in the figures represents the mean from three replicates. The bacterial number at the undetectable level ($<10^1$ CFU/ml for free cells and $<10^2$ for immobilized cells) was considered to be zero.

($P < 0.05$) in viability between immobilized and free cells was observed at the end of weeks 5, 6 and 7.

3.7. Resistance of immobilized and free cells to simulated gastric juice

Viability of immobilized and free cells of *B. infantis* ATCC 15697 in simulated gastric juice, pH 2.5, and pH 2.0 was evaluated (Fig. 3). Significant protection ($P < 0.0001$) of *B. infantis* ATCC 15697 by immobilization was observed at both the pH values tested. At pH 2.5, the viable count of free cells dropped to an undetectable level ($< 10^1$ CFU/ml) in 30 min, while the viable counts of immobilized cells decreased only by 0.67 log cycles in the same time period, and 6.3×10^5 CFU/ml still remained after 120 min (Fig. 3). At pH 2.0, the population of both immobilized and free cells decreased to an undetectable level in 30 min, however, higher viability of immobilized cells was still observed after 5 and 15 min (Fig. 3). A similar result was observed at pH 1.5 (data not shown), except that the population of both immobilized cells and free cells decreased to an undetectable level within 15 min.

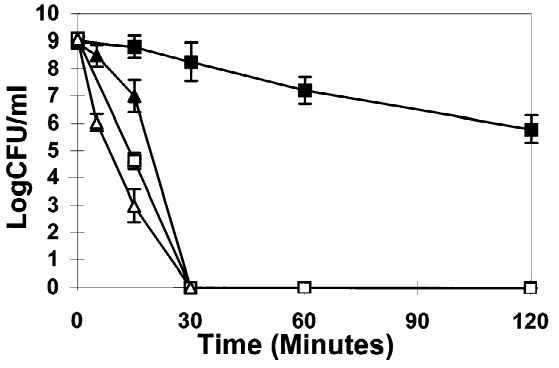

Fig. 3. Viability of immobilized and free cells of *B. infantis* ATCC 15697 in simulated gastric juice at pH 2.5 (■;□) and at pH 2.0 (▲;△). Immobilized (solid symbol) and free cells (open symbol) were incubated in prewarmed simulated gastric juice at 37°C, collected and put into 0.05 M sodium phosphate buffer, pH 7. Bifidobacterial cells were enumerated by plating. Each point in the figures represents the mean of three replicates. The bacterial number at the undetectable level ($< 10^1$ CFU/ml for free cells and $< 10^2$ CFU/ml for immobilized cells) was considered to be zero.

4. Discussion

To effectively evaluate the survival of bifidobacteria encapsulated in beads, a method of releasing the cells from gellan–xanthan beads was developed. Enumeration of viable cells in beads involved steps of immobilizing bifidobacteria in beads, exposing the beads to phosphate buffer, and grinding the beads in a mortar with a pestle. As the original bifidobacterial cell concentration in both 1 ml of beads and 1 ml of free cells was the same, the viable count difference between immobilized and free cells indicated a loss of viable cells during those procedures. Usually, dissolution of calcium alginate beads involved incubation of the beads at room temperature with pH 7.0 sodium phosphate buffer for a few hours (Fraser and Bickerstaff, 1997). This method effectively released the cells from alginate beads, but did not work well for gellan–xanthan beads. Cells immobilized in k-carrageenan–locust bean gum beads can be released by soaking the beads in 0.85% saline solution, and shaking with glass beads at 45°C (Audet et al., 1988). In our experiment, the gellan–xanthan beads could not be melted at 45°C. Furthermore, most bifidobacterial cells were killed at this temperature (data not shown). By softening the beads in sodium phosphate at room temperature, and then grinding them with a mortar and pestle, the method to release bifidobacterial cells from gellan–xanthan beads was unique, and effective.

When stored in a milk-based medium (pH 3.7–4.3), some acid-resistant strains of bifidobacteria survived well for 42 days (Lankaputhra et al., 1996). In contrast, recent work of Ellenton (1998) showed that cells of *B. infantis* ATCC 15697 were not detectable after 15 days of refrigerated storage in MRS broth at pH 4.3. However, the present study showed that *B. infantis* ATCC 15697 survived well in peptone water, pH 4, during 6 weeks of storage at 4°C. Thus, the survival of bifidobacteria in acidic environments was affected by both pH and the components of the storage medium.

Although the survival of immobilized cells and free cells showed no significant difference in peptone water, pH 4, a difference was found in yogurt after 5 weeks. Yogurt, as a fermented product, is a more complicated system than peptone water. The starter cultures, and antibacterial metabolic products such as lactic acid, H_2O_2, and bacteriocin, may affect surviv-

al 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 exerts a protective effect on bifidobacterial cells in simulated gastric juice. The formation of the pH gradient in beads was dependent on the bead size and the exposure time of the beads in simulated gastric juice. When placed into simulated gastric juice, free cells were exposed to the extreme low pH immediately, while the cells in the core and the surface of the beads were subjected to a different pH. In this study, about 5×10^6 CFU/ml of bifidobacterial cells would be alive, when 1×10^9 CFU/ml of cells in beads were kept in simulated gastric juice (pH 2.5) for 120 min. Generally, the average time to empty half of the stomach contents is 90 min, and the pH of the human stomach is 1–3 (Giannella et al., 1972). Although some protection may be afforded from the food matrix itself, the significantly higher number of viable cells in beads than free cells in simulated gastric juice suggested that the immobilization would further protect bifidobacteria from the extreme acidity in the human stomach. As soon as the bifidobacteria in beads leave the stomach, the low pH around the beads will be neutralized by intestinal juice, where the beads will be softened at higher pH, and might be broken by the peristalsis of the small intestine, causing bifidobacteria to be released from the beads.

5. Conclusions

Immobilization of bifidobacteria in beads comprised of gellan–xanthan gum mixtures increases their tolerance of high acid environments. This approach may be useful in delivery of probiotic cultures to the gastrointestinal tract of humans and animals. A further increase in acid resistance may be observed if the biomass within the beads is increased by growth of the encapsulated bacteria. This technique is currently under investigation, but preliminary results suggest that an increase in cell density by growth of the organism in the beads does not markedly affect its ability to tolerate conditions of low pH.

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