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Survival and stability of bifidobacteria loaded in alginate poly-*l*-lysine microparticles

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Abstract

Bifidobacteria-loaded alginate microparticles were prepared by spraying a mixture of alginate and bifidobacteria culture using an air atomization method. Survival and stability of bifidobacteria loaded in microparticles were then evaluated. Survival of bifidobacteria from alginate poly-*l*-lysine microparticles was significantly increased when MRS broth or yeast extract was added in simulated intestinal fluid (pH 6.8). The number of bifidobacteria gradually increased for 8 h (10^8 cfu/g) and then reached about 10^9–10^{10} cfu/g when incubated over 12 h in intestinal fluid containing 0.5% yeast extract and 0.05% L-cysteine. The survival of bifidobacteria was highly dependent on the pH of the exposing media. When the bifidobacteria was immobilized with alginate or even poly-*l*-lysine treatment, the survival of bifidobacteria was highly enhanced in the low pH conditions (ca. $> 10^8$ vs. $< 10^3$ cfu/g). The stability of free flowing bifidobacteria-loaded alginate poly-*l*-lysine microparticles was significantly improved during storage at 4°C in a refrigerator when compared to bifidobacteria cultures. The bifidobacteria-loaded alginate poly-*l*-lysine microparticles could be applied to various dairy products. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Bifidobacteria; Survival; Gastric acid resistance; Alginate-poly-*l*-lysine microparticles; Free flowing

1. Introduction

Bifidobacteria are anaerobic, rod shaped, Gram-positive bacteria, which are normal inhabitants of the human colon (Rasic and Kurmann, 1983; Simon and Gorbach, 1984). They have been shown to play important beneficial roles in human health by increasing humoral immunity (De Simone et al., 1992) and maintaining the balance of intestinal microflora (Benno and Mitsuoka, 1992). Therefore, bifidobacteria have been widely incorporated in various dairy products. The population

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of bifidobacteria in human intestinal microflora decreases with age, and is affected by factors such as gastrointestinal disorders and antibiotic therapies (Drasar and Hill, 1974; Bornside, 1978). Ingested bifidobacteria must survive transit through the gastric environment and reach the colon in large quantities to facilitate colonization (Kim, 1988). The low pH of the stomach environment is detrimental to their survival (Gianella et al., 1972; Rao et al., 1989; Berrada et al., 1991; Lee et al., 1999). We have shown large variations in the survival of bifidobacteria in various Korean dairy products when exposed to artificial gastric fluid (Lee et al., 1999).

Microencapsulation techniques have been widely utilized to protect microorganisms, cells or tissues from environmental and physiological degradation (Lim and Moss, 1981; Kim et al., 1988; Kwok et al., 1992). The biological nature of entrapped material means that the choice of method is determined by the need to avoid harmful organic solvents, heat or high shear forces (Abraham et al., 1996; Lee et al., 1998). The polysaccharide sodium alginate has been most widely used as an immobilizing vehicle. It forms a gel when in contacted with calcium and multivalent cations. Alginate beads (or microparticles) are stable in low pH conditions but swell in weak basic solutions followed by disintegration and erosion (Lee and Min, 1996; Lee et al., 1996). The size of alginate microparticles is one factor that must be considered for pharmaceutical applications. Alginate poly-*l*-lysine microparticles containing entrapping microorganisms have been produced using an air atomization device known as a Turbotak (Kwok et al., 1991, 1992; Abraham et al., 1996). This device is an air-assisted nebulizer, which produces a high velocity spray, resulting in a fine mist of alginate mixtures. This atomization device can produce small microparticles, ranging from 5 to 200 μm by adjusting gas and liquid flow and operating pressure. The alginate gel structure of the microparticles is further strengthened by the addition of cationic poly-*l*-lysine, resulting in alginate poly-*l*-lysine microparticles (Lim and Moss, 1981; Kwok et al., 1991, 1992; Liu and Krishnan, 1999). The resultant small microparticles are free flowing after freeze-drying.

In this study, alginate poly-*l*-lysine small microparticles containing bifidobacteria were prepared using an air atomization method. Survival of bifidobacteria loaded in alginate poly-*l*-lysine microparticles was then evaluated as a function of dissolution media, pH, incubation time and poly-*l*-lysine concentration. Stability of the free flowing bifidobacteria-loaded alginate poly-*l*-lysine microparticles was also determined during storage at 4°C in a refrigerator.

2. Materials and methods

2.1. Materials

Sodium alginate was purchased from Junsei (Tokyo, Japan). Calcium chloride was purchased from Shinyo (Osaka, Japan). poly-*l*-lysine (MW 38 500) and L-cysteine HCl were purchased from Sigma (St. Louis, MO. USA). A freeze-dried strain of *Bifidobacterium bifidum* was purchased from Christian Hansen's Laboratories (Horsholm, Denmark). The culture is primarily used in the production of probiotic milk products or yogurt. The manufacturer specifies *Bifidobacterium bifidum*, based on purity and a minimum cell concentration of 5×10^{10} colony forming units (cfu)/g when incubated on MRS agar culture at 37°C for 3 days. Bifidobacteria culture was used intact as supplied by the manufacturer. A commercially available bifidobacteria-loaded capsules (Doctor Capsule) was obtained from Binggre (Seoul, Korea). Lactobacilli MRS broth, Bacto-Agar and yeast extract were purchased from Difco Laboratories (Detroit, MI, USA). Anaerobic GasPak® jars, disposable H_2/CO_2 generator envelopes, catalysts and indicator strips were purchased from Becton Dickinson and Microbiology Systems (BBL, Cockeysvile, MD, USA). All other chemicals were of reagent grade and used without further purification.

2.2. Preparation of bifidobacteria-loaded alginate microparticles

The formulation for the preparation of bifidobacteria-loaded alginate microparticles is

shown in Table 1. The mixture was sprayed into a bath containing 500 ml 0.2 M $CaCl_2$ solution. An air atomizing device (Turbotak, Waterloo, Ont.) was used under aseptic conditions at a pressure of 0.75 bar and a delivery rate of 8 ml/min using a peristaltic pump. All solutions, including sodium alginate and $CaCl_2$, were autoclaved using a steam sterilizer at 121°C for 20 min before use. A piece of dry sterilized cotton was placed inside the air tube connected to the atomizer to filter contaminating microorganism from the air. The sodium alginate solution was fed through the air atomizer from the top with the pressurized air fed in from the side. The pressurized air/solution mixture was forced into tiny liquid droplets through the orifice of the atomizing nozzle. The droplets were gelled when they came in contacted with calcium chloride solution. The microgel droplets were cured for 15 min and filtered through two layers of unsterile filter paper (11 cm, circles, Whatman, Maidstone, UK) in a Buchner funnel under vacuum. Thereafter, the filtered microgel droplets were washed twice with deionized water and suspended in filter-sterilized 0.02% poly-*l*-lysine solution for 5 min to cross-link the microparticles.

The resulting poly-*l*-lysine treated microparticles were again separated by filtration under vacuum on a Buchner funnel and washed twice again deionized water. They were then frozen at −37°C for 2 h. The final free flowing bifidobacteria-loaded alginate poly-*l*-lysine microparticles were obtained after freeze-drying using a freeze dryer (Ilshin, Seoul, South Korea) at −52°C under a pressure of 8 mmHg for 14–16 h.

Table 1
Formulation for the preparation of bifidobacteria-loaded alginate microparticles

Composition	Amount (g)
Sodium alginate	1.5
Bifidobacteria culture[a]	0.5
Yeast extract	0.5
Glycerol	5.0
$NaHSO_3$	0.5
$Mg_3(PO_4)_2$	1.0
Water	q.s. to 100

[a] Used intact as supplied by the manufacturer.

2.3. Determination of survival of bifidobacteria

The survival of bifidobacteria was determined according to previously reported experiments with minor modification (Duby and Mistry, 1996; Lee et al., 1999). The bifidobacteria-loaded alginate microparticles (1 g) were incubated at 37°C for 12 h in 200 ml of simulated intestinal fluid (pH 6.8 without pancreatin) containing 0.5% yeast extract and 0.05% L-cysteine HCl. Samples (1 ml) were withdrawn and aseptically diluted in a solution containing 5.5% MRS broth and 0.05% L-cysteine HCl in sterile water (100 ml). The diluted samples were plated on MRS agar culture, and incubated at 37°C in an anaerobic GasPak® jar containing a generator envelope, indicator strip and catalyst for 48–72 h. The MRS agar culture consists of MRS broth (5.5 g), L-cysteine (0.05 g), Bacto-Agar (1.5 g) in sterile water (100 ml). The cfus were counted to assess survival. Average survival of bifidobacteria was determined from at least more than three separately diluted and plated samples. Thereafter, data were expressed as mean ± S.D.

2.4. Effect of dissolution media

Samples (1 g) of bifidobacteria-loaded alginate poly-*l*-lysine microparticles were added to 200 ml of the following three testing media: simulated intestinal fluid (pH 6.8) with or without 5.5% MRS broth, or 0.5% yeast extracts, respectively. L-Cysteine HCl (0.05%) was then added to each suspension, which were incubated at 37°C for 2 h. The survival of bifidobacteria was determined by plating suitable dilutions in triplicate as described previously.

2.5. Survival of bifidobacteria loaded in alginate poly-*l*-lysine microparticles in simulated gastric and intestinal fluid

One gram of bifidobacteria-loaded alginate poly-*l*-lysine microparticles was added to 200 ml simulated gastric fluid (pH 1.5 without pepsin) containing 0.5% yeast extract and 0.05% L-cysteine HCl. The resulting suspension was incubated at 37°C for 30, 60, 90 and 120 min and then

immediately filtered to remove the undissolved microparticles, which were subsequently placed in 200 ml of simulated intestinal fluid and then adjusted to pH 6.8 with 0.1 N NaOH. The solution in the flasks was then incubated at 37°C for 12 h and shaken periodically. Aliquots of 1.0 ml were withdrawn and assayed for bifidobacteria as described previously.

Survival of bifidobacteria loaded in alginate poly-*l*-lysine microparticles was also investigated as a function of time in 200 ml simulated intestinal fluid (pH 6.8) containing 0.5% yeast extract and 0.05% L-cysteine HCl. After incubating for 2, 4, 6, 8, 12 and 14 h in intestinal fluid, the suspension was filtered to remove the undissolved microparticles. Survival of bifidobacteria was then determined as described previously.

2.6. Effect of pH

The bifidobacteria-loaded alginate poly-*l*-lysine microparticles (1 g) were added to 200 ml of buffer systems containing 0.5% yeast extract and 0.05% L-cysteine HCl. A 7.4% HCl–NaCl buffer of pH 1.5 or 2.0, a 0.01 M acetate buffer of pH 4.0 and a 0.01 M phosphate buffer of pH 6.8 or 7.4 were used, respectively. After incubation at 37°C for 120 min, survival of bifidobacteria was measured as described previously. Solutions of pH 1.5, 2.0 or 4.0 were adjusted to pH 6.8 with 0.1 N NaOH immediately after stopping incubation.

2.7. Comparison of survival of bifidobacteria products

To compare the gastric resistance of various bifidobacteria products, 1 g of bifidobacteria cultures, or either bifidobacteria-loaded alginate or alginate poly-*l*-lysine microparticles was added to 200 ml simulated gastric fluid (pH 1.5 without pepsin) supplemented with 0.5% yeast extract and 0.05% L-cysteine HCl. After incubation at 37°C for 120min, the survival of bifidobacteria was determined as described previously and statistically compared. Survival of bifidobacteria in a commercially-available yogurt preparation containing bifidobacteria-loaded capsules (Binggre, Seoul, South Korea) was also determined.

For comparison, samples of bifidobacteria culture or bifidobacteria-loaded microparticles were also incubated in simulated intestinal fluid (pH 6.8) containing 0.5% yeast extract and 0.05% L-cysteine HCl for 12 h.

2.8. Stability of bifidobacteria-loaded alginate poly-l-lysine microparticles during storage at 4°C

A sample of free flowing bifidobacteria-loaded alginate poly-*l*-lysine microparticles were stored over 20 weeks in a refrigerator at 4°C. At predetermined time intervals, 1 g of samples was collected and then incubated in 200 ml of simulated intestinal fluids (pH 6.8) for 12 h to determine survival of bifidobacteria as described previously.

3. Results and discussion

3.1. Characterization of bifidobacteria loaded in alginate poly-l-lysine microparticles

To ensure survival under gastric conditions, a method for the microencapsulation of bifidobacteria was developed. An air atomization process produced free-flowing and small sized microparticles after a freeze drying process without using harmful organic solvents. The size and surface morphology of alginate microparticles containing bifidobacteria were dependent on the formulation compositions and processing conditions. Alginate microparticles had an irregular spherical or elliptical shape. Geometric mean size of alginate poly-*l*-lysine microparticles was reproducibly ranged from about 80 to 130 μm.

It was also known that the trapping efficiency of drugs in alginate systems was dependent on curing time, $CaCl_2$ concentration along with drug solubility (Lee et al., 1999). Because the process is operated under aqueous condition, spraying and gelling time must be also considered. When the 0.5 g of bifidobacteria suspension as shown in Table 1 was sprayed, about 2.3 g of alginate microparticles were produced. Because average survival of bifidobacteria culture and alginate mi-

croparticles per g was also determined as 1.2×10^{11} and 3.2×10^{9} cfu/g, respectively, the trapping efficiency of bifidobacteria was estimated to be 12.3%.

3.2. Effect of dissolution media

Survival of bifidobacteria is affected by various factors such as pH, nature of the dissolution media, presence of nutrients and incubating conditions (Rao et al., 1989; Louis et al., 1990; Berrada et al., 1991; Lee et al., 1999). Freeze-dried bifidobacteria-loaded products will contain both unharmed or damaged cells. Under suitable conditions the injured cells may repair and become viable, i.e. capable of colony formation on suitable media (Vandez et al., 1985). Bifidobacteria are very labile in low pH condition but quite stable at physiological pH.

The type of media and nutrients for selective enumeration of bifidobacteria can have an affect on growth characteristics (Rao et al., 1989; Duby and Mistry, 1996). The MRS broth and yeast extract may act as useful nutrients (Rao et al., 1989; Duby and Mistry, 1996). L-Cysteine is also used as an antioxidant. They are commonly used in bifidobacteria culture media. The survival of bifidobacteria was tested in simulated intestinal fluid supplemented with these components.

The survival of bifidobacteria-loaded alginate poly-*l*-lysine microparticles in different dissolution media at 37°C for 2 h is shown in Fig. 1. In the simulated intestinal fluid without nutrient, the survival was relatively low. The survival of bifidobacteria was significantly increased when MRS or yeast extract was added to the intestinal fluid (pH 6.8). The survival of bifidobacteria showed 10^{8} cfu/g when supplemented with 0.5% yeast extracts. Bifidobacteria could survive longer when cocultured with MRS broth and yeast extract. Although cell division of immobilized bifidobacteria in alginate microparticles was negligible during 2 h incubations in the simulated intestinal fluid, addition of some nutrients in the simulated intestinal fluid could protect bifidobacteria, resulting in an increase of survival when incubated on MRS agar.

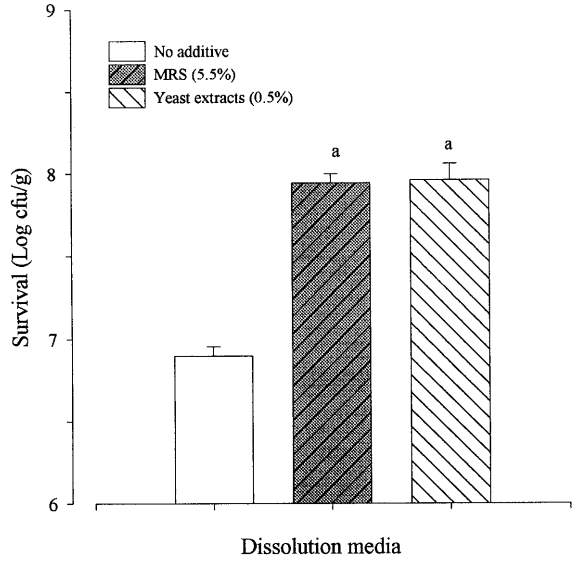

Fig. 1. Survival of bifidobacteria loaded in alginate poly-*l*-lysine microparticles in different dissolution media at 37°C for 2 h. Bar represents the S.D. from at least more than three different experiments. $^{a}P < 0.05$ compared to no additive.

3.3. Survival of bifidobacteria loaded in alginate poly-l-lysine microparticles in gastric and intestinal fluid

It is known that alginate microparticles are stable in gastric fluid but erode and disintegrate in higher pH conditions (Lee and Min, 1996; Lee et al., 1996). Therefore, the maximal survival of the bifidobacteria can be determined after completely dissolving alginate microparticles in simulated intestinal fluid. The release profiles of entrapping materials from the alginate microparticles can be changed by various factors, i.e. the solubility, type of entrapping materials and alginate, curing time and reinforcement of the alginate membranes (Lee and Min, 1996; Lee et al., 1996). The survival of bifidobacteria loaded in alginate poly-*l*-lysine microparticles as a function of time in simulated intestinal fluid (pH 6.8) at 37°C is shown in Fig. 2. The number of bifidobacteria gradually increased for 8 h (10^{8} cfu/g) and then reached about 10^{9}–10^{10} cfu/g when incubated over 12 h. From this finding, the bifidobacteria-loaded alginate poly-*l*-lysine microparticles could be completely dissolved over 12 h in intestinal fluid without

losing their activities. Free or viable bifidobacteria released from alginate microparticles containing immobilized bifidobacteria are hard to divide in intestinal fluid containing yeast extract and L-cysteine during incubation. Incubation time in intestinal fluid did not have an affect on survival of bifidobacteria. Under the appropriate MRS culture media, multiplication of bifidobacteria released from non- or fully-disintegrated microparticles can be possible as mentioned previously.

Due to stability of alginate microparticles in gastric fluid, bifidobacteria can be protected when exposed to gastric fluid without losing their survival significantly. Survival of bifidobacteria loaded in alginate poly-*l*-lysine microparticles in simulated gastric fluid (pH 1.5 without pepsin) as a function of time is shown in Fig. 3. The survival of bifidobacteria gradually declined as the incubation time increased due to detrimental effect of low pH on bifidobacteria. Survival was maintained at 4.5×10^7 cfu/g (1–3% of the original survival) after treatment for 2 h in simulated gastric fluid. It was evident that the survival of bifidobacteria decreased to some extent when exposed to gastric fluid even though alginate microparticles could protect bifidobacteria from gastric fluid. It seemed likely that gastric fluid entered the microparticles through the surface pinholes resulting in loss of viability. The strengthening of surface membrane and size control of microparticles are also an alternative for further enhancement of gastric resistance. Nevertheless, the survival of bifidobacteria could be highly enhanced by microencapsulation using alginate as compared to bifidobacteria culture ($<10^3$ cfu/g). The survival of bifidobacteria can also be affected by physiological factors such as time of ingestion, type of food, transit time and gastrointestinal conditions (Dressman et al., 1990; Berrada et al., 1991; Lee et al., 1999).

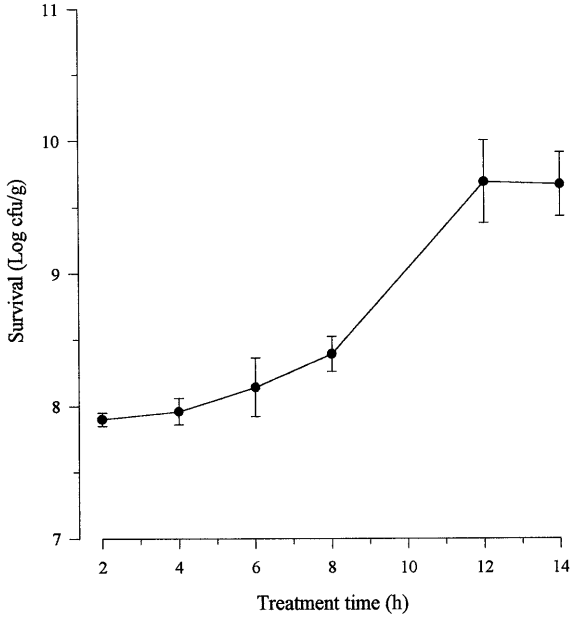

Fig. 2. Survival of bifidobacteria loaded in alginate poly-*l*-lysine microparticles as a function of time in simulated intestinal fluid (pH 6.8) at 37°C. Bar represents the S.D. from at least more than three different experiments.

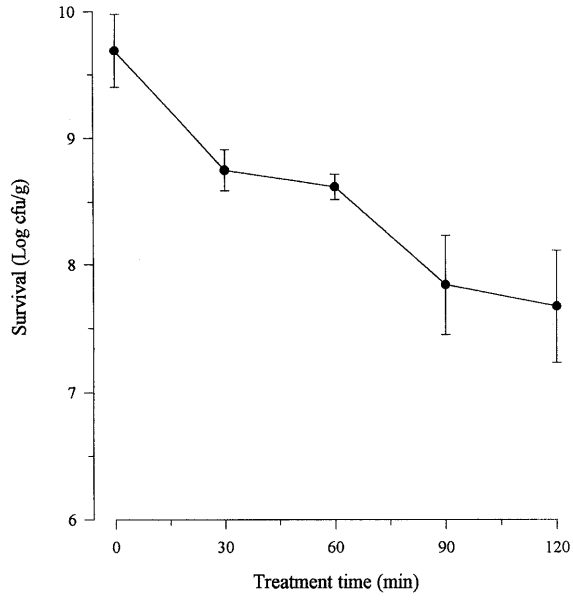

Fig. 3. Survival of bifidobacteria loaded in alginate poly-*l*-lysine microparticles in simulated gastric fluid (pH 1.5) as a function of time. Bar represents the S.D. from at least more than three different experiments.

3.4. Effect of pH

It is known that bifidobacteria are very susceptible to pH conditions. Even though bifidobacteria can be protected by microencapsulation using

alginate, the survival is also varied by pH of the exposing medium. The effect of various pHs on the survival of bifidobacteria loaded in alginate poly-*l*-lysine microparticles is shown in Fig. 4. The survival of bifidobacteria (2.67×10^9 cfu/g) was highest at pH 6.8. There were no significant differences at pH 7.4 and pH 4.0. However, the survival of bifidobacteria loaded in alginate poly-*l*-lysine microparticles was reduced when exposed to pH 1.5 and pH 2.0 for 2 h, but was maintained at 5.0×10^7 and 8.7×10^7 cfu/g, respectively.

It was evident that the survival of bifidobacteria was highly dependent on the pH of the exposing media. Survival was maintained in neutral pH conditions but rapidly decreased in low pH (Rasic and Kurmann, 1983). However, the bifidobacteria could survive in a relatively large quantity even in low pH because alginate or alginate poly-*l*-lysine microparticles significantly improved the gastric acid resistance of bifidobacteria as compared with intact bifidobacteria cultures (see Fig. 5).

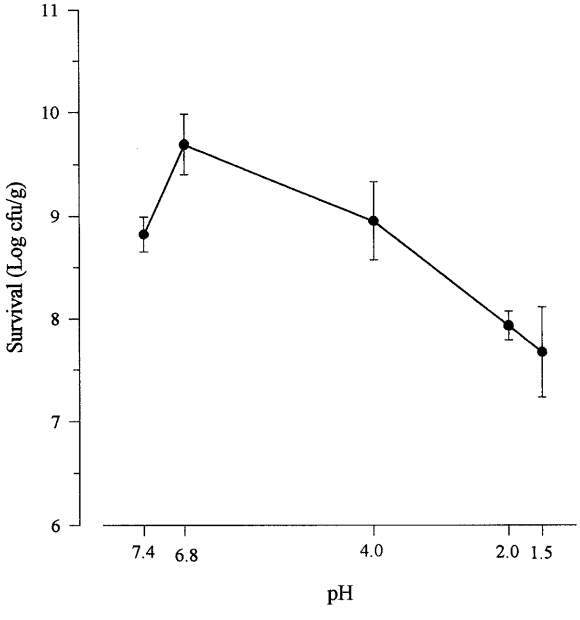

Fig. 4. Effect of pH on the survival of bifidobacteria loaded in alginate poly-*l*-lysine microparticles after exposure to different pH media for 2 h. Data expressed as mean ± S.D.

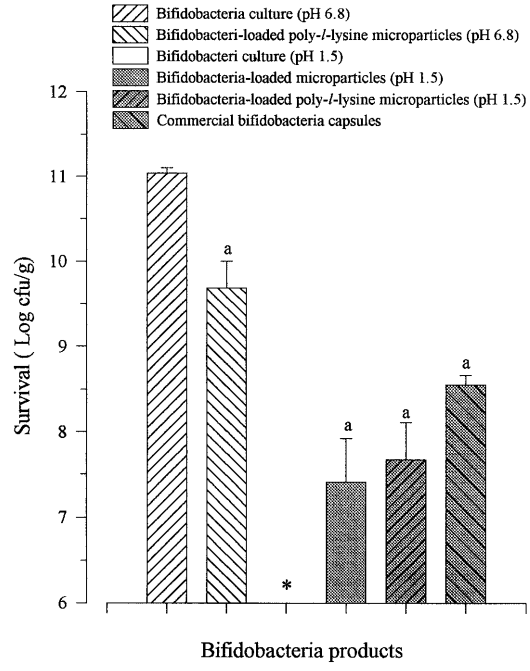

Fig. 5. Comparison of survival of various bifidobacteria products after exposure to simulated gastric fluid (pH 1.5) for 2 h or intestinal fluid (pH 6.8) for 12 h. Bar represents the S.D. from at least more than three different experiments. $^aP < 0.05$ compared to bifidobacteria culture (pH 1.5) as a control. *The survival was too low to show ($< 10^3$ cfu/g).

3.5. Comparison of survival of bifidobacteria products

Survival of various bifidobacteria products after exposure to simulated gastric fluid for 2 h or intestinal fluid for 12 h is compared in Fig. 5. The microencapsulation process resulted in a decrease of survival and showed about 10^9 cfu/g at pH 6.8 condition as compared with bifidobacteria cultures (10^{11} cfu/g). Survival of commercial bifidobacteria cultures rapidly declined from 10^{11} cfu/g to less than 10^3 cfu/g when exposed to simulated gastric fluid for 2 h. This suggested that unprotected (intact) bifidobacteria died rapidly when exposed to low pH condition. Survival of bifidobacteria loaded in alginate or alginate poly-*l*-lysine microparticles was maintained at approximately 10^7 cfu/g and 10^8 cfu/g, respectively, when exposed to pH 1.5 for 2 h. On the other hand, bifidobacteria-loaded capsules (Doctor Capsules)

as claimed by the manufacturer in South Korea (Binggre) also showed about 10^8 cfu/g survival. It was evident that microencapsulated bifidobacteria could be protected from gastric fluid due to the stability in low pH environments and was statistically significant when compared with bifidobacteria culture (pH 1.5) (<0.05). Furthermore, poly-*l*-lysine treatment of bifidobacteria-loaded alginate microparticles resulted in a further increment of survival when exposed to simulated gastric fluid because the membrane of alginate can be more tightly constructed by complexing with cationic poly-*l*-lysine (Kwok et al., 1992; Liu and Krishnan, 1999).

3.6. Stability of bifidobacteria-loaded alginate poly-l-lysine microparticles during storage at 4°C

Stability of free flowing bifidobacteria-loaded alginate poly-*l*-lysine microparticles during storage in a refrigerator at 4°C is also shown in Fig. 6. Survival of bifidobacteria loaded in alginate poly-*l*-lysine microparticles had a tendency to decline during storage. The survival was maintained at approximately 10^7 cfu/g after 16 weeks storage. The stability of free flowing bifidobacteria-loaded alginate poly-*l*-lysine microparticles significantly improved after immobilization with alginate when compared to bifidobacteria cultures. Survival of bifidobacteria in various commercially available South Korean yogurts gave over 10^8–10^9 cfu/g during storage at 4°C for 10 weeks in a refrigerator even though the gastric resistance was quite different (Lee et al., 1999). It was known that the dairy products containing bifidobacteria have an expiry date of at least over 3 weeks when stored in a refrigerator at 4°C (Shah et al., 1995; Lee et al., 1999). In addition to gastric resistance, the stability of bifidobacteria can be also considered after incorporating in various dairy products during storage.

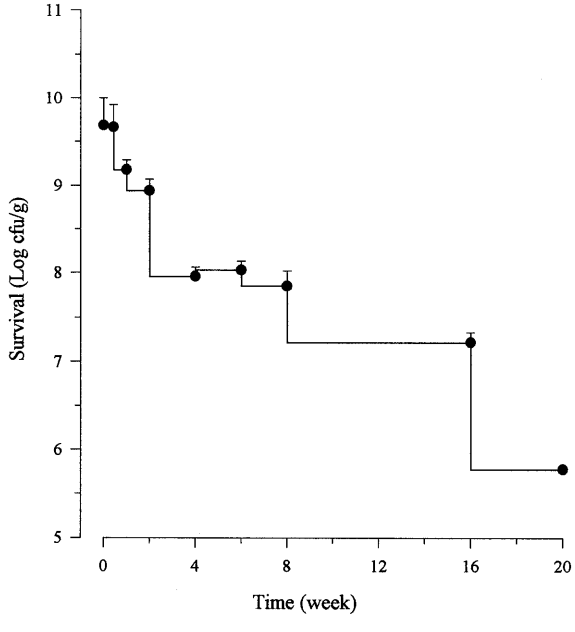

Fig. 6. Stability of free flowing bifidobacteria-loaded alginate poly-*l*-lysine microparticles during storage in a refrigerator at 4°C. The data are expressed as mean ± S.D.

4. Conclusions

The survival of bifidobacteria loaded in alginate poly-*l*-lysine microparticles was highly dependent on dissolution media, pHs and incubation time. When the bifidobacteria was immobilized with alginate or even poly-*l*-lysine treatment, the survival of bifidobacteria was highly enhanced under low pH conditions. The free flowing bifidobacteria-loaded alginate poly-*l*-lysine microparticles also maintained their survival at over 10^7 cfu/g during 16 weeks storage at 4°C. The bifidobacteria-loaded alginate poly-*l*-lysine microparticles with smaller size ranges could be applied to various dairy products without significantly losing viability under the low pH conditions.

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