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Effect of Skim Milk-Alginate Beads on Survival Rate of Bifidobacteria

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Abstract In this study, an attempt was made to increase the survival rate of bifidobacteria entrapped in alginate in the gastrointestinal tract, and to investigate the potential industrial applications, for example lyophilized capsules and yogurt. First, the protective effect of various food additives on bifidobacterial survivability was determined after exposure to simulated gastric juices and bile salts. The additives used in this study were skim milk (SM), poly dextrose (PD), soy fiber (SF), yeast extract (YE), chitosan (CS), κ-carageenan (κ-C) and whey, which were added at 0.6% concentration (w/v) to 3% alginate-bifidobacterial solution. In the simulated gastric juices and bile salts, the protective effect of 0.6% skim milk-3% alginate (SM-A) beads on the survival rate of bifidobacteria proved to be higher than the other additives. Second, the hydrogen ion permeation was detected through SM-A vessel without bifidobacterial cells at different SM concentrations (0.2%, 0.4%, 0.6%, 0.8%, and 1.0%). There were no differences in terms of the pH decrease in SM-A vessels at 0.6%, 0.8%, and 1.0% (w/v) SM concentrations. The survival rate of bifidobacteria in SM-A beads would appear to be related to the SM buffering capacity against hydrogen ions and its tendency to reduce the pore size of bead. In this experiment, the survival rate of bifidobacteria entrapped in beads containing 0.6% SM showed the highest viability after exposure to simulated gastric juices for 3 h, thereby indicating that 0.6% SM is the optimum concentration for 3% alginate bead preparation. Third, the effect of SM-A beads on the freeze-drying and yogurt storage for 10 days was investigated. SM-A beads were found to be more efficient for freeze drying and yogurt storage than untrapped cells and the alginate bead. Consequently, the survival rate of bifidobacteria entrapped in SM-A beads was increased in simulated gastric juices, bile salts and probiotic products, such as lyophilized capsules and yogurt, SM-A beads can be expected to produce high value probiotic products.

Keywords : bifidobacteria, bile salts, lyophilized adjuncts, simulated gastric juices, SM-A bead, survival rate, yogurt

INTRODUCTION

The human colon is known to be a complex ecosystem with more than 400 different types of bacteria [1]. Bifidobacteria were first isolated by Tissier in 1889 as a predominant flora in breast-fed infants, which are gram positive anaerobes with special nutritional requirements and produce acetic and lactic acid at a molar ratio of 3:2, along with a small amount of formic acid, ethanol and succinic acid through the F6PPK pathway [2]. Bifidobacteria are related to human health because they have the probiotic ability to prevent constipation, suppress lactose intolerance, reduce the risk of colon cancer, lower blood cholesterol levels and improve the immune system [3,4]. Also bifidobacteria adopt a 'barrier' role that inhibits potentially pathogenic microorganisms by decreasing the pH in the colon and promoting a healthy microbial balance in the gastrointestinal tract, when present in sufficient numbers [5]. However, the number of bifidobacteria is disturbed by many factors, such as antibiotics, aging, stress and diet [6,7]. Therefore, as mentioned by Fuller, probiotics represent a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance [8-10]. For this reason it is necessary to supply live bifidobacteria to the human intestine. However, a high percentage of ingested bifidobacteria lose their viability during delivery through the gastrointestinal tract because of the free hydrochloric acid in the stomach and the bile salts secreted by the gall bladder into the duodenum [11]. In order to increase viability, lactic acid bacteria have been immobilized using various biopolymers, such as alginate, chitosan, κ-carageenan, gellan gum, xanthan gum etc [12-15]. Alginate is frequently used for the immobilization of lactic acid bacteria due to its ease of handling, non-toxic nature, safety as a food additive [16-18]. The pore size of the alginate bead is reflected by the viscosity of the carrier, due to the size of the alginate molecule, and its concentration can affect the diffusion of the substrates or products

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and limit the reaction rate of the entrapped cells and enzymes [19]. Lee and Heo [20] reported that the survival rate of bifidobacteria immobilized in a Ca-alginate bead is strongly dependent on various parameters including, the alginate concentration, bead size, initial cell number, and bacterial strains. However, as the alginate concentration becomes higher, the preparation of the bead becomes more difficult due to its high viscosity and, therefore, more expensive. Also, if a larger bead size is prepared, consumers acceptability suffers and the product value decreases.

In this study, an attempt was made to increase the survival rate of bifidobacteria entrapped in alginate in simulated gastric juices and bile salts by adding various materials to the alginate, also the optimum concentration of skim milk in SM-A beads was investigated. Finally, the protective effect of SM-A beads against freeze drying and yogurt storage was examined.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The strains included in this study, *Bifidobacterium longum* ATCC 15707, *Bifidobacterium infantis* ATCC 25962, and *Bifidobacterium breve* ATCC 15700 were obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA. These strains were routinely prepared as an inoculum by incubation in an anaerobic system (Forma Scientific Inc., USA) filled with mixed gases, consisting of N_2 (75%), H_2 (10%), and CO_2 (5%) for 20 h at 37°C. Strains were then subcultured in TPY broth after inoculation in a trypticase-proteose peptone-yeast extract (TPY) broth containing 0.5% glucose as the only carbohydrate source. High concentration fermentation for bifidobacterial cell entrapment was conducted with 20% (w/v) Na_2CO_3 buffer solution so as to control the at pH 5.5 under anaerobic conditions for 20 h at 37°C in a 2.5 L fermenter containing 1 L of TPY (2% glucose) broth to which at 2% (v/v) of fresh inoculum had been added.

Bacterial Cell Entrapment

Bifidobacterial cells were harvested in the late exponential phase (for 20 h at 37°C) and centrifuged for 15 min at 3000 × *g*. After washing twice with a 0.85% (w/v) NaCl saline solution, the cell pellet was resuspended in 500 mL of the same solution. The cell suspension was poured into a sterile disposable bag (Seward Co., UK) and 15 g of autoclaved alginate powder (medium viscosity, Sigma Chemical Co., USA) was added. This solution was then mixed using a laboratory blender (Stomacher 400, Seward Co., UK) to produce a final concentration of 3% (w/v). In the case of the preparations, various materials were added, such as poly dextrose (Jiwon Technical Co., Ltd.), soy fiber (Jiwon Technical Co., Ltd.), skim milk (Maeil Co., Korea), yeast extract (Difco), κ-carageenen (Sigma Chemical Co., USA), chitosan (Sigma Chemical Co., USA) and whey (Sigma Chemical Co., USA). The autoclaved powders 0.6% (w/v) were mixed with 3% alginate solution including bifidobacterial cells. These solutions were dropped into sterile 0.1 M $CaCl_2$ solution through a 24G blunt-ended needle using compressed air (2.3 kg/cm^2) and filtered through a sequence of 5 μm, 1 μm, and 0.5 μm air filters set in an air compressor [21]. The beads were gently stirred using a magnetic stirrer, hardened for 1 h in this solution, recovered using a sieve (1 mm pore size) and finally washed with sterile saline prior to use in order to remove any excess calcium ions and untrapped cells. The diameters of the calcium alginate beads were measured using an eyepiece micrometer on an optical microscope at a magnification of 100×, and their mean diameter (Dm) was calculated by measuring at least a 20 bead/sample.

Enumeration of Entrapped Cells

10 beads were transferred into 5 mL of 0.1 M sodium citrate and vortexed until the beads were completely dissolved in order to completely release the bifidobacterial cells. This solution was then used for the viable cell count. To enumerate the living cells, 100 μL of a tenfold diluted solution in physiological saline was spread on TPY agar gel and incubated in an anaerobic system for 48 h at 37°C, and cell colonies were counted.

Determination of Hydrogen Ion Permeation

An SM-A vessel without cells was prepared to determine its hydrogen ion permeability [22]. A glass tube was independently coated with sodium alginate (3%) solution containing different SM concentrations (0.2%, 0.4%, 0.6%, 0.8% and 1.0%) on several occasions in 0.1 M $CaCl_2$ solution for 1 h. The mean membrane thickness of the Ca-alginate vessel was about 1.5 mm using a vernier caliper (Mitutoyo Co., Japan). Individual alginate vessels were tightened at glass column, which was then filled with 5 mL of distilled water. Thereafter, a pH electrode was placed into the alginate vessel through the glass column, which was then placed in simulated gastric juices (pH 1.55). The pH changes inside the vessel were recorded using an X/Y recorder (1 cm/min) to determine the time base.

Survival Rate of Entrapped Cells

Reaction with Simulated Gastric Juices and Bile Salts

The acid tolerance of the bifidobacteria immobilized in the beads was determined by measuring the viability of the bacteria after being exposed for 3 h to simulated gastric juices (pH 1.55), which mainly consisted of 0.08 M HCl and 0.2% NaCl . The reaction in simulated gastric juices was conducted by exposing 10 beads to 3 mL, 37°C, 100 rpm for 3 h. After this time, 10 beads were collected, washed and dissolved in 0.1 M sodium citrate for enumeration of the live bacteria. The bile salt solution was made from 0.6% Oxgall (Difco) and the bead

treatment was similar to that used for the gastric juices reaction except that the contact time was extended to 6 h.

Effect of Freeze Drying

The beads samples were soaked in cryoprotectant (20% (w/v) skim milk) for 1 h and 10 beads were placed in a 1.5 mL test tube with 0.5 mL cryoprotectant. Free cells were centrifuged at 3000 × *g* for 15 min then the harvested cells were suspended in 0.85% (w/v) NaCl saline solution containing 0.05% (w/v) cystein HCl. After recentrifuging, cells were resuspended in 100 mL of a physiological solution. 0.1 mL of this cell suspension was then mixed with 0.5 mL of cryoprotectant in a 1.5 mL test tube for 1 h and frozen with liquified nitrogen for 10 sec. These preparations were then dried using a freeze-dryer (EYELA Co., Japan) for 16 h.

Preparation of Pasteurized Yogurt and Storage Condition

In order to investigate the stability of entrapped bifidobacteria in fermented milk over a product storage time, pasteurized yogurt was prepared. 10% (w/v) of skim milk was added to 200 mL of fresh milk and then autoclaved at 90°C for 30 min. An ABT941 starter (YC-180, Hansen's Lab., Denmark) in lyophilized form was inoculated and incubated at 37°C for 8 h. This yogurt, with a final pH of 4.4 was sterilized by autoclaving at 90°C for 30 min. The different bead type were then soaked in the pasteurized yogurt, stored at 4°C, and the viable cell count was determined after 2, 4, 6, 8, and 10 days.

RESULTS AND DISCUSSION

Preparation of Beads

In the case of bifidobacterial cell entrapment by alginate, various factors need to be considered, such as the alginate concentration, air pressure and needle size. These factors all influence the shape and size of the beads. Uniform spherical 3% alginate beads with a mean diameter (Dm) of about 1.5 mm were prepared without no tails under 2.3 kg/cm^2 of compressed air pressure through a 2.4 G needle. In order to standardize the numeral distribution of bifidobacteria in each bead, a homogeneous cell-alginate mixture was prepared using a stomacher, as described by Woo *et al.* [21].

Survival of Entrapped Bifidobacteria in Simulated Gastric Juices and Bile Salts

The entrapped bifidobacteria were exposed *in vitro* to simulated gastric juices without pepsin (pH 1.5). The survival rate of untrapped bifidobacteria species in simulated gastric juices showed that all species suffered a similar decrease, and were uncountable after 3 h (data not shown). However, in the case of 3% alginate cell entrapment, there was a small difference in the survival rate. The entrapped *B. longum* was the most resistant species, which decreased from initial 1.28×10^7 cfu/bead to 5.93×10^5 cfu/bead after exposure (Table 1). Various food additives were mixed with the cell-alginate solution in order to select the most protective material against the high acidic conditions. When the food additives were added to 3% alginate at a concentration of 0.6%, differences in the stability of the alginate beads were observed. The survival rate of the bifidobacteria entrapped in beads prepared with 0.6% SM exhibited the highest resistance to the simulated gastric juices. The viable cell numbers of *B. longum* decreased from 8.75×10^6 cfu/bead to 1.96×10^6 cfu/bead after 3 h (22.39% survival rate). *B. breve* entrapped in SM-A beads showed the highest survival rate of 23.61%. *B. infantis* showed a 19.37% survival rate which was the lowest rate recorded among the species examined. However, this difference is negligible in view of the overall picture, because the survival rate was signifycantly improved by SM as compared to the results of 3% alginate alone. These results indicate that the entrapping material is more potent protective factor than the acid resistance of the bacterial species in terms of the survival of the entrapped bifidobacteria. In the case of beads prepared with additions of 0.6% YE, 0.6% PD, 0.6% κ-C and 0.6% CS, the survival of bifidobacteria in the presence of simulated gastric juices was higher than that of the bacterial cells in beads containing only 3% alginate. However, these results were lower than that obtained using beads containing 0.6% SM, and their percent survival range varied between 5% and 15%. Other materials including 0.6% SF and 0.6% whey failed to improve cell survival compared to the 3% alginate beads, and SF actually reduced cell survival. It appears that this phenomenon results from ineffective blocking of the pores in the alginate beads. In order to investigate the effect of bile salts in the small intestine, entrapped bifidobacteria were exposed to 0.6% (w/v) oxgall solution for 6 h, which has a similar composition to human bile. The percent of viable cells entrapped with only 3% alginate lay between 19.36% and 24.15%, that is bile salts affected cell viability but to a lesser extent than gastric juices. In addition, there were no discernable differences in survival rate after bile salt exposure, with the exception of SM and CS. In the case of CS-A beads, the uptake of bile acid into bead was attributable to the electrostatic interaction between an amine group of the chitosan and the carboxylic groups of the bile acid, therefore it appears reasonable that an ion-exchange reaction accompanies insoluble complex formation between the chitosan and bile acid in the calcium alginate gel matrix [23], which may perturb the reaction between bile acid and the entrapped cells. The survival rate of bifidobacteria entrapped with SM was as high as CS. In summary, the majority of materials examined in this study improved the survival rate of bifidobacteria, this synergistic effect would seem to be attributable to two reasons. First, the pore size is blocked by adding materials into the alginate solution. Alternatively, the added materials have a buffering capacity that inhibits the effect of the hydrogen ions. The

Table 1. Survivability of bifidobacteria entrapped with various food additives in combination with alginate after exposure to simulated gastric juices (3 h) and bile salts (6 h)

Kinds of beads [a]	Species	Diameter of beads (mm) [b]	Reaction in simulated gastric juices (3 h) [c]			Reaction in bile salts (6 h) [c]		
			Before (log cfu/bead)	After (log cfu/bead)	Survival rate (%)	Before (log cfu/bead)	After (log cfu/bead)	Survival rate (%)
Alginate(A)	*B. longum*	1.550	7.108 ± 0.085	5.773 ± 0.151	4.62 ± 0.25	7.330 ± 0.038	6.737 ± 0.016	24.15 ± 1.29
	B.breve	1.453	7.354 ± 0.010	5.928 ± 0.081	3.79 ± 0.69	7.241 ± 0.003	6.528 ± 0.005	19.36 ± 0.16
	B. infantis	1.475	7.321 ± 0.003	5.870 ± 0.002	3.54 ± 0.04	7.530 ± 0.004	6.859 ± 0.014	21.34 ± 0.82
A+PD	*B. longum*	1.545	7.017 ± 0.076	5.743 ± 0.003	5.41 ± 1.20	6.953 ± 0.007	6.387 ± 0.003	27.17 ± 0.63
	B.breve	1.432	7.341 ± 0.004	6.262 ± 0.010	8.34 ± 0.18	6.825 ± 0.009	6.305 ± 0.005	30.20 ± 0.63
	B. infantis	1.536	7.397 ± 0.007	6.225 ± 0.009	6.73 ± 0.13	7.017 ± 0.006	6.420 ± 0.044	25.40 ± 2.92
A+SF	*B. longum*	1.470	7.983 ± 0.005	6.354 ± 0.002	2.35 ± 0.02	7.913 ± 0.006	7.336 ± 0.024	26.53 ± 1.89
	B.breve	1.493	8.013 ± 0.027	6.072 ± 0.008	1.15 ± 0.08	7.994 ± 0.006	7.342 ± 0.005	22.28 ± 0.05
	B. infantis	1.502	7.842 ± 0.026	6.025 ± 0.002	1.52 ± 0.01	7.837 ± 0.007	7.152 ± 0.011	20.66 ± 0.52
A+YE	*B. longum*	1.435	6.850 ± 0.004	5.977 ± 0.002	13.40 ± 0.08	6.752 ± 0.007	6.217 ± 0.003	29.18 ± 0.44
	B.breve	1.536	6.777 ± 0.010	5.874 ± 0.005	12.50 ± 0.19	6.702 ± 0.008	6.179 ± 0.005	29.99 ± 0.18
	B. infantis	1.528	7.023 ± 0.007	6.053 ± 0.006	10.72 ± 0.07	6.950 ± 0.005	6.391 ± 0.009	27.61 ± 0.25
A+SM	*B. longum*	1.532	6.942 ± 0.003	6.292 ± 0.003	22.39 ± 0.24	6.932 ± 0.004	6.513 ± 0.003	38.11 ± 0.44
	B.breve	1.506	6.660 ± 0.004	6.033 ± 0.011	23.61 ± 0.79	6.650 ± 0.004	6.276 ± 0.002	42.27 ± 0.54
	B. infantis	1.498	7.112 ± 0.007	6.399 ± 0.008	19.37 ± 0.62	7.017 ± 0.004	6.610 ± 0.002	39.18 ± 0.45
A+κ-C	*B. longum*	1.636	7.130 ± 0.004	6.127 ± 0.007	9.93 ± 0.24	7.021 ± 0.009	6.471 ± 0.003	28.19 ± 0.43
	B.breve	1.522	6.694 ± 0.003	5.680 ± 0.011	9.70 ± 0.20	6.565 ± 0.010	5.965 ± 0.001	25.12 ± 0.55
	B. infantis	1.542	6.745 ± 0.003	5.791 ± 0.016	11.12 ± 0.41	6.542 ± 0.003	6.030 ± 0.006	30.77 ± 0.63
A+CS	*B. longum*	1.436	7.213 ± 0.021	6.370 ± 0.002	14.36 ± 0.64	7.138 ± 0.013	6.731 ± 0.003	39.19 ± 1.28
	B.breve	1.368	7.029 ± 0.071	5.978 ± 0.005	8.98 ± 1.57	7.002 ± 0.008	6.606 ± 0.006	40.19 ± 1.25
	B. infantis	1.463	7.252 ± 0.021	5.968 ± 0.032	5.20 ± 0.22	7.135 ± 0.016	6.718 ± 0.001	38.30 ± 1.29
A+Whey	*B. longum*	1.485	6.362 ± 0.004	4.936 ± 0.003	3.75 ± 0.01	6.333 ± 0.004	5.735 ± 0.004	25.24 ± 0.38
	B.breve	1.553	7.190 ± 0.008	5.770 ± 0.003	3.80 ± 0.07	7.192 ± 0.004	6.643 ± 0.004	28.25 ± 0.43
	B. infantis	1.511	7.019 ± 0.003	5.648 ± 0.009	4.26 ± 0.06	6.985 ± 0.006	6.367 ± 0.008	24.10 ± 0.11

[a] Aliginate concentration : 3% Food additive concentration : 0.6%, [b] Mean diameter based on 20 beads
[c] Data are mean values ± standard deviations

high survival rate of bifidobacteria entrapped in SM-A beads presumably was more likely due to the latter reason.

Stability of Skim Milk-Alginate Beads with Different Skim Milk Concentrations

Hydrogen Ion Protection

In order to examine the effect of hydrogen ion permeation through the SM-A gel matrix, alginate vessel (membrane mean thickness = 1.5 mm) containing SM at concentrations of 0.2%, 0.4%, 0.6%, 0.8%, and 1.0% (w/v) were prepared and the pH changes were measured inside the vessel using an X/Y recorder, as described above. All types of vessels indicated that initially the pH increased within 1-2 min and then sharply decrease for 5 min (Fig. 1). The alginate vessel without SM showed a rapid pH reduction to 3 within 10 min, whereas the alginate vessel containing a higher SM concentration showed a slight pH decline and then an increase to a constant pH level between 10 min and 20 min. No differences were observed with the 0.6%, 0.8%, and 1.0% (w/v) SM concentrations. These results indicate that SM was effective in stabilizing the alginate beads yet no additional advantage was obtained by increasing the concentration beyond 0.6% into 3% alginate solution.

Survival of Bifidobacteria under Acidic Condition

Bifidobacteria entrapped with different SM concentrations were exposed to simulated gastric juices for 3 h to confirm the above results (Fig. 2). The survival rate of bifidobacteria entrapped with 0.6% SM was the highest, although bacterial cells entrapped with 0.8% and 1.0% SM exhibited very similar rates. Therefore, it was concluded that 0.6% SM was the optimum concentration for bead preparation.

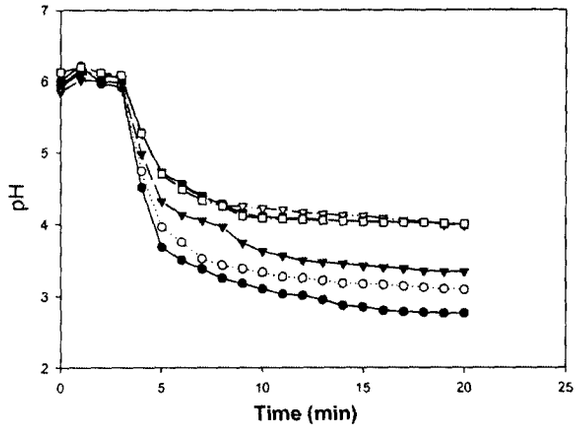

Fig. 1. Changes of pH in the alginate vessels containing different skim milk concentrations on exposure to simulated gastric juices. ● : 3% A, ○ : 3% A+ 0.2% SM, ▼ : 3% A+ 0.4% SM, ▽ : 3% A+ 0.6% SM, ■ : 3% A + 0.8% SM, □ : 3% A + 1.0% SM.

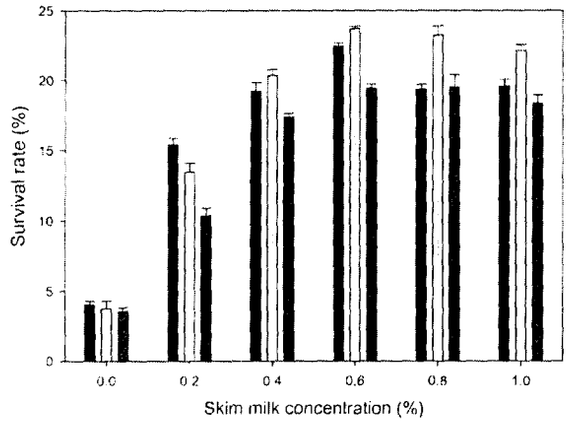

Fig. 2. Survival rate of bifidobacteria entrapped by alginate containing different skim milk concentrations after exposure to simulated gastric juices for 3 h. ■ : *B. longum* ATCC 15707, □ : *B. breve* ATCC 15700, ▩ : *B. infantis* ATCC 25962.

Effect of Freeze-drying

The survival rates of bifidobacteria were examined after freeze-drying, an industrial production requirement for probiotic powder or capsules, based on comparing untrapped cells, alginate and SM-A beads (Fig. 3). The untrapped bifidobacteria showed the lowest survival rate 6.73-8.24% among the three samples, the survival rate of the bacterial cells entrapped with only 3% alginate increased 25.32-29.31%, and the beads containing 0.6% SM showed 10% more viable cells than alginate. These results indicate that alginate beads containing SM are more stable than pure alginate at the same concentration in the freeze-drying process. As such, this type of beads can be recommended for probiotic powder or tablets.

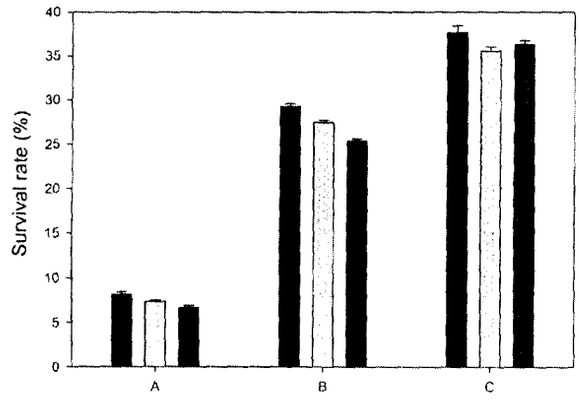

Fig. 3. Survival rate of bifidobacteria in various conditions upon freeze-drying. Type of beads : A ; Untrapped cell, B ; 3% Alginate bead, C ; 0.6% SM-3% A bead. ■ : *B. longum* ATCC 15707, □ : *B. breve* TCC 15700, ▩ : *B. infantis* ATCC 25962.

Survival of Entrapped Bifidobacteria in Pasteurized Yogurt

The survival rate of bifidobacteria in pasteurized yogurt at 4°C were investigated for 10 days (Table 2). The viable count of untrapped *B. infantis* dropped from 9.64 × 10^8 cfu/mL to 1.07 × 10^8 cfu/mL, and that of *B. infantis* entrapped in 3% alginate beads, with and without 0.6% SM, hardly changed during the storage periods. *B. longum* and *B. breve* had almost the same tendency as *B. infantis*. Thus, bifidobacteria entrapped in beads appeared to maintain their viability better than untrapped cells over a 10 days storage period.

CONCLUSION

The majority of materials examined in this study improves the survival rate of bifidobacteria, and this protective synergy would seem to be attributable to two reasons. Firstly, the pore size is blocked by adding materials into the alginate solution, thereby preventing the permeation of hydrogen ions into the beads, and secondly, the added materials appeared to have a good buffering capacity, thereby protecting the cells from penetrating hydrogen ions. SM proves to have the greatest buffering capacity of the materials examined, plus it is relatively cheap. The stability of the bifidobacteria entrapped in the SM-A beads was higher than that in the other beads in both the model of the human gastrointestinal tract and in practical applications, including lyophilized capsules and yogurt. Consequently, it is considered that SM-A beads are very suitable for producing probiotic adjuncts and high value yogurt.

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Table 2. Survival of bifidobacteria in pasteurized-fermented milk during storage at 4°C

Type of beads	Species	Storage time (days) [c]					
		0	2	4	6	8	10
Untrapped cells [a]	*B. longum,*	9.348 ± 0.002	8.985 ± 0.004	8.915 ± 0.002	8.820 ± 0.006	8.685 ± 0.004	8.651 ± 0.004
	B. breve	9.154 ± 0.002	9.012 ± 0.009	8.735 ± 0.005	8.656 ± 0.003	8.311 ± 0.004	8.215 ± 0.005
	B. infantis	8.984 ± 0.003	8.531 ± 0.005	8.617 ± 0.005	8.342 ± 0.005	8.210 ± 0.001	8.030 ± 0.004
3% Alginate [b]	*B. longum,*	7.217 ± 0.002	7.215 ± 0.04	7.212 ± 0.003	7.193 ± 0.004	7.190 ± 0.003	7.189 ± 0.008
	B. breve	7.354 ± 0.004	7.352 ± 0.006	7.353 ± 0.002	7.350 ± 0.005	7.350 ± 0.004	7.348 ± 0.007
	B. infantis	7.532 ± 0.005	7.533 ± 0.002	7.526 ± 0.006	7.525 ± 0.002	7.527 ± 0.006	7.523 ± 0.002
0.6%SM-A [b]	*B. longum,*	7.344 ± 0.005	7.340 ± 0.012	7.340 ± 0.002	7.338 ± 0.003	7.337 ± 0.004	7.337 ± 0.002
	B. breve	7.625 ± 0.004	7.625 ± 0.005	7.624 ± 0.004	7.623 ± 0.001	7.624 ± 0.007	7.622 ± 0.002
	B. infantis	7.157 ± 0.003	7.155 ± 0.004	7.154 ± 0.004	7.156 ± 0.005	7.156 ± 0.003	7.155 ± 0.005

[a] Viable cell count log cfu/mL, [b] Viable cell count log cfu/bead, [c] Data are mean values ± standard deviations

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