

## Survival of Double-Microencapsulated *Bifidobacterium breve* in Milk in Simulated Gastric and Small Intestinal Conditions

Ji-Kang Jung<sup>1</sup>, Jeung-Ha Kil<sup>1</sup>, Sang-Kyo Kim<sup>2</sup>, Jung-Tae Jeon<sup>2</sup> and Kun-Young Park<sup>1†</sup>

<sup>1</sup>Department of Food Science and Nutrition, Pusan National University, Busan 609-735, Korea

<sup>2</sup>R&D Center, Vilac Company LTD., Busan 616-821, Korea

### Abstract

**Bifidobacteria** are probiotic organisms that provide both flavor and health benefits when incorporated as live cultures into commercial dairy products. Because bifidobacteria are very sensitive to environmental conditions (acids, temperature, oxygen, bile salts, the presence of other cultures, etc.), their viability in human gastrointestinal tract is limited. The microencapsulation of bifidobacteria is a process to protect them against harsh environmental conditions, thereby increasing their viability while passing through human gastrointestinal tract. To confirm the survival rate of microencapsulated *Bifidobacterium breve* CBG-C2 in milk, their survival rate was compared with several kinds of free bifidobacteria and lactic acid bacteria in commercial yogurt products under simulated gastric and small intestinal conditions. Double-microencapsulation of the bacteria was employed to increase the survival rate during digestion. The outer layer was covered with starch and gelatin to endure gastric conditions, and the inner layer was composed of a hard oil for the upper small intestinal regions. Almost all microencapsulated bifidobacteria in the milk survived longer than the free bifidobacteria and lactic acid bacteria in the commercial yogurt products under the simulated gastric conditions. Numbers of surviving free bifidobacteria and lactic acid bacteria in the commercial products were significantly reduced, however, the viability of the microencapsulated bifidobacteria in the milk remained quite stable under gastric and small intestine conditions over 3~6 hrs. Thus double-microencapsulation of bifidobacteria in milk is a promising method for improving the survival of bifidobacteria during the digestive process.

**Key words:** survival, bifidobacteria, microencapsulation, gastric and small intestinal condition

### INTRODUCTION

Bifidobacteria are anaerobic, rod shaped Gram-positive bacteria, that normally inhabit the human colon (1,2). Beneficial effects of bifidobacteria including improvement of intestinal flora by preventing colonization of pathogens, activation of the immune system, increased protein digestion and amelioration of diarrhea or constipation have been reported (3). Therefore bifidobacteria have been used in foods as a probiotic adjunct, with some of the most commonly used bifidobacterial strains being *B. bifidum*, *B. breve*, *B. infantis*, and *B. longum*, *B. adolescentis*. Recently, many kinds of food products containing these bifidobacteria such as yogurt, beverages, and cheese have entered the market.

The population levels of lactic acid bacteria initially contained in commercial dairy products are commonly  $10^7 \sim 10^8$  CFU/mL or more. However, bifidobacteria are very sensitive and intolerant of acid, oxygen, temperature, bile salt and the presence of other cultures (4). Therefore the population levels of bifidobacteria in com-

mercial dairy products at time of consumption may be only  $10^5 \sim 10^6$  CFU/mL or less. Furthermore, during their passage through the human gastrointestinal tract, the viability of the remaining bifidobacteria is again greatly decreased.

The minimum level of viable bifidobacteria in commercial dairy products to exert beneficial effects on human health is known to be approximately  $10^5 \sim 10^7$  CFU/mL (5,6). Microencapsulation techniques have been utilized to protect microorganisms such as bifidobacteria (7-9), and the encapsulation of bifidobacteria to protect them against adverse effects of gastric and small intestinal conditions has been attempted and reported by various investigators (10-15).

Double-microencapsulation has a more protective effect than single-microencapsulation (16). It was designed so that the outer layer can be resistant to gastric acid and bile salts, and the inner layer can be broken down by lipase in the lower region of small intestine enabling viable bifidobacteria to reach the colon.

Prebiotics are indigestible food ingredients that benefi-

<sup>†</sup>Corresponding author. E-mail: kunypark@pusan.ac.kr  
Phone: +82-51-510-2839, Fax: +82-51-514-3138

cially affect the host by selectively stimulating growth and/or activity of one or a number of health-promoting colon bacteria (17). Inulin is a plant (chicory, onion, asparagus, etc.)-derived carbohydrate with the benefits of soluble dietary fiber. It is not digested or absorbed in the small intestine, but is fermented in the colon by the beneficial bacteria. The average dietary intake of inulin by humans is estimated to be 1~4 g/day (18). Functioning as a prebiotic, inulin has been used to promote the proliferation of beneficial bacteria in the gastrointestinal system. Chicory derived inulin showed prebiotic effects from the proximal to distal colon. Additionally, supplementing inulin or oligofructose increases colonic Ca, Mg and Fe absorption and enhances bone calcium stores in rats and humans (19-22). In addition, it influences blood glucose levels, and reduces the levels of cholesterol and serum lipids (23,24). The addition of inulin to the microencapsulated bifidobacteria supplemented milk was designed to act as a prebiotic in the colon.

This study was conducted to determine the survival of the double-microencapsulated bifidobacteria in milk under human gastrointestinal conditions. Microencapsulated bifidobacteria supplemented milk (MBM) was designed to contain 0.3% double-microencapsulated *Bifidobacterium breve* CBG-C2 as a probiotic and 0.5% chicory inulin as prebiotic. The survival behavior of bifidobacteria was examined when they were exposed to simulated gastric conditions (pH 2.0, 0~3 hours) and small intestinal conditions (pH 8.0, 0~6 hours) after staying for different time (0~3 hours) at the gastric condition. Survival of the microencapsulated bifidobacteria in milk was compared with the survival of free bifidobacteria and other lactic acid bacteria in 6 different kinds of commercial yogurt products available in Korea.

## MATERIALS AND METHODS

### Preparation of the MBM

Microencapsulated bifidobacteria supplemented milk (MBM) was provided from Vilac Co. LTD., Busan, Korea. The 0.3% of double-microencapsulated *Bifidobacterium breve* CBG-C2 was added to milk. Fig. 1 shows the structure of the double-microencapsulated bi-



Fig. 1. Structure of double-microencapsulated bifidobacteria.

fidobacteria used in this study. Microencapsulation was carried out using starch, gelatin, gum, hard oil, glycerin esters of fatty acids and *Bifidobacterium breve* CBG-C2. The mixture was sprayed into a bath containing an aseptic water solution. The microencapsulation was optimized for formation of a double microcapsule using W/O/W type emulsions containing the *Bifidobacterium breve* CBG-C2. The inner layer of the double-microcapsule was covered with hard oil made from coconut oil and the outer layer was composed of starch and gelatin for protection in the upper region of the small intestine and stomach, respectively. The milk was also supplemented with 0.5% water soluble dietary fiber, inulin from chicory, as a prebiotic.

### Preparation of commercial yogurt products

Six kinds of commercial yogurt products (CP) were purchased at a market in Busan, Korea, all of which contained bifidobacteria. Different strains of lactic acid bacteria were also used in the commercial yogurt products (CP), *L. acidophilus* in CP1 (S Co.), *L. bulgaricus* in CP2 (N Co.), LGG in CP3 (M Co.), 5 complex lactic acid bacteria in CP4 (B Co.), *L. delbrückii subsp.*, *L. bulgaricus* and *S. thermophilus* in CP5 (P Co.), *L. acidophilus*, *L. casei* and *S. thermophilus* in CP6 (H Co.).

### Survival test for bifidobacteria and total lactic acid bacteria under simulated stomach and small intestinal conditions

The simulated gastric condition was prepared by suspending pepsin (1 mg/mL, Sigma Chemical Co., St. Louis, MO, USA) in distilled water and adjusting the pH to 2.0 with 1 N HCl. To mimic small intestine conditions, cholic acid (0.02 M) and deoxycholic acid (0.02 M) (bile salt), lipase (0.05 mg/mL) and pancreatin (0.2 mg/mL) (Sigma Chemical Co., St. Louis, MO, USA) were suspended in distilled water and adjusted to pH 8.0 with 1 N HCl and NaOH (Fig. 2) (25).

One milliliter of MBM and 6 kinds of CP was added to 9 mL simulated gastric fluid and vortexed for 20 sec, and samples taken at 0 hr, 1 hr, 2 hr and 3 hr after mixing to determine viability of bifidobacteria and lactic acid bacteria in the test samples.

One milliliter each of the remainders of the mixtures after incubating for 0, 1, 2 and 3 hr were added to 9 mL simulated small intestinal fluids respectively and vortexed for 20 sec. The samples were taken at 0 hr, 2 hr, 4 hr and 6 hr after the mixing to determine viability of bifidobacteria and total lactic acid bacteria.

In this experimental process, all test tubes containing the mixtures were incubated at 37°C.

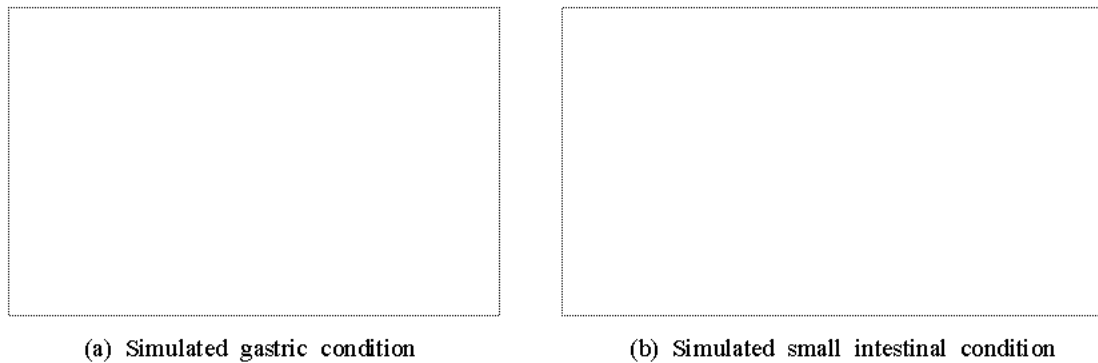


Fig. 2. Simulated gastric and small intestinal conditions.

#### Enumeration of bifidobacteria and total lactic acid bacteria

Two types of culture media were prepared. BS (bifidobacteria selective) medium was prepared by mixing 58 g BL agar (Difco, Sparks, MD 21152, USA), 15 g sodium propionate, 3 g lithium chloride (Junsei Chemical Co. LTD., Tokyo, Japan), 0.05 g paromomycin sulfate, 0.1 g neomycin and 0.04 g bromocresol purple (Sigma Chemical Co., St. Louis, MO, USA) in 1,000 mL distilled water and sterilizing at 121°C for 15 min.

BCP medium for lactic acid bacteria was prepared by mixing 24.6 g BCP plate count agar (Difco, Sparks, MD, USA) and in 1,000 mL distilled water and sterilizing at 121°C for 15 min (5).

Samples taken at each time point were serially diluted with sterilized physiological saline solution and inoculated on BS and BCP agar medium, and the former was kept in an anaerobic package (Nicepack Co., Korea) with Anaerogen (Oxid Limited, Wade Road, Basingstoke, Hampshire, England). Colonies were counted (CFU/mL)

after incubation at 37°C for 72 hr.

## RESULTS AND DISCUSSION

#### Survival of the bacteria in MBM and CP in simulated gastric condition

To be used as food adjuncts, bifidobacteria must survive transit through the human gastrointestinal tract (7, 26). The minimum level of viable bifidobacteria in commercial dairy products needed to exert beneficial effect on human health is approximately  $10^5 \sim 10^7$  CFU/mL (5, 27).

Fig. 3 (A) shows the viability of microencapsulated bifidobacteria in MBM and free bifidobacteria in 6 kinds of CP after initial, 0, 1, 2 and 3 hour of exposure to the simulated gastric condition. The commercial yogurt products contained more lactic acid bacteria ( $\sim 10^{10}$ ) than bifidobacteria ( $\sim 10^6$ ); therefore, the survival level of microencapsulated bifidobacteria in MBM were compared with total lactic acid bacteria found in CP (Fig.

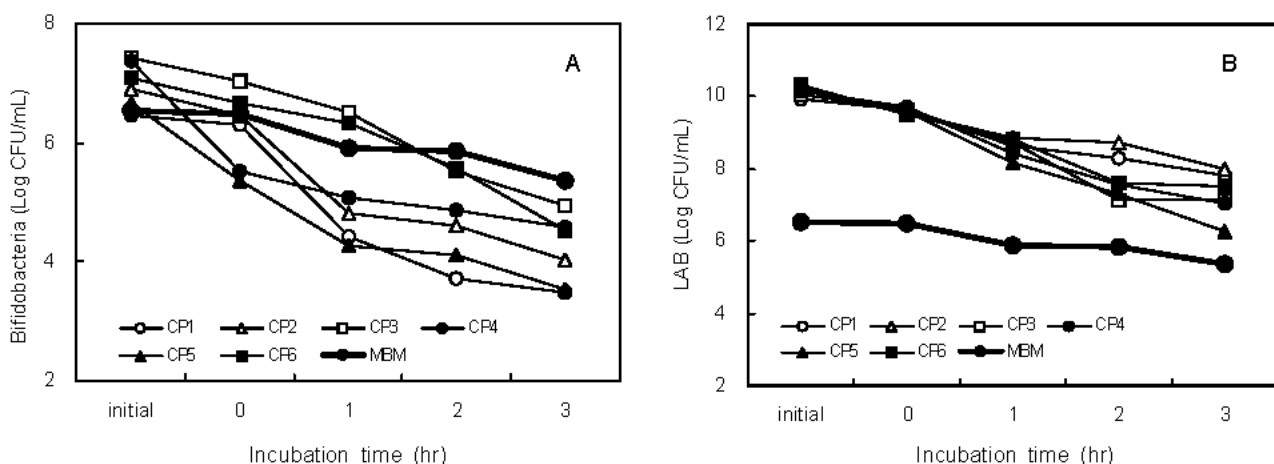


Fig. 3. Survivals of bifidobacteria and total lactic acid bacteria (LAB) in microencapsulated bifidobacteria supplemented milk (MBM) and 6 kinds of commercial yogurt products after exposure to simulated gastric conditions (pH 2.0) as a function of treatment time. Data of MBM in B is bifidobacteria count shown as a control.

3 (B)).

The microencapsulated bifidobacteria in MBM were more resistant to simulated gastric conditions than free bifidobacteria in CP. More than  $10^5$  bifidobacteria in MBM survived after 3 hr and decreased by less than one logarithm unit (initial content was  $10^6$  in the simulated gastric condition). The bifidobacteria in the 6 kinds of CP were much less resistant, as indicated by the 2~3 logarithm unit decrease in the population after the incubation. Since the outer layer of microencapsulation consisted of starch and gelatin, the gastric enzyme could not break the layer. This is why most of the bifidobacteria in MBM survived.

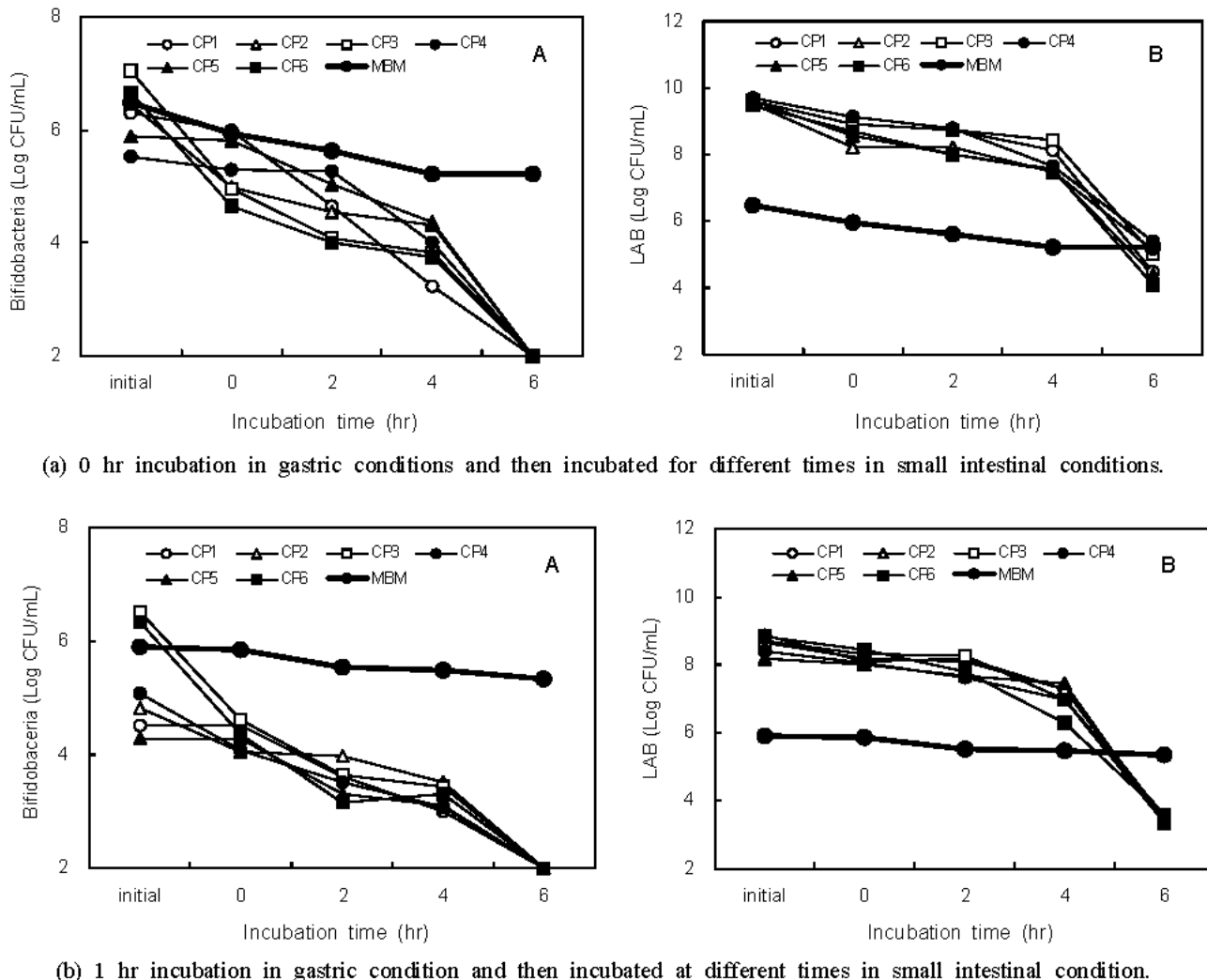
The total lactic acid bacteria counts declined a little more than did bifidobacteria. At the beginning, the lactic acid bacteria levels ( $10^9 \sim 10^{10}$ ) in CP were remarkably

higher than bifidobacteria ( $10^6$ ) in MBM (Fig. 3 (b)). The lactic acid bacteria level were considerably decreased to  $10^6 \sim 10^8$  in CP, but the counts only decreased to  $10^5$  in MBM after 3 hr in simulated gastric condition.

Thus the double-microencapsulated bifidobacteria in MBM exhibited a better survival rate than free bifidobacteria and lactic acid bacteria in CP under the simulated gastric conditions.

#### Survival of the bacteria in MBM and CP in simulated small intestinal conditions

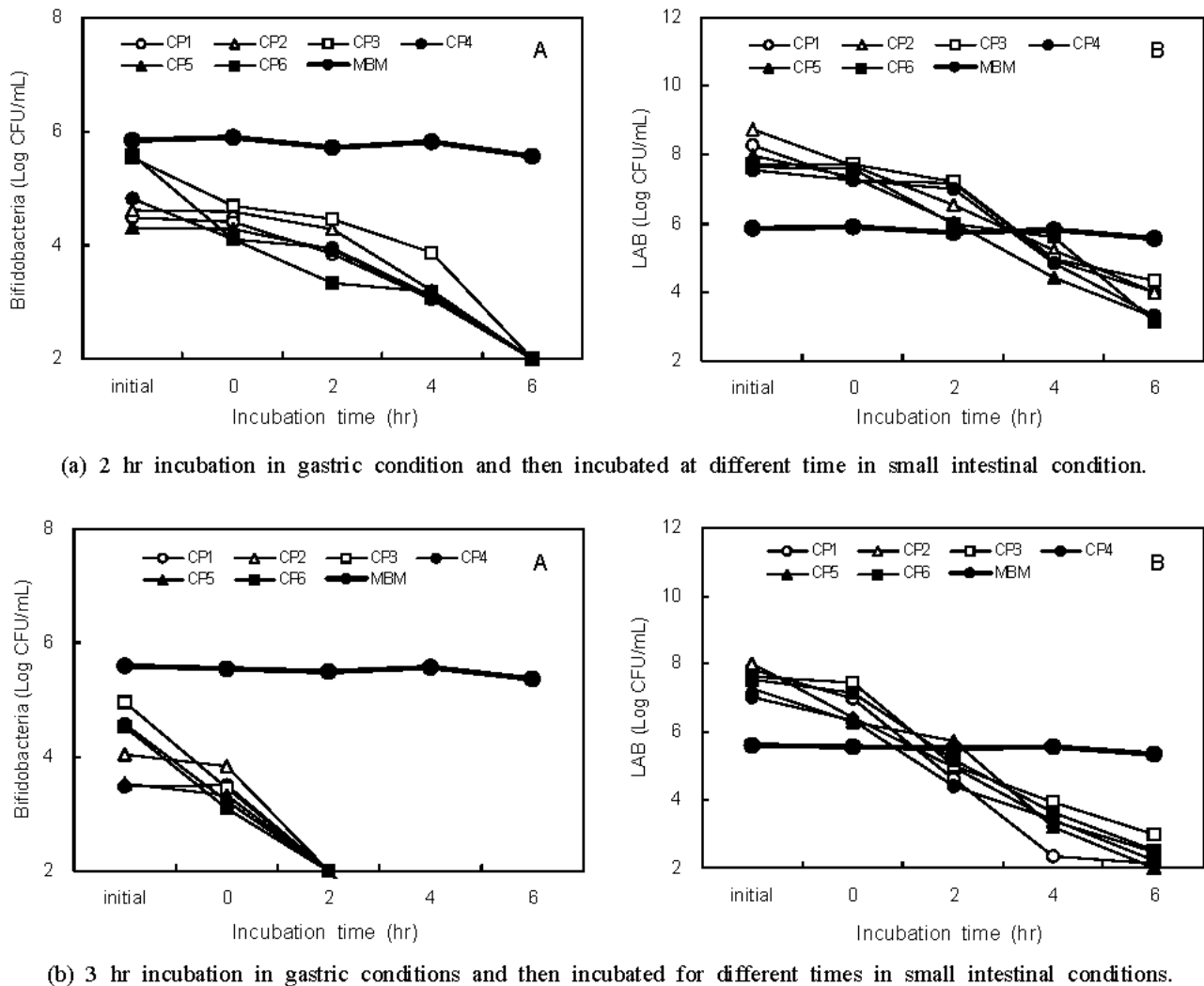
Survival curves of microencapsulated bifidobacteria in MBM and free bifidobacteria and total lactic acid bacteria in CP while exposed to simulated small intestinal condition during 6 hr after a brief exposure to the gastric conditions (0 (a) and 1 (b) hour) are shown in Fig. 4.



**Fig. 4.** Survivals of bifidobacteria and total lactic acid bacteria (LAB) in microencapsulated bifidobacteria supplemented milk (MBM) and 6 kinds of commercial yogurt products while exposed to simulated small intestinal condition (pH 8.0) after exposure to simulated gastric condition during short time (0 and 1 hour). Data of MBM in B is bifidobacteria count for shown as a control.

Viable bifidobacteria cells decreased very rapidly in CP. The initial cells were  $10^6$  in MBM and  $10^5 \sim 10^7$  in CP after exposure gastric condition for 0 hr. However, 4 hr later the viable bifidobacteria cells in small intestinal condition were  $10^5$  in MBM and  $10^3 \sim 10^4$  in CP, and 6 hr later less than  $10^2$  remained in CP but viable cells in MBM remained at  $10^5$ . The bifidobacteria counts in CP were  $<10^2$  CFU/mL 6 hr after exposure to the gastric condition with exposures of either 0 or 1 hour. On the other hand, microencapsulated bifidobacteria in MBM were very stable. Free lactic acid bacteria were more resistant to simulated gastric and small intestinal condition than free bifidobacteria as shown in Fig. 4. However, the rate of decrease in viability of microencapsulated bifidobacteria in MBM was slower than

that of the total lactic acid bacteria in CP. The CP bifidobacteria cells that survived gastric conditions for 2 hr were affected similarly by small intestinal condition as the 0 or 1 hr, although the starting levels of total lactic acid bacteria were considerably decreased, and when they were first treated for 3 hr in the gastric conditions and for 2 hr in the simulated small intestinal conditions the counts were  $<10^2$  or not detected at all (Fig. 5). However, bifidobacteria in MBM under the same conditions were decreased only by 4%. The viable cells of lactic acid bacteria in CP very rapidly declined, the initial cell number were  $10^7 \sim 10^8$  in CP, and 4 hr later the numbers had decreased to  $10^2 \sim 10^4$ , and finally the survival cell numbers 6 hr later were  $<10^2$  (Fig. 5). The rate of decrease was dependent on time in gastric and



**Fig. 5.** Survival of bifidobacteria and total lactic acid bacteria (LAB) in microencapsulated bifidobacteria supplemented milk (MBM) and 6 kinds of commercial yogurt products while exposed to simulated small intestinal conditions (pH 0.8) after exposure to simulated gastric conditions for long times (2 and 3 hours). Data of MBM in B is bifidobacteria count is shown as a control.

small intestinal conditions. This experiment demonstrated that the inner and outer layer of microencapsulation was still stable in the small intestinal conditions.

Microencapsulated bifidobacteria in MBM appear to have better resistance against adverse conditions of the stomach and small intestine. Therefore the microencapsulated bifidobacteria in milk has good efficacy as a probiotic in foods like yogurt, with high survival rate. This is the first product developed that gives both milk and yogurt function. The inner layer of the double-microencapsulation was designed to be broken in the end of small intestine so that it can be colonized in the colon. When the bifidobacteria reach the colon, the chicory inulin in milk was able to help the colonization and growth of bifidobacteria as a prebiotic.

However, further studies are needed in *in vitro*, *in vivo* and in human trials to elucidate the mechanisms and mode of action of the microencapsulated bifidobacteria in the digestive tracts and to evaluate its ability to colonize the colon.

## ACKNOWLEDGMENT

This research was supported by Vilac Company, LTD, Busan, Korea.

## REFERENCES

1. Rasic LJ, Kurmann JA. 1983. *Bifidobacteria and their role*. Birkhauser, Bern, Switzerland. p 8-34.
2. Simon GL, Gorbach SL. 1984. Intestinal flora in health and disease. *Gastroenterology* 86: 174-193.
3. Ishibashi N, Shimamura S. 1993. Bifidobacteria: research and development in Japan. *Food Technol* 6: 126-136.
4. Favaro-trindade CS, Grosso CR. 2002. Microencapsulation of *L. acidophilus* (La-05) and *B. lactis* (Bb-12) and evaluation of their survival at the pH values of the stomach and in bile. *J Microencapsul* 19: 485-494.
5. Cui JH, Shim JM, Lee JS, Lee BJ. 2000. Gastric acid resistance of *Lactobacilli* and *Bifidobacteria* in commercial drink and lipid yogurts. *Kor J Microbiol* 36: 161-165.
6. Chen RM, Wu JJ, Lee SC, Huang AH, Wu HM. 1999. Increase of intestinal *bifidobacterium* and suppression of coliform bacteria with short-term ingestion. *J Dairy Sci* 82: 2308-2314.
7. Kim HS, Kamara BJ, Good IC, Enders GL. 1988. Method for the preparation of stable microencapsulated lactic acid bacteria. *J Ind Microbiol Biotechnol* 3: 253-257.
8. Lim F, Moss RD. 1981. Microencapsulation of living cells and tissues. *J Pharm Sci* 70: 351-354.
9. Kwok KK, Groves MJ, Burgess DJ. 1992. A novel method for the determination of sterility of microcapsules and measurement of viability of encapsulated organisms. *Pharm Res* 9: 410-413.
10. Rao A, Shwinnarain VN, Maharaj I. 1989. Survival of microencapsulated *Bifidobacterium pseudolongum* in simulated gastric and intestinal juice. *Can Inst Food Technol J* 22: 345-349.
11. Modler HW, McKeller RC, Yaguchi M. 1990. Bifidobacteria and Bifidogenic factors. *Inst Food Sci Technol J* 23: 29-41.
12. Hughes DB, Hoover DG. 1991. Bifidobacteria: their potential for use in American dairy products. *Food Technol* 45: 74-83.
13. Cui JH, Goh JS, Kim PH, Choi SH, Lee BJ. 2000. Survival and stability of bifidobacteria loaded in alginate poly-L-lysine microparticles. *Int J Food Pharm* 210: 51-59.
14. Sun W, Griffiths MW. 2000. Survival of bifidobacteria in yogurt and simulated gastric juice following immobilization in gellan-xanthan beads. *Int J Food Microbiol* 61: 17-25.
15. Siuta-Cruce P, Goulet J. 2001. Improving probiotic survival rates. *Food Technol* 55: 36-42.
16. Park BG. 2004. Optimization of condition for the double-microencapsulation of lactic acid bacteria. *MS Thesis*. Seoul Natl Univ of Technol, Seoul, Korea.
17. Gibson GR, Roberfroid MB. 1995. Dietary modulation of the human colonic microbiota-Introducing the concept of prebiotics. *J Nutr* 125: 1401-1412.
18. Vanloo J, Coussemont P, Deleenheer L, Hoebregs H, Smit G. 1995. On the presence of inulin and oligofructose as natural ingredients in the western diet. *Crit Rev Food Sci Nutr* 35: 525-552.
19. Griffin IJ, Davila PM, Abrams SA. 2002. Non-digestible oligosaccharides and calcium absorption girls with adequate calcium intake. *British J Nutr* 87: S187-S191.
20. Ohta A, Ohtsuki M, Uehara M, Hosono A, Hirayama M, Adachi T, Hara H. 1998. Dietary fructooligosaccharides prevent post gastrectomy anemia and osteopenia in rats. *J Nutr* 128: 485-490.
21. Roberfroid MB, Cumps J, Devogelaer JP. 2002. Dietary chicory inulin increases whole-body bone mineral density in growing male rats. *J Nutr* 132: 3599-3602.
22. Van den Heuvel EGHM, Scaafsma G, Muys T, Van Dokkum W. 1998. Nondigestible oligosaccharides do not interfere with calcium and nonheme-iron absorption in young healthy men. *Am J Clin Nutr* 67: 445-451.
23. Coudray C, Bellanger J, Castiglia-Delavaud C, Remesy C, Vermorel M, Rayssiguier Y. 1997. Effect of soluble or partly soluble dietary fibers supplementation on absorption and balance of calcium, magnesium, iron and zinc in healthy young men. *Eur J Clin Nutr* 51: 375-380.
24. Niness KR. 1999. Inulin and oligofructose: what are they? *J Nutr* 129: 1402S-1406S.
25. Lee BJ. 2003. Microencapsulation of vitamin C and its effect on iron bioavailability in iron fortified milk. *MS Thesis*. Sejong Univ., Seoul, Korea.
26. Gilliland SE. 1989. Acidophilus milk product a review of potential benefits to consumers. *J Dairy Sci* 72: 2483-2494.
27. Lian WC, Hsiao HC, Chou CC. 2003. Viability of microencapsulated bifidobacteria in simulated gastric juice and bile solution. *Int J Food Microbiol* 86: 293-301.

(Received January 29, 2007; Accepted March 2, 2007)