Dual Coating Improves the Survival of Probiotic Bifidobacterium Strains during Exposure to Simulated Gastro-Intestinal Conditions

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Probiotics have been reported to benefit human health by modulating immunity, lowering cholesterol, improving lactose tolerance, and preventing some cancer. Once ingested, probiotic microorganisms have to survive harsh conditions such as low pH, protease-rich condition, and bile salts during their passage through the gastro-intestinal (GI) tract colonize and proliferate to exert their probiotic effects. The dual coating technology, by which the bacteria are doubly coated with peptides and polysaccharides in consecutive order, was developed to protect the ingested bacteria from the harsh conditions. The aim of the study was to evaluate the viable stability of a doubly coated blend of four species of Bifidobacterium by comparing its bile/acid resistance and heat viability in vitro with that of the non-coated blend. After challenges with acid, bile salts, heat, and viable cell counts (VVCs) of the dual coated and non-coated blend were determined by cultivation on agar plates or flow cytometric measurement after being stained with the BacLight kit™. The results showed that the dual coated blend was much higher resistant to the acidic or bile salt condition than the non-coated blend and heat viability was also higher, indicating that the dual coating can improve the survival of probiotic bacteria during their transit through the GI tract after consumption.

Keywords: Bifidobacterium, dual coating technology, duolac, probiotics

In today’s society, there has been increasingly interested in their personal health and functional food. Probiotic products are an important functional food as they represent about 65% of the world functional food market, and the market for probiotic products continues to expand (Agrawal, 2005; Jankovic et al., 2010). Probiotics are defined as 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2002). Researchers have reported to play a therapeutic role by modulating immunity, lowering cholesterol, improving lactose tolerance and preventing some cancer (Kailasapathy and Chin, 2000; Sanders et al., 2007). Probiotics are orally administrated and are available in various forms such as food products, capsules, sachets, or tablets. Ingested probiotics have to survive adverse conditions such as low pH, protease-rich condition, and bile salts during their passage through the gastro-intestinal (GI) tract to be able to influence the human gut microflora (Weichselbaum, 2009; Burgain et al., 2011). However some probiotic bacteria are sensitive to oxygen, and many require media ingredients or modified gas environments to enable their growth (Dave and Shah, 1996; Talwalkar and Kailasapathy, 2004). Especially, Bifidobacterium strain that are the most widely used probiotic bacteria and are included in many products and functional foods vary greatly in their sensitivity to the harsh acidic environment of the stomach and many foods (Clark and Martin, 1994; Lankaputhra and
Shah, 1997; Charteris et al., 1998; Truestrup et al., 2002; Guarner and Malagelada, 2003; Annan et al., 2008). For this reason, many researchers reported that there is poor survival of probiotic bacteria in products containing free probiotic cells (De Vos et al., 2010). Various technologies of encapsulation such as emulsification, spray drying, spray cooling, and freeze drying have been developed for protection live cells in the food industry (De Vos et al., 2010; Burgain et al., 2011), and it has been reported these technologies of encapsulation improved the viability of probiotic bacteria in the GI tract (Krasaekoopt et al., 2003; Picot and Lacroix, 2004; Sohail et al., 2011; Su et al., 2011; Saarela et al., 2011).

Dual coating technology is so-called fourth generation coating technology for protection of LAB during the passage through the GI tract and manufacturing process and was patented in Korea (patent no. 0429495), Japan (patent no. 3720780), and Europe (patent no. 1514553B) (Burgain et al., 2011; Cha et al., 2011). The technology was developed to protect the ingested bacteria from the harsh conditions. In the technology, bacteria cells are coated with peptides and subsequently with polysaccharide matrix (Figs. 1 and 2). A polypeptide behaves in a pH-dependent way, and there exists a pH gradient along the stomach and intestine. Thanks to the coating layers, the doubly coated cells remain mostly uncoated at pH 4.0, which is the pH of stomach after meal and begin to be released from the coats at pH 6.0 and are fully released at pH 7.0, the pH in the intestine. The polysaccharide matrix protects from moisture, heat, and physical pressure, and so stability is increased. Therefore the dual coated bacteria after ingestion are able to reach the intestine alive and in good condition to colonize and proliferate while uncoated bacteria that is prone to be damaged in the gastro-intestinal environment.

In this study, we evaluated in vitro acid/bile resistance and heat viability of a probiotic blend consisting of four Bifidobacterium species which were doubly coated or non-coated and compared the resistances of the dual coated blend with those of the non-coated blend.

Materials and Methods

Bacteria

Dual coated Bifidobacterium bifidum BF3 (KCTC 12199BP), B. infantis BT1 (KCTC 12859BP), B. longum BG7 (KCTC 12200BP), and B. rhamnosus BR3 (KCTC 12201BP) were blended in equal proportions and tested to assess the resistance to acid or bile salts. The same tests were applied to a blend containing the same kind of species of non-coated Bifidobacterium. Comparison of acid, bile, and heat resistance between the two formulations was made.

Resistance to acid and bile salt

For acid tolerance test, the blend of dual coated Bifidobacterium or non-coated Bifidobacterium was inoculated in BL broth (around 10^7 CFU/ml) which was adjusted to pH 2.0, 3.0, 5.0, or 7.0 using 0.1 M HCl or 0.1 M NaOH. Samples were taken at various time points (0, 0.5, 1, 3, and 8 h) and subjected to viable cell count using BL agar plate with 0.005% bromocresol purple or LIVE/DEAD BacLight kit™ (Invitrogen, USA).

For bile tolerance test, the blends were inoculated into acidified BL broth (pH 4.0) (approximately 10^7 CFU/ml) containing oxgall (BD, USA) at the concentration of 0, 0.1, 0.3, or 1% (w/v). Samples were taken at various time points (0, 3, 6, 9, 12, and 24 h) and subjected to viable cell count with the same methods, as described above.

Staining of bacterial cells

Cells collected at the time points were adjusted to be 10^6-7 CFU/ml in potassium phosphate buffer (PBS, pH 7.2), treated with the reagents in the BacLight kit as recommended by the manufacturer, and gently shaken for 15 min in dark condition.
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First labeling was confirmed by the use of a fluorescent microscopy (Olympus, Japan) set to accept fluorescence intensity at a wavelength (emission 1; green and emission 2; red).

**Flow cytometric measurements (FCM)**

Flow cytometric measurements were performed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, USA) equipped with a 15-mW, 488-nm, air-cooled argon ion laser and a cell-sorting catcher tube. Cell samples were diluted to approximately 10⁵ cells/ml and delivered at the low flow rate, corresponding to 150 to 500 cells/sec. FSC, SSC, and three fluorescence signals were measured. A band pass filter of 530 nm (515 to 545 nm) was used to collect the green fluorescence (FL1), a band pass filter of 585 nm (564 to 606 nm) was used to collect the yellow-orange fluorescence (FL2), and a long pass filter of 670 nm was used to collect the red fluorescence (FL3). FSC was collected with a diode detector. SSC and the three fluorescence signals were collected with photomultiplier tubes. All signals were collected by using logarithmic amplifications. A combination of FSC and SSC was used to discriminate bacteria from background.

**Heat stability**

For stability test, we studied the differences in the viability values between non-coated and dual coated *Bifidobacterium* during 2 weeks storage period at 40°C. The blend of dual coated *Bifidobacterium* or non-coated *Bifidobacterium* were kept for 2 weeks at 40°C for subsequent microbial counts. Later, samples were randomly taken before and after 1, 3, 7, and 14 days during the 2 weeks storage period. Test samples were rehydrated to the original volume with 0.1% peptone for 10 min at room temperature, and appropriate dilutions were poured in BL agar (Difco, USA). Plates were incubated anaerobically at 37°C for 48 h. After that, the number of CFUs was counted.

**Results**

**Acid tolerance**

To assess acid tolerance of the four bifidobacterial species in each blend, the cells were challenged with various acidic conditions (pH 2.0, 3.0, 5.0, or 7.0) up to for 8 h and sampled at 0, 0.5, 1, 3, and 8 h after incubation. There was little difference in viable cell counts (VCCs) determined using agar plate between the blends of dual coated or non-coated bifidobacteria under conditions of pH 7.0 and 5.0 over the test period (Figs. 3A and 3B). Differences in acid tolerance between them began to appear when incubated for 30 min at pH 3.0 during which the VCCs of the non-coated blend dropped by 1.4 log-fold compared to the initial counts whereas the dual coated blend showed a small decrease in the VCCs, from 7.7±0.12 log-CFU/ml to 7.5±0.14 log-CFU/ml (Fig. 3C). Incubation at pH 2.0 severely affected the VCCs of both blends. For the non-coated blend, the initial viable counts dropped from 7.3±0.19 log-CFU/ml to 5.3±0.11 log-CFU/ml (2 log-fold drop) only after 30 min and to under the lower detection limit, 2.3±0.0 log-CFU/ml, after 8 h (5 log-fold drop or greater). By contrast, the dual coated blend was much less affected under the same pH condition. Its VCCs were determined to be 6.9±0.19 log-CFU/ml after 30 min incubation, showing a small decrease, and 4.9±0.06 log-CFU/ml even after 8 h incubation (Fig. 3D).

**LIVE/DEAD BacLight kit™** was used to monitor visually the fraction of live or dead bacterial cells in the test samples as live and dead cells fluoresce green and red lights, respectively, when stained with the kit. When the cells in the dual coated or non-coated blend were incubated for 3 h in pH 7.0 condition, most of the cells were live (green) and no distinct differences between the samples were observed (Figs. 4A and 4B). However, most of the non-coated bacteria incubated for 3 h in pH 2.0 condition were severely damaged (yellow and orange) or dead (red). By contrast, half of the dual coated bacteria were visually found to be live (Figs. 4C and 4D).

In order to quantify the live and dead cells in the samples, the green and red bacterial counts were analyzed by FCM (Fig. 5). The percentages of green and red bacteria for the non-coated blend at 3 h in pH 3.0 condition were 28.3% (live cell) and
Fig. 4. Fluorescent microscopic images of cells of the non-coated *Bifidobacterium* blend or dual coated *Bifidobacterium* blend stained with the Live/Dead BacLight kit after challenge with acid. The non-coated bacteria (A, C) and dual coated bacteria (B, D) were incubated at pH 7.0 (A and B, respectively) or at pH 2.0 (C and D, respectively) for 3 h. Live cells, green; dead cells, red; yellow and orange, injured.

64.3% (dead cell), respectively (Fig. 5A). Under the same condition, live and dead cells for the dual coated blend were determined to be 53.7% and 34.7% respectively (Fig. 5C). In the 3 h samples at pH 7.0, the percentages of live and dead cells in the non-coated blend were 87.1% (live cell) and 2.5% (dead cell), respectively (Fig. 5B). On the other hand, the respective percentages for the dual coated blend were 89.0% for live cells and 0% for dead cells (Fig. 5D). Therefore the result further supports that the dual coated bacteria are more resistant to acid than the non-coated bacteria.

**Bile tolerance**

Bile tolerance of the bacteria in the non-coated or dual coated blend was investigated by incubating them in BL broth (pH 4.0) containing oxgall at the final concentration of 0, 0.1, 0.3, or 1.0% (w/v). The pH of BL broth was adjusted to 4.0 because such acidic condition is assumed to be closer to the human proximal intestinal condition (Succi et al., 2005). At 0.1% oxgall, bacterial cells of both blends proliferated over time, and no large differences were observed in the VCCs between the blends (Fig. 6). However, the growth of the non-coated bacteria began to be inhibited at 0.3% oxgall, and the VCCs dropped by 1.7 log fold after 24 h incubation while the growth of the dual coated bacteria was barely affected (Fig. 6C). Distinct differences in the VCCs were seen when they were incubated at 1.0% oxgall. Under the condition, the viability of the non-coated bacteria dropped sharply from 7.6±0.10 log-CFU/ml to 2.3±0.0 log-CFU/ml after 24 h incubation whereas the dual coated bacteria were less affected and their VCCs after 24 h were much higher than those of non-coated bacteria (5.0±0.28 log-CFU/ml vs 2.3±0.0 log-CFU/ml) (Fig. 6D).

Bacterial cells in the non-coated or dual coated blend incubated at 1.0% oxgall for 3 h were subjected to staining with the BacLight kit and examined under the fluorescent microscope.

Fig. 5. Flow cytometric analysis of bifidobacterial cells incubated in acidic conditions. All bacteria were previously stained with SYTO Green I and propidium iodide. Gates indicate the position and concentration of intact cells on the plots. The strains of non-coated *Bifidobacterium* blend incubated at pH 3.0 (A) or at pH 7.0 (B) for 3 h. The dual coated *Bifidobacterium* blend incubated at pH 3.0 (C) or at pH 7.0 (D) for 3 h. Q1 and Q2, dead cell; Q3, injured cell or un-staining cell; Q4, live cell. The counts of live cells (E) and dead cells (F) for the dual coated blend were compared with non-coated blend.
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Fig. 6. Time course analysis of viable cell counts of the non-coated *Bifidobacterium* blend and dual coated *Bifidobacterium* blend incubated at different concentrations of oxgall (0, 0.1, 0.3, and 1%). Viable cell counts were determined by collecting cells at the time points, which were then plated on BL agar plates and incubated at 37°C anaerobically.

The result was that a great number of the non-coated bacteria were found to be dead (red in color) from the fluorescent observation while half of the dual coated bacteria were found to be live (green) (Fig. 7).

When the stained bacteria were further analyzed by FCM, the percentages of live and dead cells of the non-coated *Bifidobacterium* blend incubated at pH 4.0 and 0% oxgall for 3 h were 71.5% (live cell) and 20.3% (dead cell), respectively (Fig. 8A). Under the same condition, live and dead cells of the dual coated blend were 78.2% and 11.3% respectively (Fig. 8C). In the 3 h samples at 1.0% oxgall, the percentages of live and dead cells of the non-coated blend were 26.2% and 66%, respectively (Fig. 8B). By contrast, the respective percentages of the dual coated bacteria were 67.5% for live cells and 21.0% for dead cells (Fig. 8D).

**Heat stability**

To measure the heat resistance, non-coated or dual coated bacteria were kept at 40°C for 14 days. Figure 9 shows the viability of non-coated or dual coated bacteria at the end of storage at 40°C for 14 days. The initial counts of non-coated...
and dual coated bacteria ranged from 10.57±0.08 log-CFU/ml and 10.13±0.05 log-CFU/ml and there was little difference in viable cell counts between them (Fig. 9). However, non-coated bacteria showed a tendency to decrease highly throughout the storage period. After 1 day, non-coated blend counts decreased from 10.57±0.08 log-CFU/ml to 9.38±0.2 log-CFU/ml and kept decreasing at the end of storage period (Fig. 9). The viable counts of non-coated blend declined by about 44% after 2 weeks compared to the initial viable count. By contrast, dual coated blend showed a small decrease. Its VCCs were determined to be 10.10±0.02 log-CFU/ml after 1 day and 9.31±0.11 log-CFU/ml even after 2 weeks (Fig. 9). These results suggested that dual coated bacteria are more resistant to heat than non-coated bacteria.

**Discussion**

The viability of probiotics in functional foods is the most important requirement, because it has a direct bearing on effects of functional foods. For this reason, many researchers studied methods for protection of live cells and various technologies of encapsulation have been developed. The demands of successful encapsulation are protection against adverse environmental conditions such as low pH, biliary salts, and proteases during passage through the GI tract and efficient release of the probiotics bacteria. The encapsulation technologies depend on the capsule material, particle size, and bacterial strain (Burgain et al., 2011). It has been reported that various probiotics encapsulation technologies improve the viability of bacteria during exposure to simulated gastro-intestinal conditions such as alginate-coated gelatin microspheres (Annan et al., 2008), alginate–human-like collagen (Su et al., 2011) and in alginate gel microbeads (Sohail et al., 2011). Alginate is a representative encapsulating material and extensively used, but they are sensitive to the acidic environment and very difficult to scale up (Mortazavian et al., 2008). Many researchers have attempted to remedy disadvantages in various materials and ways. Among the probiotic encapsulation technologies, the spray coating technology is easy to scale up and is adapted to give multilayer coatings (Burgain et al., 2011).

We have developed the dual coating technology which is so-called fourth generation coating technology. The dual coating system is based on a pH-dependant release mechanism which protects the cells against acidic environments in the stomach and releases the bacteria from coating in the neutral pH environment of the intestines. The dual coated blend consisting of *B. bifidum* BF3 (KCTC 12199BP), *B. infantis* BT1 (KCTC 12859BP), *B. longum* BG7 (KCTC 12200BP), and *B. rhamnosus* BR3 (KCTC 12201BP) was found to be highly resistant to acid or bile salt compared to the non-coated counterpart in this study. These results indicate that the dual coating technology can improve the survival of probiotic bacteria during their transit through the GI tract after consumption. In the case of probiotic encapsulation, the objective is not only to improve the survival of probiotic bacteria through the GI tract, but also to protect the cell against adverse environment. It is estimated that daily consumption of 10^7 CFU/ml of live probiotic cells are needed to confer health benefits to the consumer (Ouwehand and Salminen, 1998; Shah, 2000). However, probiotic is affected by storage environments such as temperature, and humidity, and it might contribute to reductions in viable cell counts during storage period. Weinbreck et al. (2010) reported that viable counts of unencapsulation bacteria declined dramatically during 2 weeks when stored at 37°C, whereas encapsulation bacteria were much less affected under the same condition. Likewise, the role of the encapsulation also is important at the end of storage period. The dual coating technology improved the viability of *Bifidobacterium* blend during 2 weeks storage period at 40°C compared to the non-coated counterpart in this study. These findings demonstrate the dual coating technology can improve stability and viability of probiotic bacteria more effectively from harsh environments.
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