Encapsulation of *Bifidobacterium pseudocatenulatum* G7 in gastroprotective microgels: Improvement of the bacterial viability under simulated gastrointestinal conditions

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**Abstract**

The oral delivery of probiotics to the colon is often challenging because probiotics tend to lose viability when exposed to the harsh conditions in the upper gastrointestinal tract, such as the highly acidic gastric fluids. Properly designed encapsulation technologies can be used to protect probiotics during their transit through the human gut. In this study, an anaerobic probiotic (*Bifidobacterium pseudocatenulatum* G7 or BPG7) was encapsulated within alginate microgels that also contained antacid agents to control their internal pH within the stomach. Probiotic-loaded microgels were exposed to a simulated gastrointestinal tract (GIT) model to establish the impact of gastric and small intestinal conditions on their physicochemical properties and cell viability. In the absence of antacids, no live probiotic cells were detected in the microgels after exposure to gastrointestinal conditions. Conversely, in the presence of antacids, there was only a 1.5 log CFU decrease in cell viability after incubation in simulated gastric fluids for 2 h. Subsequently, after the antacid microgels were incubated in simulated intestinal fluids, considerable amount of viable probiotic cells were still detected when CaCO3 was used as an antacid but not when Mg(OH)2 was used. Overall, these results indicated that alginate microgels containing CaCO3 as an antacid were efficient at protecting the probiotic during passage through the upper GIT. This novel encapsulation technology utilizing all food grade materials may be useful for the oral delivery of probiotics to the gut in the form of functional foods or supplements.

**1. Introduction**

The human gastrointestinal tract (GIT) typically contains hundreds to thousands of different bacterial species, forming a complicated microbial ecosystem (Turnbaugh et al., 2007). The genus *Bifidobacterium* is one of the most common bacteria in the feces (3.2%) and cecum (5.2%) of humans (Marteau et al., 2001; Matsuki, Watanabe, Fujimoto, Kado, Takada, Matsumoto, et al., 2004; Matsuki, Watanabe, Fujimoto, Takada, & Tanaka, 2004). *Bifidobacterium* species are regarded as probiotics due to their potential health benefits and have been generally recognized as safe (GRAS) by the US government (Picard et al., 2005). There are more than 50 species belonging to the genus *Bifidobacterium*, with *Bifidobacterium pseudocatenulatum* (*B. pseudocatenulatum*) being one of the most dominant species. As a probiotic, *B. pseudocatenulatum* has been investigated for its effect in the treatment and prevention of a broad spectrum of human and animal gastrointestinal disorders. It has been reported to attenuate abnormal vascular function in obese mice (Mauricio, Serna, Fernandez-Murga, Porter, Aldasoro, Valles, et al., 2017), to partially restore neuroendocrine function in obese mice (Agusti et al., 2018), and to reduce inflammation in mice with cirrhosis (Moratalla, Gomez-Hurtado, Moya-Perez, Zapater, Peiro, Gonzalez-Navajas et al., 2016). However, probiotics have to reach their site of action at a sufficiently high level before they can exhibit their potential health benefits. Unfortunately, environmental challenges encountered in the human GIT, such as gastric acids, digestive enzymes, and bile salts, have limited the application of probiotics in vivo (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012).

Encapsulation of probiotics in colloidal delivery systems can
improve the shelf life and viability of probiotics during storage and in the GIT (Gonzalez-Ferrero, Irache, & Gonzalez-Navarro, 2018; Yao et al., 2017; Yeung, Ucok, Tiani, McClements, & Sela, 2016). Encapsulation of Lactobacillus casei 01 in alginate-starch beads improved cell viability during freeze-drying and storage (Chan, Wong, Lee, Lee, Ti, Zhang, et al., 2011). Encapsulation of B. longum in alginate microgels coated with chitosan improved their survival during passage through a simulated GIT (Yeung et al., 2016). However, there are only a few studies showing that colloidal delivery systems can successfully preserve the viability of probiotics after 2 h exposure to gastric conditions (Lee & Heo, 2000; Yeung et al., 2016). The main reason for the poor gastric stability of probiotics encapsulated in microgels is that hydrogen ions (H+) can diffuse through the hydrogel network easily and inactivate the encapsulated bacteria. Previously, we prepared alginate microgels loaded with an antacid agent, Mg(OH)2, that could neutralize hydrogen ions when they entered the microgels and thereby maintain a neutral internal pH even when they were incubated with the acidic gastric fluids (Zhang, Zhang, Sun, Park, & McClements, 2017b). These antacid microgels may therefore be particularly useful for increasing the cell viability of probiotics for oral delivery.

In this study, we encapsulated a strain of example probiotic bacteria, B. pseudocatenulatum G7 (BPG7) isolated from healthy human feces in alginate microgels loaded with two different kinds of antacids, either Mg(OH)2 or CaCO3. Both Mg(OH)2 and CaCO3 are widely used food-grade antacids, which are insoluble at neutral and basic pH but dissolve at acidic pH thereby releasing hydrogen ions (OH−). We hypothesized that these antacid microgels would maintain an internal neutral pH under acidic gastric conditions so as to enhance the viability of the probiotic in the gastrointestinal tract. However, the two antacids have different physicochemical characteristics, such as pKa values, solubilities, and ionic constituents, which we hypothesized would impact their ability to protect the probiotics. The results obtained from the current study should, therefore, provide valuable information for optimizing the performance of probiotic-loaded delivery systems for applications in functional foods and beverages.

2. Material and methods

2.1. Bacteria propagation and general growth conditions

The probiotic (BPG7) was stored at −80°C in deMan, Rogosa, Sharpe (MRS) broth (Difco Laboratories, Sparks, MD) containing 50% glycerol (Sigma, St Louis, MO, USA). After growing on an MRS agar plate at 37°C for 48 h, a single colony was selected and propagated in fresh 40 mL MRS for 24 h, anaerobically. Anaerobic conditions were maintained using anaerobic chamber with an airlock (82% N2, 10% CO2 and 7% H2; Whitley A35 Anaerobic Workstation, Microbiology International, Frederick, MD).

2.2. Probiotic microencapsulation

Cells were obtained by centrifugation (4000 g × 10 min, 4°C) and then washed twice with sterile phosphate water. The washed cells were re-suspended in 20.0 mL sterile deionized water. BPG7-loaded microgels were prepared by mixing the bacteria with 2% sodium alginate solution in the absence of presence of an antacid agent (either Mg(OH)2 or CaCO3) (1:1, v/v) (Zhang, Zhang, Zou, & McClements, 2016). As shown in Fig. 1, the polymeric matrix was agitated to uniformly distribute the bacterial cells throughout the mixture. Then, the mixture was subject to microgel preparation with an automated encapsulation device (Büchi B-390 Encapsulator, Flawil, Switzerland). The microgels were fabricated based on a method described previously (Zhang, Zhang, Sun, Park, & McClements, 2017a), with some slight modifications. An injection nozzle with a diameter of 200 μm was used and standard operating conditions were utilized: vibration frequency = 800 Hz, electrode potential = 800 V, and driving pressure = 500 mbar. The microgels were collected in 60 mL of 10% calcium chloride solution (Sigma, St Louis, MO, USA). The microgels were then vacuum-filtered and washed with sterile deionized water.

2.3. Characterization of encapsulated calcium alginate microgels

2.3.1. Surface potential

The surface potential (ζ-potential) of the microgels was determined using an electrophoretic light scattering device (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK). For each sample, filtered microgels (~0.1 g) were re-suspended in corresponding pH-adjusted solutions before measurements to limit multiple scattering effects. Specifically, phosphate buffer (5 mM, pH 7.0) was used for the dilution of initial and small intestine samples, while acidic distilled water (pH 2.5) was used for stomach samples.

2.3.2. Particle size

The particle size distribution of samples collected at different stages was detected using a static light scattering instrument ( Mastersizer S, Malvern Instruments, Worcestershire, UK). Again, initial and small intestine samples were diluted in phosphate buffer (5 mM, pH 7.0) while stomach samples were diluted in acidic distilled water (pH 2.5) to avoid multiple scattering effects. The particle sizes are reported as the surface-weighted mean diameter ($d_{43}$).

2.3.3. Cold-stage scanning electronic microscopy (cryo-SEM)

Microgels were fixed by immersion in 2.5% (v/v) glutaraldehyde in 0.5 M sodium cacodylate-hydrochloric acid buffer (pH 7.2) for 1 h at room temperature. The fixed samples were then washed three times in deionized water. The alginate microgels were then placed on an aluminum SEM stub and a second stub was placed on top to create a cap on top of the alginate microgels. This sandwich was then plunged frozen in liquid nitrogen. Once frozen, the aluminum stubs were quickly pulled apart, which fractured the microgels. The two aluminum stubs were then placed in a vacuum lyophilizer and completely dried overnight. The next morning the fractured and dried alginate microgels were mounted to carbon tape on a SEM stub and sputter coated with 6 nm of Au/Pd (80/20). The mounted and coated specimens were examined and digitally imaged at various magnifications using a scanning electron microscope (FEI Quanta 200 MKII FESEM, FEI Company, Hillsboro, Oregon, USA).

2.4. Cell viability tests

2.4.1. Confocal scanning laser microscopy (CLSM)

Cell viability and distribution were characterized using confocal scanning laser microscopy (CLSM). The probiotic was labelled with fluorescent molecular probes using a LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher Scientific, MA, USA). Dye SYTO 9 is a green-fluorescent nucleic acid stain for both live and dead cells, whereas propidium iodide (PI) is a red-fluorescent nucleic acid stain that only interacts with cells that have damaged membranes, leading to a reduction in the SYTO 9 fluorescence signal. Therefore, live cells fluoresce green, while dead cells fluoresce red. Equal volumes of the SYTO 9 and PI dyes were mixed together. Then, the dye mixture (3 μL) was added into 1 mL microgel solution and incubated for 15 min in dark at room temperature prior to visualization with CLSM (Keen, Slater, & Routh, 2012). Images were obtained with a 20-objective lens (Nikon D-Eclipse CI 80i, Nikon, Melville, NY, USA). For the SYTO 9 dye, an excitation wavelength of 488 nm was used and an emission wavelength of 590 nm/50 nm. For the PI dye, an excitation wavelength of 488 nm was used and an emission wavelength of 650 nm/LP. The confocal fluorescent images were analyzed using image analysis software (NIS-Elements, Nikon, Melville, NY, USA).
2.4.2. Enumeration of viable cells

To determine the viable cell counts of encapsulated bacteria, alginate microgels (0.10 g) were homogenized with 9.9 mL of 10% sodium citrate dihydrate solution (pH 8.2; Fisher, Fairlawn, NJ, USA) for 10 min at room temperature to dissociate them. After serial dilutions of the dissociated microgels ($10^{-2}$–$10^{-5}$), the number of surviving cells was counted by plate culture on MRS agar in duplicate and anaerobically incubated at 37 °C for 48 h. All the samples were counted initially, after the simulated stomach phase, and after the simulated stomach and small intestinal phase.

2.5. Simulated gastrointestinal fate of microgels

Samples with the same concentrations of free or encapsulated BPG7 cells and the same total volumes were prepared by diluting the original samples with buffer solution (5 mM PBS, pH 7). The samples were then passed through an in vitro GIT model that simulated the stomach and small intestine phases. Specifically, simulated gastric fluids (SGF) was prepared by adding sodium chloride (2 g) and 6 M hydrochloric acid (7 mL) into 1-L of distilled water and then filter sterilized. The simulated intestinal fluids (SIF) was prepared by dissolving calcium chloride (0.25 M) and sodium chloride (3.75 M) in phosphate buffer (5 mM PBS, pH 7). A bile salts solution was prepared by dissolving 1.5 g porcine bile extract in 28 mL phosphate buffer (5 mM PBS, pH 7), and 3.5 mL of the bile salts solution was added to each intestinal phase. All the simulated stock solutions were subjected to autoclaving before the experiments.

Free and encapsulated BPG7 cells were sequentially added to SGF (45 mL, pH adjusted to 2.5) for 2 h, and then incubated with SIF (45 mL, pH adjusted to 7.0) for 2 h. All the incubation experiments were carried out in an anaerobic chamber set at 37 °C. Dilutions ($10^{-0}$–$10^{-5}$) of the released cells were plated on MRS agar for samples collected initially, after 2 h exposure to a simulated gastric phase, or after 4 h exposure to simulated gastric and then intestinal phases. Then MRS plates were incubated anaerobically for 48 h at 37 °C for viable cell counting.

2.6. Statistical analysis

Experiments on the physicochemical characteristics of the microgels were repeated in duplicate. Experiments on viable cells counts were repeated at least six times independently. Data were expressed as mean ± standard deviation (SD). Difference among samples were evaluated with one-way ANOVA and p < 0.05 was considered significant.

3. Results and discussion

3.1. Particle size analysis

Alginate is an anionic polysaccharide that has been widely used for the microencapsulation of probiotics because of its ability to form microgels using a simple injection process. Two different antacid agents, either Mg(OH)2 or CaCO3, were co-encapsulated with the probiotics inside the alginate microgels to enhance their viability during gastrointestinal transit (Fig. 1). A series of controls was also studied: alginate microgels containing no antacids or probiotics; Mg(OH)2-loaded microgels with no probiotics; and, CaCO3-loaded microgels with no probiotics. Changes in the particle size of the microgels were then determined as they passed through various stages of the simulated GIT. Incubation under oral conditions was not simulated in the current study because the short duration and neutral pH conditions in the human mouth would not have been expected to impact cell viability.

The general shape of the particle size distribution of all the microgels remained fairly similar throughout the entire GIT (Fig. 2), suggesting that they retained their overall integrity when exposed to upper gastrointestinal conditions. There was a slight decrease in the mean particle diameter ($d_{43}$) for all the samples after exposure to the simulated stomach phase (Table 1), which has been previously attributed to a reduction in the electrostatic repulsion between the anionic alginate chains when the carboxyl groups become partially protonated under acidic conditions. Interestingly, the CaCO3-loaded microgels were initially larger (665 μm) than the Mg(OH)2-loaded ones (540 μm). This may have been because some of the solid CaCO3 particles partially dissolved and released a few calcium ions (Ca$^{2+}$) that cross-linked the alginate and increased the solution viscosity (Smidsrød & Skja, 1990).
3.2. Surface potential analysis

The electrical characteristics of the microgels were also determined during the simulated GIT process. All the microgels initially had a strong negative charge of around $-25 \text{ mV}$ (Fig. 3), which can be attributed to the presence of anionic carboxylic acid groups ($\text{COO}^-$) from the alginate molecules being present at the hydrogel surfaces. An appreciable reduction in the magnitude of the $\zeta$-potential occurred for all the microgels after exposure to the simulated stomach phase. This effect is due to the fact that the simulated gastric fluids have a low pH and high ionic strength, which reduced the ionization of the anionic charged groups and caused electrostatic screening effects. This result is consistent with the observed shrinkage of the microgels under simulated stomach conditions, as mentioned in the previous section. After exposure to the small intestine phase, all the microgels again had relatively high negative charges. This was due to the deprotonation of the carboxyl groups on the alginate molecules under simulated intestine conditions (pH 7), as well as the presence of anionic species such as bile salts. Overall, all the antacid-loaded microgels had fairly similar charge characteristics throughout the simulated GIT, indicating that encapsulation of the antacids did not change their surface potentials.

3.3. Cryo-SEM for hydrogel beads with presence or absence of antacid agent

Cryo-SEM was used to probe the internal structure of fractured alginate microgels in the absence or presence of antacid agents (Fig. 4). Cryo-SEM allows detailed observation of microgels in the frozen hydrated state. Compared with conventional SEM, potential artifacts like shrinkage produced by dehydration and fixation are avoided using cryo-SEM (Allan-Wojtas, Hansen, & Paulson, 2008). To study their inner structure, alginate microgels were cracked by cryo-fracturing and then the cracked surfaces were visualized by SEM. The probiotics were clearly observed at the surfaces of the antacid-free microgels (Fig. 4a). Both probiotics and irregular shaped particles (presumably antacid crystals) were observed in the probiotic-loaded microgels containing Mg(OH)$_2$, which were indicative of the presence of antacid crystals (Fig. 4b). Probiotics were visible in clusters at the surfaces of the probiotic-loaded microgels containing CaCO$_3$, which appeared to have a...
highly variegated surface morphology (Fig. 4c).

In the absence of probiotics, the surfaces of the antacid-free microgels appeared smoother than in the two antacid-loaded microgels (Fig. 4d-f). The irregular surfaces of the antacid-loaded microgels was probably because of the presence of antacid crystals embedded inside them (Xie et al., 2010). The SEM images suggested that the size of the Mg(OH)₂ crystals were considerably smaller than those of the CaCO₃ crystals. In addition, the structure of the hydrogel network in the microgels appeared to be different when they contained different antacids. This effect might have been caused by the presence of different ions (magnesium versus calcium) that altered the cross-linking between the alginate chains in the hydrogel network (Sultana et al., 2000).

3.4. Viability of encapsulated BPG7 under simulated digestion

Previous studies have shown that probiotics tend to be inactivated during their passage through the upper GIT and therefore do not reach the colon intact (Nazzaro, Orlando, Fratianni, & Coppola, 2012). Therefore, we examined the impact of microgel encapsulation on cell viability when exposed to simulated GIT conditions. BPG7 cells encapsulated in alginate microgels in the absence or presence of antacids (Mg(OH)₂ or CaCO₃) were subjected to simulated stomach or stomach/small intestine conditions (Li, Hu, Du, Xiao, & McClements, 2011). The level of viable cells that survived was measured before and after exposure to the gastric fluids (Fig. 5). A live/dead fluorescent stain method was used to visualize probiotic viability, where viable bacteria with intact cell membranes fluoresce green and dead bacteria with damaged cell membranes fluoresce red. The confocal microscopy images showed that all the free bacteria lost their viability after 2 h incubation in the gastric fluids (Fig. 5). Similarly, none of the cells inside the antacid-free alginate microgels survived after exposure to the simulated stomach phase, indicating that hydrogen ions (H⁺) from the gastric fluids rapidly diffused into the microgels and deactivated the probiotic bacteria (Figs. 5 and 6). Conversely, many of the cells remained viable in the microgels containing Mg(OH)₂ or CaCO₃ after exposure to the stomach phase (Fig. 5). After 2 h gastric digestion, there was a 1.5 log₁₀ CFU reduction in the number of viable bacteria in the microgels containing Mg(OH)₂ and a 1.0 log₁₀ CFU reduction for the microgels containing CaCO₃ (Fig. 6). This result highlighted the ability of the antacids to protect the probiotics against acidic gastric conditions. Our previous study showed that the pH inside Mg(OH)₂-loaded alginate microgels remained close to neutral when they were incubated in acidic gastric fluids for 2 h (Zhang et al., 2017b). This suggests that the level of insoluble Mg(OH)₂ inside the microgels was sufficient to neutralize the gastric fluids throughout the entire incubation time. This phenomenon was probably because the Mg (OH)₂ particles only dissolved slowly in the gastric fluids (Zhang et al., 2017b). The microgels containing CaCO₃ gave even better gastric protection than the ones containing Mg(OH)₂ (Fig. 6). After exposure to the intestinal phase, no viable cells were detected for the microgels containing Mg(OH)₂, which corresponded to a greater than 5.43 log₁₀ CFU loss in cell viability. On the other hand, the microgels containing CaCO₃ only suffered a 2.5 log₁₀ CFU reduction. These results suggest that CaCO₃ also had a better protective effect on cell viability than Mg (OH)₂ in the small intestinal phase.

The origin of the different protective effects of Mg(OH)₂ and CaCO₃ is still unclear. There are several possible reasons that might contribute for this observation. Firstly, the slowly released calcium ions might interact with bile salts or intestinal enzymes and thereby reduce cell injury. The bile salts in the small intestine are known to have a strong antimicrobial activity due to their ability to disrupt the structure of the cell membrane and trigger DNA damage (Ruiz, Margolles, & Sanchez, 2013). Secondly, the cryo-SEM images suggested that CaCO₃ crystals had a larger particle size than Mg(OH)₂ crystals, which may have reduced their dissolution rate or sterically hindered the ability of hydrogen ions to diffuse through the hydrogels (McClements, 2015). Third, the released calcium ions may have cross-linked the alginate molecules in the hydrogel thereby reducing the pore size and inhibiting diffusion. Fourth, the size of the microgels containing CaCO₃ was larger than the ones containing Mg(OH)₂, which would have also slowed
down diffusion processes (Chen et al., 2018). Clearly, further work is required to work out the detailed physicochemical mechanisms underlying the difference between the two types of antacids.

It should be noted that gastrointestinal systems of animals and humans is much more complicated that the simple in vitro model used in this study. In particular, we did not use digestive enzymes in our simulated GIT, which may have impact probiotic viability. Moreover, we did not study the release of the probiotic cells in the colon, which would be important for their ability to colonize the human gut. For this reason, it would be useful to investigate the performance of the antacid microgels using animals models in future studies. It would also be informative to identify the relative importance of the different protective effects postulated earlier, as this knowledge may inform the design of more effective delivery systems.

4. Conclusion

In order to colonize the colon, probiotics must survive the harsh conditions they experience during passage through the upper gastrointestinal tract. In particular, they must resist deactivation by the deleterious actions of acids and bile salts within the gut. In this study, we showed that antacid-loaded microgels could improve probiotic survival under both stomach and small intestine conditions. It was postulated that the antacids helped maintain neutral pH conditions within the interior of the microgels as they passed through the highly acidic gastric fluids, thereby protecting the probiotics trapped inside them. CaCO₃ proved to be a more effective antacid than Mg(OH)₂ at protecting the probiotics for reasons that are currently unknown. Overall, the microgels developed in this work may be useful for encapsulating, protecting, and delivering sensitive probiotics in functional foods, supplements, and pharmaceuticals.

It should be noted that we used a relatively simple GIT model in this study that did not include oral, gastric or pancreatic enzymes, which may also have deactivated the probiotics. In future studies, it would therefore be useful to focus on the impact of digestive enzymes on the viability of encapsulated and non-encapsulated probiotics. Moreover, future work should focus on determining the mucoadhesion properties of the encapsulated probiotics within the intestinal mucosa, which will require animal or human studies. This research is important because probiotics have to colonize the colon if they are going to be effective.

Fig. 5. Fluorescence confocal microscopy images of viable bacteria in different systems after exposure to successive gastrointestinal track stage (stained with LIVE/DEAD BacLight Bacterial Viability Kit). Red indicates live cells, while green indicates cell with damaged membrane. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
and there are distinct differences between the abilities of different probiotics to achieve this. Our research is currently investigating a number of these issues.

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