Survival of probiotics in pea protein-alginate microcapsules with or without chitosan coating during storage and in a simulated gastrointestinal environment

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Abstract Pea protein-alginate microcapsules with or without a chitosan coating and containing *Lactobacillus rhamnosus* R0011 and *L. helveticus* R0052 were produced by extrusion and tested for survivability during storage and in an *in vitro* gastrointestinal environment. Both microcapsule formulations provided significant protection for cells incubated in synthetic stomach juice at 37°C for 2 h, followed by 3 h in simulated intestinal fluid, relative to non-encapsulated bacteria. However, evaluation of cell viability during 9 weeks of storage at room temperature revealed that chitosan coating significantly improved microcapsule performance compared to non-coated microcapsules. Refrigerated storage had no negative impact on the microcapsule protection ability of both types of microcapsules. Notably, chitosan-containing microcapsules showed much higher bacterial survival counts during challenge tests even after storage. Moreover, the addition of chitosan to the microcapsule formulation did not increase the microcapsule size.

Keywords: encapsulation, pea protein, alginate, probiotic, chitosan coating

Introduction

In 2001, FAO/WHO experts defined probiotics as living microorganisms that confer a health benefit when administered in adequate amounts (1). Although numerous studies describing the potential of probiotics in the prevention and treatment of multiple gastrointestinal (GIT) disorders have been conducted, the efficacy of these microbial strains is still arguable. Probiotic-based strategies for therapeutic and prophylactic use against GIT diseases have been recently reviewed by our group (2). Apart from the challenge of finding the right species that would “confer a health benefit” to its host, there are technical issues such as delivery of the probiotic to a certain part of the GIT where it could perform its function. One way to circumvent this issue is through the immobilization of bacteria in a protective polymer matrix. Microencapsulation of probiotic bacteria has been widely and successfully used in the protection of acid-sensitive bacteria during storage and/or passage through a simulated GIT environment (3–5). With respect to materials used for encapsulation, it is preferable to choose plant proteins, such as whey and pea, and natural biopolymers, such as alginate or carrageenan, that are generally recognized as safe (GRAS) and are able to provide good protection to bacterial cells (6–8). However, since all such materials do not provide sufficient protection by themselves, there has been a growing

interest in using various mixtures, coatings, and layers of different biopolymers to improve microcapsule performance.

Sodium alginate and pea protein isolate, used for micro-encapsulation in this study, have GRAS status. Sodium alginate is a polymer of algal origin, consisting of (1→4)-linked β-D-mannuronic (M) and α-L-guluronic acid (G) residues, and undergoes gelation in the presence of divalent calcium ions, making it ideal for an extrusion-based encapsulation process (9). The use of pea protein isolate (PPI) in the food industry is gaining popularity as an alternative to animal-derived proteins and soy, which is a known allergen. Pea proteins are mainly comprised of globulin-type proteins with molar masses of 320–400 kDa (legumin) and 150 kDa (vicilin) and albumin proteins (10). To the best of our knowledge, chitosan has yet to receive GRAS status but has already been extensively tested for safety and toxicity on different species in order to support its use in food. Moreover, it has an oral LD₅₀ of over 16 g/kg in mice (11,12). Chitosan (β-(1→4)-poly-D-glucosamine) is a biodegradable, biocompatible, nontoxic, and non-allergenic linear cationic polysaccharide derived from chitin via deacetylation (13). It is widely used in medicine, agriculture, functional foods, biotechnology, and cosmetics (14). For encapsulation, typically the gel-like capsule matrix is initially formed, followed by the addition of the coating material. The binding of the coating material in the proposed capsule

formulations is attributed to polyelectrolyte complexation involving the reaction of positively charged chitosan with negatively charged alginate (15).

Our previous research shows that PPI-alginate microcapsules have the potential to protect acid-sensitive bacteria *in vivo* (6). In an attempt to improve microcapsule formulation, we added chitosan to the mixture because this polymer improves the protective properties of microcapsules made of alginate (16). The bacteria used in the study were *L. rhamnosus* R0011 and *L. helveticus* R0052 (mixed in a ratio of 95:5; Lacidofil[®]; Lallemand Health Solutions, Montreal, QC, Canada), whose probiotic potential in suppressing *Citrobacter rodentium* infection has previously been reported (17–19). The goal of this study was to examine the ability of chitosan coating to improve the protective properties of PPI-alginate microcapsules and determine how different storage conditions affect the viability of bacteria entrapped in both types of microcapsules.

Materials and Methods

Materials PPI was donated by Nutri-Pea Ltd. (Portage La Prairie, Canada). Alginic acid sodium salt, calcium chloride dehydrate, Tween 80, and chitosan (low molecular weight) were purchased from Sigma-Aldrich (St. Louis, MI, USA). Probiotic strains *L. rhamnosus* R0011 and *L. helveticus* R0052 were received from Lallemand Health Solutions (Montreal, Canada).

Bacteria and culture conditions Probiotic strains were stored at 80°C in a 1:2 (v/v) suspension of glycerol and de Man, Rogosa, and Sharpe broth (MRS; Sigma-Aldrich). Prior to bacterial encapsulation, frozen stocks were plated on MRS medium containing 1.5% (w/v) agar and then incubated for 24 h at 37°C under anaerobic conditions (80% N₂, 10% CO₂, and 10% H₂) in an anaerobic chamber (Model 1025 (1029); Forma Scientific Inc., Marietta, GA, USA). Pure colonies of *L. rhamnosus* R0011 and *L. helveticus* R0052 were then transferred from the plates to 50 mL tubes containing 45 mL of MRS broth for incubation under the same conditions for 24 h. Next, bacterial cells were recovered by centrifugation (2,000×g for 12 min) (Sorvall SS-1; Sorvall Inc., Newtown, CT, USA), washed twice with 1.0% alkaline peptone water (APW; 10% (w/v) peptone, 10% (w/v) sodium chloride; pH 8.5), and used for the preparation of microcapsules.

Preparation of bacteria-containing microcapsules Encapsulation and extrusion techniques used in this study were based on the methodology described by Klemmer *et al.* (20), Krasaekoopt *et al.* (21), and Chávarri *et al.* (16), with modifications. The composition of the capsule material was as follows: 2.0% PPI+0.5% (w/w) sodium alginate. The concentration of PPI was adjusted to the protein content of the isolate (85%). PPI was dissolved at 85°C and pH 9.0 for 2 h under constant mechanical stirring. The pH was then adjusted to 7.0 and sodium alginate powder was added to the mixture. After

complete dissolution of the polysaccharide (1–1.5 h at 85°C), the solution was cooled to room temperature (21–23°C), corrected for evaporative water loss with the addition of sterile water, and mixed with a 2.0% (v/v) bacterial slurry preparation (*L. rhamnosus* R0011 and *L. helveticus* R0052 in a ratio of 95:5). Bacterial cells were recovered by centrifugation from 24 h bacterial broth culture (2,000×g for 12 min) (Sorvall SS-1; Sorvall Inc.). The final mixture of protein, polysaccharide, and bacteria was then extruded using a syringe and 18 G (G, gauge) needle into 200 mL of dd-H₂O containing Ca²⁺ ions (crosslinking agent) and Tween 80 (emulsifier). The concentrations of Ca²⁺ (CaCl₂) and Tween 80 were 1.0% (w/v) each (based on (6)). A portion of microcapsules was extruded into a crosslinking solution of the same composition but with the addition of chitosan (0.4% w/v). The concentration of chitosan in lyophilized capsules was calculated to be less than 50 g/kg, which proves that the capsules are non-toxic (LD₅₀ of chitosan in mice is 16 g/kg). After extrusion, the microcapsules were left in the crosslinking solution on a shaker for 15 min, removed, and air dried for another 15 min. Prepared microcapsules were immediately freeze dried (Labconco FreeZone; Labconco Corp. Kansas City, MO, USA) for 15 h in the absence of cryoprotectants and used in storage and survival studies according to the methods described below.

Viability of bacteria in capsules during storage Both types of microcapsules—with and without chitosan coating (labeled “PAC” and “PA,” respectively)—were stored for 9 weeks in bags (200 × 300 mm) of plastic barrier film (75 microns nylon/polyethylene, oxygen transmission rate: 63 cc/sq. m/24 h at 23°C) in triplicate under four different conditions: 4, 20, 4°C+vacuum (KOMET Vakuum-Verpacken; KOMET Maschinenfabrik GmbH, Plochingen, Germany), and 20°C+vacuum. Aliquots of microcapsules (0.1 g) were removed from the bags every week, homogenized (Omni-mixer; Sorvall Inc.) at 250×g for 2 min, serially diluted, plated on MRS agar in triplicate, incubated at 37°C for 48 h, and enumerated. Results were presented as log CFU/g of microcapsules.

Survival of probiotics under simulated stomach conditions For survival studies, 0.01 g aliquots of non-encapsulated bacteria were added to centrifuge tubes (2 mL) containing 0.99 mL of synthetic stomach juice (SSJ; 8.3 g/L proteose peptone, 3.5 g/L glucose, 2.05 g/L NaCl, 0.6 g/L KH₂PO₄, 0.11 g/L CaCl₂, 0.37 g/L KCl, 0.05 g/L bile salts, 0.1 g/L lysozyme, 13.3 mg/L pepsin; pH 1.8) (5,22) and incubated at 37°C. Starting at time 0, every 5 min over the first 30 min and subsequently every 30 min (2 h in total), tubes of each microcapsule type (with or without chitosan coating) were removed from the incubator, washed with 1.0% APW, homogenized (Omni-mixer; Sorvall Inc.) at 250×g for 2 min, serially diluted with 1.0% APW, and plated on MRS agar. The same method without exposure to SSJ was used to quantify the initial cell counts within the microcapsules. The plates were incubated at 37°C in an anaerobic chamber for 48 h and enumerated.

To determine the survival of encapsulated bacteria before and after the storage period (on weeks 0 and 9), 0.1 g aliquots of microcapsules were added to 0.9 mL of SSJ and incubated for 2 h at 37°C. They were then transferred to 0.9 mL of simulated intestinal fluid (SIF; 1.25% (w/v) NaHCO₃, 0.60% (w/v) bile salts, 0.09% (w/v) pancreatin; pH 6.5) and incubated for another 3 h at 37°C. By the end of the incubation period, all the microcapsules were completely dissolved in SIF and the viable cell counts were determined by serial dilutions in 1.0% APW and plating on MRS agar. All experiments were conducted in triplicate.

Morphology and size of microcapsules Lyophilized microcapsules were cut in half using a scalpel, stained, and immediately immobilized on the surface of a cover glass using molten 0.8% (w/v) agar tempered to 40–45°C. After the agar solidified, the cover glass was inverted and the agar was transferred to a glass chamber made from glass slides. The chamber was then filled with tempered liquid agar. Staining of the microcapsules was performed using SYTO 9 and propidium iodide (5 mM solutions in DMSO; Life Technologies, Carlsbad, CA, USA) according to the guidelines provided by Life Technologies™ (SYTO 9 and propidium iodide manuals, 2011). Dyes (1 µL each) were added to 2 mL water, vortexed, and directly applied to the microcapsule surface. The microcapsules were then incubated for 15 min in dark conditions. Bright-field and fluorescent images of stained microcapsules were obtained using a Nikon C2 confocal laser scanning microscope (CLSM; Nikon, Tokyo, Japan) using 488 and 543 nm wavelength excitation in conjunction with an oil immersion, 60X Plan Apo VC (1.4 N.A., Nikon) objective lens. Three microcapsule locations were randomly selected for obtaining optical thin sections representative of the microcapsule surface and stained bacteria. Each image was taken with a xy-resolution of 512 by 512 pixels and a pinhole radius of 20 µM.

Microcapsule size was measured using an electronic digital caliper (Model 62379-531; Control Company, Friendswood, TX, USA). The sizes of 15 microcapsules of each capsule type were measured and averaged.

Statistics Mean values, standard deviation, *t*-values, and *p*-values within each group, as well as between groups, were determined using Welch *t*-test and Two-Way ANOVA (One-Way ANOVA in case of microcapsule size analysis). The level of statistical significance was set at *p*<0.05. All statistical analyses were performed using the SPSS software (Ver. 17.0, SPSS Inc., Chicago, IL, USA).

Results and Discussion

Protective properties of microcapsules during storage Viability of probiotic bacteria within the microcapsules was monitored over a period of 9 weeks to determine the effect of temperature and vacuum packaging on their survival for both PA and PAC

Fig. 1. Viability of probiotic bacteria in pea protein alginate (A) and pea protein-alginate chitosan (B) microcapsules during storage at 4 and 22°C without and under vacuum over a 9-week duration. Data represent the mean±standard deviation (*n*=3).

microcapsules (Fig. 1). Results showed that cell viability in both PA and PAC microcapsules was significantly (*p*=0.001) improved when they were stored at 4°C compared to when they were stored at 22°C. When stored at room temperature, PAC microcapsules provided better protection for probiotic cells than PA microcapsules. There was a decrease in the bacterial viability of 0.7 log CFU/g (from initial 8.10 log CFU/g) in PAC microcapsules stored at 22°C by week 9, whereas the viable cell counts in PA microcapsules stored under the same conditions reached 6.68 log CFU/g by week 5 (Fig. 1). By week five, the viable cell counts continued to decrease but started to fluctuate considerably. These are not shown on the graph (Fig. 1A). Vacuum sealing did not have any major effect on cell survival in either PA or PAC microcapsules stored at room temperature (*p*>0.05; Fig. 1A and 1B). However, storage of PAC microcapsules under vacuum at 4°C led to no reduction in the viable cell counts (Fig. 1B).

These findings are in accordance with other studies, where it was reported that the stability of dried cultures decreased significantly when stored at room temperature (23,24). Obradović and Krnić (25) also observed greater survival of bacteria in chitosan-coated alginate beads, compared to non-coated ones, during 21 days of refrigerated storage (4°C). Chávarri *et al.* (16) reported that the viable bacterial counts in PAC microcapsules decreased by 2.0 log CFU/g over 28 days of storage at 4°C. However, our results showed considerably less decrease in the viable cell counts in PAC pea protein-alginate microcapsules, which was only 0.3 log CFU/g during 9 weeks of refrigerated storage (Fig. 1B). Improved protective properties might be caused by the addition of PPI to the formulation. According to the results, the best way to preserve viable probiotic cells of *L. rhamnosus* R0011 and *L. helveticus* R0052 for 9 weeks was encapsulating them in PPI-alginate with chitosan coating and storing at 4°C under vacuum.

Survival of probiotic bacteria in microcapsules during simulated GIT conditions Free non-encapsulated bacterial cells were incubated in SSJ for 2 h as a control, where viable cells became undetectable by

Fig. 2. Decrease in the viable cell counts of non-encapsulated probiotic bacteria (*L. rhamnosus* R0011 and *L. helveticus* R0052) during incubation for 2 h in synthetic stomach juice (SSJ). Data represent the mean±standard deviation ($n=3$).

Fig. 3. Decrease in the viable cell counts within freshly prepared pea protein-alginate (PA) or pea protein-alginate-chitosan (PAC) microcapsules after incubation for 2 h in SSJ followed by 3 h in simulated intestinal fluid (SIF). Data represent the mean±standard deviation ($n=3$). Different letters next to error bars represent significant differences between treatments.

plating after 60 min (*L. rhamnosus* R0011) and 90 min (*L. helveticus* R0052) of incubation (Fig. 2). To assess the survival of immobilized bacteria under simulated GIT conditions after 9 weeks of storage, PA and PAC microcapsules were incubated for 2 h in SSJ and then for 3 h in SIF. Cell viability within freshly prepared PA and PAC microcapsules (control) and after incubation for 2 h in SSJ and 3 h in SIF revealed that PAC microcapsules provided significantly better protection, with a decrease in viable counts of only 0.7 log CFU/g as compared to a 1.5 log CFU/g decrease in PA microcapsules after the 5 h test ($p<0.05$) (Fig. 3).

Survivability tests were repeated after 9 weeks of storage under different temperatures (4 or 22°C) and atmospheric (with and without vacuum) conditions (Fig. 4). Microcapsules were tested for cell viability before (control) and after incubation within SSJ (2 h) followed by SIF (3 h). PA microcapsules stored at room temperature provided the least protection for immobilized probiotics, with a 4.1 log CFU/g decrease in viable counts (Fig. 4). Storage at 22°C under

Fig. 4. Decrease in the viable cell counts in PA and PAC microcapsules after 9 weeks of storage at 4 and 22°C without and under vacuum and after incubation for 2 h in SSJ followed by 3 h in SIF. Data represent the mean±standard deviation ($n=3$). Different letters next to error bars represent significant differences between treatments.

vacuum did not improve their performance (Fig. 4). When stored at 4°C, cell viability was improved relative to room temperature storage, and losses in viable cell counts were only 1.3 and 1.9 log CFU/g with and without vacuum, respectively (Fig. 4). The addition of a chitosan coating to the PA microcapsules led to significantly improved cell viability ($p<0.05$), where no loss in viable cell counts was observed after treatment with SSJ and SIF in samples stored under different temperatures (4 or 22°C) or atmosphere (with or without vacuum) conditions (Fig. 4). Zanjani *et al.* (26) reported that chitosan coating played a significant role in the protection of *L. casei* ATCC 39392 and *Bifidobacterium bifidum* ATCC 29521 immobilized in calcium-alginate-gelatinized starch microcapsules during simulated gastric and intestinal juices challenge test. Chitosan-coated alginate beads similarly provided good protection for *L. acidophilus* and *L. casei* cells during survival tests as reported by Krasaekoopt *et al.* (27).

Protection of bacterial cells immobilized in PA microcapsules is mainly provided by an outer membrane that is formed upon extrusion of the microcapsules material into a crosslinking solution containing divalent cations. The improvement in the protective properties of PAC microcapsules compared to PA ones can be attributed to an additional polyelectrolyte complex membrane that forms on the surface of negatively charged alginate when positively charged chitosan diffuses into its three-dimensional network (15).

Microscopy and size of microcapsules The morphology of the surface of PA and PAC microcapsules were imaged using CLSM (Fig. 5). No discernable differences could be seen with the addition of chitosan because the coating thickness was too small to be visualized by this technique, which has a minimum resolving power of 0.2 microns. On average, the size of PA microcapsules was ~2.4 mm,

Fig. 5. Confocal laser scanning microscope images of the surface of PA (left) and PAC (right) microcapsules stained with propidium iodide and SYTO 9. Arrows indicate entrapped bacteria. Scale bar: 50 µm.

Fig. 6. Diameter of PA and PAC microcapsules before and after incubation for 2 h in SSJ. Data represent the mean±standard deviation ($n=15$ for each microcapsule type).

where the addition of the chitosan coating had very little impact on microcapsule diameter (Fig. 6). Alginate (only) microcapsules have previously been shown to shrink at low pH (28,29). Alginate molecules have a pKa of ~3.5 and therefore lose 50% of their charge at pH values less than 5.5, leading to a dense structure and shrinkage of alginate-based microcapsules (30).

To conclude, PPI-alginate microcapsules formulated with and without a chitosan coating enhanced the survival of probiotic cells during refrigerated (4°C) storage for 9 weeks. In contrast, storage at 22°C worsened the performance of both types of microcapsules. While addition of chitosan to the microcapsule formulation improved storage and viability during simulated GIT challenges, the coating was so thin that it could not be visualized using CLSM. Vacuum packaging did not have any significant impact on the protective properties of microcapsules stored at room temperature but improved cell survival at 4°C in PAC microcapsules. These results suggest that PPI-alginate microcapsules coated with chitosan can be successfully used to preserve high viable counts of *L. rhamnosus* R0011 and *L. helveticus* R0052 during long-term refrigerated storage.

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