Delivery of Probiotic and Prebiotic Synergy Using Microencapsulation

DY Ying, S Parkar, XX Luo, R Seelye, JC Sharpe, J Saunders, R Schroeder
Health & Food, HortResearch, New Zealand (Danyang.Ying@hortresearch.co.nz)

Introduction

Probiotics have been defined as live microbial food supplements which beneficially influence the health of the host (Schrezenmeir & De Vrese 2001). Prebiotics are non-digestible food ingredients that can beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria (Gibson & Roberfroid 1995). When Gibson & Roberfroid introduced the concept of prebiotics they also speculated as to the additional benefits one might see if prebiotics were combined with probiotics to form what they called a synbiotic. They defined this as "a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implementation of live microbial dietary supplements in the gastrointestinal tract by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare" (Gibson & Roberfroid 1995).
To be effective, the probiotic bacteria must arrive at the site of action in large enough numbers to exert their effects. The Fermented Milk and Lactic Acid Bacteria Beverage Association of Japan has set a minimum of 10^7 bifidobacteria/g or ml. However, experimental evidence in which various concentrations of bacteria were fed and viable numbers counted after passage through the stomach would indicate that probiotic products may have to deliver even higher ($>10^{10}$ to 10^{11}) concentrations of microorganisms to survive the conditions of the gastro-intestinal (GI) tract (Farnworth 2001). Therefore, some protection is required.
Protection of probiotics by microencapsulation has been extensively researched (Krasaekoopt et al. 2003). Microencapsulation techniques include spray-drying, spray cooling/chilling, extrusion and co-extrusion and cocervation, among others. The materials used include Ca-alginate, a mixture of k-carageenan and locust bean gum, cellulose acetate phthalate, chitosan and gelatin (Krasaekoopt et al. 2003), gellan-xanthan (Sun & Griffiths 2000), alginate/poly-l-lysine/pectin/poly-l-lysine/alginate (Ouyang et al. 2004) and many other combinations.
This paper presents part of our work on the prebiotic effects of fruit polysaccharides and a microencapsulation technique that could deliver the synergies of the combination of probiotics and prebiotics while protecting the probiotics from the damage by the low pH environment of the human stomach.

Materials and Methods

Extraction of fruit polysaccharides
Fruit polysaccharides were extracted from two HortResearch fruit varieties (A and B) using ethanol precipitation technique. The polysaccharide obtained from fruit B was solubilised using monopotassium phosphate and re-precipitated using ethanol.

Culturing of human Caco-2 cells (colonic adenocarcinoma cells)
Caco-2 cells were cultivated as per recommendations of the American Type Culture Collection (ATCC) (Parkar et al. 2006). All tissue culture reagents were obtained from Invitrogen.

Bacterial Adhesion Assay
The cells were seeded at 5 x10^4 cells per well, in 24 well plates, and incubated at 37ºC in 5% CO_2. Medium was changed every 48 h and the cells maintained for 14 to 18 d, by which time these enterocytes were considered differentiated and displaying cell integrity similar to the actual adult gut. *Salmonella menston* and *Lactobacillus rhamnsosus* were cultured overnight in their respective media (tryptic soy broth and deMann-Rogosa-Sharpe broth respectively) from glycerol stocks, and

washed three times with phosphate buffered saline (PBS). They were diluted in MEM to obtain counts of $5x10^{10}$ cfu/ml.

The last 2 changes of media for the Caco-2 cells were made with MEM medium without any antibiotics or antimycotics. The polysaccharides were added to the wells in triplicate, while three wells were left for controls. After 1 h of incubation, either *S. menston* or *L. rhamnosus* was added to all the wells, maintaining a multiplicity of infection ratio of 100:1 (bacteria to caco-2 cells), and further incubation continued at 37ºC. After 2 h, the cells were washed twice with pre-warmed PBS and lysed for 10 min with 0.5% TritonX-100. Total bacteria adherent to or internalised by the cells, and also the initial bacterial challenge were quantified by serial dilution and plating on appropriate agar media.

Percentage change in the number of adherent and internalised bacteria (decrease or increase) was calculated as the average cfu in treated cells/average cfu in untreated control x100.

Probiotics

The probiotic bacterium used in this study was *Lactobacillus rhamnosus*. The bacterium was grown in de Man-Rogosa-Sharpe (MRS, Oxford, UK) medium in the presence of an Oxoid Anaerogen™ sachet at 37ºC for 18 h from glycerol stocks stored at -20ºC; bacterial counts were established by plating on MRS agar. This bacterium was chosen as our model bacterium because it is pH sensitive. Our aim was to develop a microencapsulation technique that will protect these bacterial cells from the harsh environment of the human stomach.

Alginate and Chitosan Encapsulents

The alginate was supplied by Sigma Aldrich (Sigma A2158). A 1.5% alginic acid sodium salt water solution was used in encapsulation. During encapsulation, a 3% calcium chloride solution was used to induce cross-linking of the alginate. The chitosan was supplied by Sigma Aldrich (Sigma C3646). A 0.08% chitosan solution in 1% acetic acid was used for coating the alginate beads.

Microencapsulation Apparatus

Equipment was designed and purpose-built for the microencapsulation of the probiotic bacteria and polysaccharide samples. In this system a nozzle was fabricated from polycarbonate and stainless steel to extrude two fluids simultaneously into an outer (shell), and inner (core) arrangement. Samples were aspirated from vessels through the nozzle at controllable flow rates and differential pressures provided by a regulated pressure air source. The tip of the nozzle contained a 150 μm diameter orifice and was shaped to allow the core-shell stream to exit under laminar flow conditions. The continuous stream was broken into regular-sized microdroplets by inducing a mechanical oscillation through the nozzle assembly to the stream. This oscillation was achieved using a piezoelectric element operating at a frequency (and therefore droplet generation rate) of 2,300 Hz. The droplets were electrostatically dispersed by applying an electric potential between the nozzle and an electrode ring that encircles the stream, in order to minimise microbead deformation from impact velocity and/or collision. Droplets were collected in a calcium chloride solution where ion-induced polymerisation of the alginate shell occurred, thereby encapsulating the core material in a microbead.

Encapsulation procedures

Three types of encapsulated samples were produced following the procedure shown in Figure 1. To produce sample 1, the lactobacilli were cultured for 18 h in MRS broth, and centrifuged at 3000 rpm for 20 min at 25ºC. This produced a pellet at the bottom of the centrifuge bottle. The pellet was mixed with a 5% polysaccharide solution. The mixture of bacteria and polysaccharide was sprayed into a liquid nitrogen bath (-196ºC). This produced frozen granules with sizes ranging from a few tens of microns to several hundred microns. The frozen granules were collected, after the liquid nitrogen was evaporated, and transferred into a freeze drier and dried for 24 h. The resulting dried granules were collected.

Sample 2 was prepared using a mixture of probiotic bacteria and polysaccharide. The mixture was co-extruded with the alginate through the co-extrusion microencapsulation system. Microdroplets were collected in a calcium chloride bath where the alginate formed a spherical shell around a core of the mixture of probiotic bacteria and polysaccharide. The product diameter was approximately 200 µm with a core/shell structure. The beads were kept in the calcium chloride solution for 30 min with continuous stirring to permit sufficient time for the polymerisation of the alginate shell to occur.

Sample 3 was prepared by starting with the procedure of Sample 2. The microbeads were then washed with distilled water and transferred into the chitosan solution with continuous stirring for 1 h. This process provided a protective coating of chitosan, a material known to offer increased resistance to acidic environments (Kosaraju 2005). The resulting coated beads were then rinsed with distilled water before viability testing.

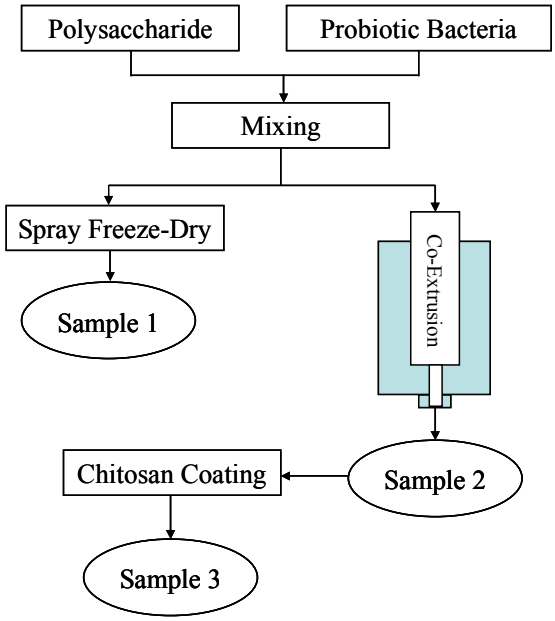

Figure 1. Diagram showing the sample preparation procedure

Simulated Gastric Fluid

Viability testing was conducted in simulated gastric fluid (SGF) (Gibson et al. 2004). The SGF was prepared using peptone water (7.5 g/L peptone in distilled water), at 3 pH levels (6.5, 3.0 and 2.0) using 1 M HCl. The SGF (9.85 ml) was aliquoted into 10 ml tubes, autoclaved, and stored in an anaerobic jar at 37ºC. Before the experiment 1.5 g/L solution of pepsin was prepared and added to SGF at a final concentration of 15 mg/L. *Lactobacilli* suspension (50 µl) or 1 g of encapsulated beads and 100 µl of pepsin stock were added into 9.85 ml of SGF. The mixture was incubated in the anaerobic jar at 37ºC for 2 h. SGF (100 µl) was then serially diluted ten-fold in peptone water (14 g/L) (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilution). All the dilutions were plated out in MRS agar in triplicates and were incubated under anaerobic condition at 37ºC for 48 h.

The three sample types were tested for viability of probiotic bacteria after exposure to SGF with pH levels of 6.5, 3.0 or 2.0 for 2 h. The encapsulated beads, after incubation with SGF, were washed twice with sterile, distilled water and then suspended into lysis solution (1% EDTA in 0.4% peptone water adjusted to pH 6.0).

Results and Discussion

The fruit polysaccharides were able to prevent the adhesion of *S. menston*, an enteropathogen, to Caco-2 cells, and to enhance the adhesion of the probiotic bacteria, *L. rhamnosus,* to the gut cells *in vitro* (Figure 2). The polysaccharide from fruit A only showed a slight effect in inhibiting the adhesion of the enteropathogen compared with the control.

The viability test showed that neither free cell nor freeze-dried mixture of lactobacilli and polysaccharide retained bacteria viability after 2 h

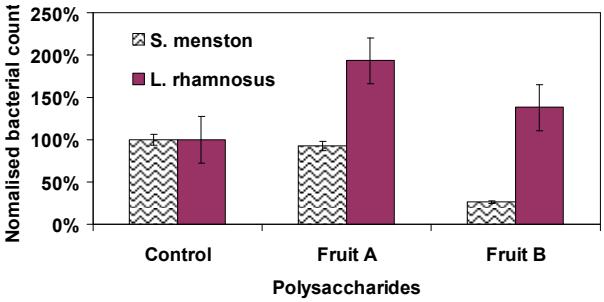

Figure 2. Effect of fruit polysaccharides on bacterial attachment to Caco-2 cells.

of incubation in SGF of pH 2.0 (Figure 3). This indicated that using polysaccharide as encapsulant alone was not sufficient to protect the probiotic bacteria against a low pH environment. Alginate encapsulation retained an efficacy of 5 log units of viable cfu/ml. Chitosan-coating the alginate beads further increased the protective effect and the chitosan-coated alginate beads retained 8 log units of viable cfu/ml counts after 2 h of incubation in the SGF of pH 2.0. Therefore, a combination of alginate and chitosan could provide a sufficient protection for the probiotic bacteria when passing through the human stomach.

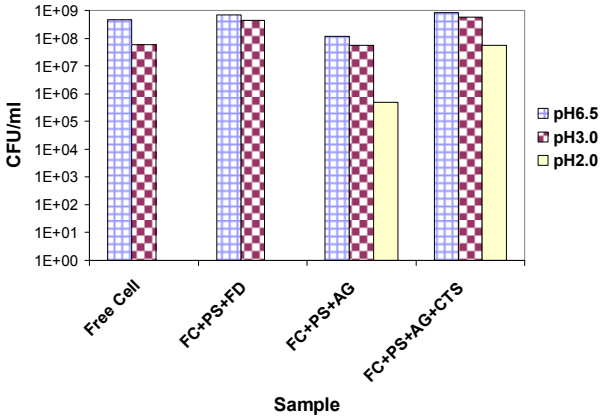

Figure 3. Bar chart show the viability of the *Lactobacilli* bacteria subjected to different treatments after exposure to different pH environment for 2 h. FC: Free Cell; PS: Polysaccharide; FD: Freeze Dried; AG: Alginate; CTS: Chitosan Coated.

Fruit polysaccharides were also found effective in increasing the proliferation of Caco-2 cells (Parkar et al. 2006) and the polysaccharide from fruit A carries natural antioxidants. Therefore, delivering the probiotic bacteria together with fruit polysaccharide using microencapsulation would not only deliver the prebiotic effect of the fruit polysaccharides to the functioning site of the human body but also protect the probiotic bacteria from the damage by the low pH environment of the human stomach.

Conclusions

Fruit polysaccharides showed prebiotic effects through preventing the adhesion of *S. menston*, an enteropathogen, to Caco-2 cells, and enhancing the adhesion of the probiotic bacteria, *L. rhamnosus,* to the gut cells *in vitro.*
Microencapsulation using alginate and chitosan could be one delivery mechanism that will protect the probiotic bacteria from the damage of the low pH human stomach environment, whilst delivering the prebiotic synergies of fruit polysaccharides in a form suitable for per oral administration.

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