Microencapsulation of probiotic *Lactobacillus plantarum* in an alginate matrix coated with whey proteins.

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1. Introduction

Over the last 20 years there has been an increase interest in the role of probiotic bacteria in human health. These bacteria defined as ″living microorganisms which, upon ingestion in certain numbers, exert health benefits beyond inherent general nutrition″ have become increasingly popular in foods industry. Yoghurt, fruit juice or chocolate products have been used as vehicle for incorporation of probiotic microorganisms, mainly bifidobacteria and lactobacilli (Picot 2003, Shima 2006). It has been recommended that foods containing such bacteria should contain at least 10^6 live microorganisms per g or ml of product at the time of consumption, in order to produce therapeutic benefits (Shah 2000). Several factors have been claimed to affect the viability of probiotic cultures in foodstuffs, including acidity, hydrogen peroxide and dissolved oxygen content. Microencapsulation has previously been reported as a technology that can provide protection to these sensitive cultures during incorporation in foods and storage (McMaster 2005) and during transit through the human gastrointestinal tract (Ainsley Reid 2005). Edible gums such as alginate, locust bean gum, k-carrageenan, xanthan and gellan are often used to immobilize bacterial cells (Mcmaster 2005, De Giulio 2005, and Krasaekoopt 2005). These polymers form a soft microcapsule for inclusion into dairy products. The aim of the present work was to provide microcapsules in which a high concentration of living lactobacilli was present, for food applications. A further objective was to establish the viability of immobilized cells when tested in conditions simulating the gastrointestinal tract.

2. Material and Methods

2.1 Ingredients and Reagents

Sodium alginate was obtained from Fluka AG (Buchs, Switzerland) and used as immobilization matrix. Whey protein isolate (WPI) containing 93% (w/w) protein was obtained from Arla Foods Ingredient (Skanderborgvej, Denmark) and used as coating agent. Calcium chloride anhydrous 93% (Alfa Aesar, Karlsruhe, Germany) was used as gelling solution. Hydrochloric acid 37% was purchased from Riedel-de Haen (Seelze, Germany), Sodium Chloride was supplied from VWR (Fontenay sous bois, France) and Sodium phosphate ($Na_2HPO_4.2H_2O$) was obtained from Merck (Darmstadt, Germany). Pepsin and bile salts were obtained from Sigma-Aldrich (Steinheim, Germany).

2.2 Bacterial strain and culture conditions

Lactobacillus plantarum 299V (from the strain collection of our laboratory) was grown in MRS broth (Biokar diagnostics, Beauvais, France).

After transfer of the test organism in MRS broth at 37°C for 16h, 0.1 ml of the activated culture was again inoculated into 100 ml of MRS broth at 37°C for 24h. Cells were harvested by centrifugation at 4000 rpm for 5 min at 6°C (Beckman GS-15R centrifuge, rotor C 0642, Hamburg, Germany), washed with cold sterile Phosphate Buffer Saline (pH 6.8), and resuspended with 2% sterile sodium alginate solution. All solutions used in our experiments were previously sterilized and handlings were carried out aseptically in a laminar flow hood.

2.3 Immobilization in Calcium chloride

1 ml of microbial suspension was introduced in a 5-ml syringe (Teruma, Leven, Belgium) fitted on a 27.5 G bevelled needle (Teruma). The suspension was dropped into the gelling solution (100 ml of 0.1M $CaCl_2$). Microdroplets formed in $CaCl_2$ solution were hardened into spheres. After 30 min, beads were separated from the solution. Microcapsules remaining were rinsed with sterile 0.05 M $CaCl_2$.

2.4 Coating procedures

Rinsed beads were weighted (0.2g) several times, and some of them were coated by immersion either into 2% whey protein isolate solution and vortex mixed or into 0.2% sodium alginate solution and vortex mixed.

2.5 Enumeration of *Lactobacillus plantarum*

Bacterial viable counts (cfu ml^{-1} or g^{-1}) were performed from serial dilutions in isotonic solution. Free cells were diluted in Nacl 0.9%. Representative 0.1 ml volumes from dilution were spread in duplicate onto MRS agar plates, and incubated at 37°C for 48h. After this period, colonies were counted, and values obtained as means of duplicate samples were expressed as log_{10}cfu ml^{-1}. To estimate numbers of *L. plantarum* in microcapsules before freezing in liquid nitrogen and freeze-drying (Christ® Freeze Dryer alpha 1-4 LSC, Fischer Bioblock, France), 0.2 g of capsules was weighted and introduced into 10 ml of Simulated Intestinal Juice (SIJ: 0.05 M sodium phosphate + 0.45% NaCl, pH 6.8, 37°C). After 20 to 30 min, standard serial dilution and plate counts were done. Freeze-dried microcapsules were directly added to Simulated Gastric Juice (SGJ: 1.5<pH<4.5) at 37°C during 2h, and then the capsules were placed automatically in SIJ (5.5<pH<7.5) at 37°C. After matrix completion, viability of *L. plantarum* was determined by serial plate dilution.

3. Results and Discussion

3.1 Physical examination of microcapsules

The size and shape of the beads were appreciated using a light microscope. The encapsulation technique used in this study resulted in bead size < 1 mm. The shape of the beads was generally spherical, sometimes oval shaped capsules were observed. Photomicrographs of alginate microspheres (results not shown) revealed encapsulated bacteria which were distributed randomly through the alginate network. (Sun 2000) using *B. infantis* reported an average bead diameter of 3 mm produced by a simple dropping of the mixture through a syringe. (Krasaekoopt 2003) reported an average diameter of 2-5 mm for capsules synthesized by extrusion. By using a smaller gauge needle with a bevelled tip, we have reduced the average size of alginate beads.

3.2 Survival of free cells in SGJ and bile salt

Previous researches reported that acid and bile resistance of free cells varies greatly among strains within a species and among species (Hansen 2002, Picot 2004).

The strain *L. plantarum 299V* has been found to be acid-sensitive in this study, and more bile resistant at the concentration of 5 g l^{-1}. (Favaro-Trindade 2002) observed a rapid decline in the number of free *L. acidophilus* into hydrochloric acid solution (pH 1.0 and 2.0) with virtually no survival after only 1h. (Sultana 2000) also reported a similar phenomenon, he observed at pH 2.0 a loss of 5 log of cfu ml^{-1} for *L. acidophilus* and *L. casei* after 3h of exposure. In addition, the author noted that an exposure of these two strains to 2% bile extract medium, for the maximum exposure up to 6h, involve a relative decrease in cell death after 2h compared to the initial cell count (decrease of 2 log cycles). Our results (Figures 1a and 1b) show the viability of free *L. plantarum* after 2h of exposure to a SGJ (pH 1.8, 3.0 and 4.5) and 0.5% bile salts.

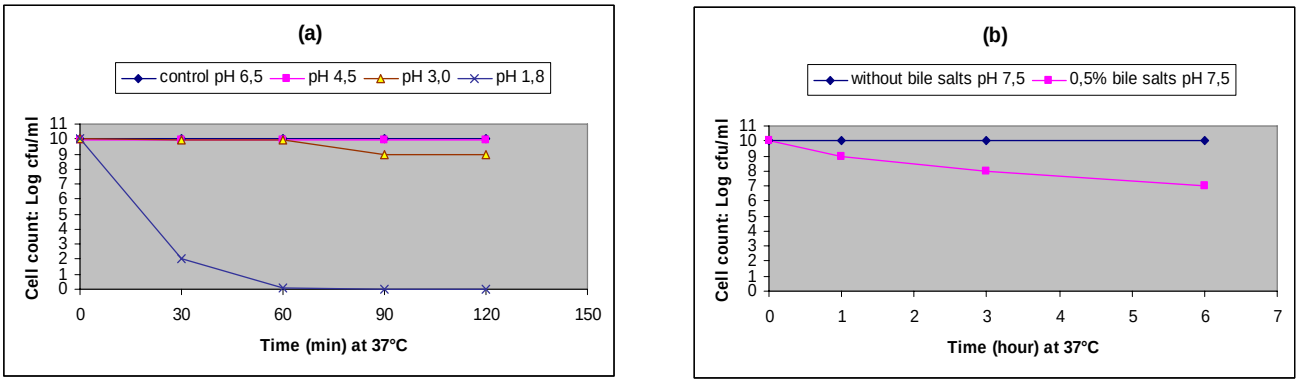

Figure 1. **Viability of free *Lactobacillus plantarum 299V* after exposure to**
a) Simulated gastric juice at different pH b) 0.5% bile salts

It was noted that no survival was obtained after 05 min of exposure at pH 1.3, but at pH 1.8, a decrease of 8 log cycles was observed after 30 min. The reduction in the survival of free cells tested was slight, less than 1.0 log cfu ml^{-1}, after exposure to the SGJ for 2h at pH 3.0 with or without pepsin. On the other hand, the survival of free *L. plantarum* at the end of exposure period did not differ at pH 4.5 with or without pepsin (no decrease of log cycle compared to the initial count). In the same way, the corresponding control samples at pH 6.5 remained at initial levels. A decrease of 3 log cycles was observed after 6h of exposure to 0.5% bile salts.

3.3 Survival of encapsulated cells in conditions simulating the gastrointestinal tract

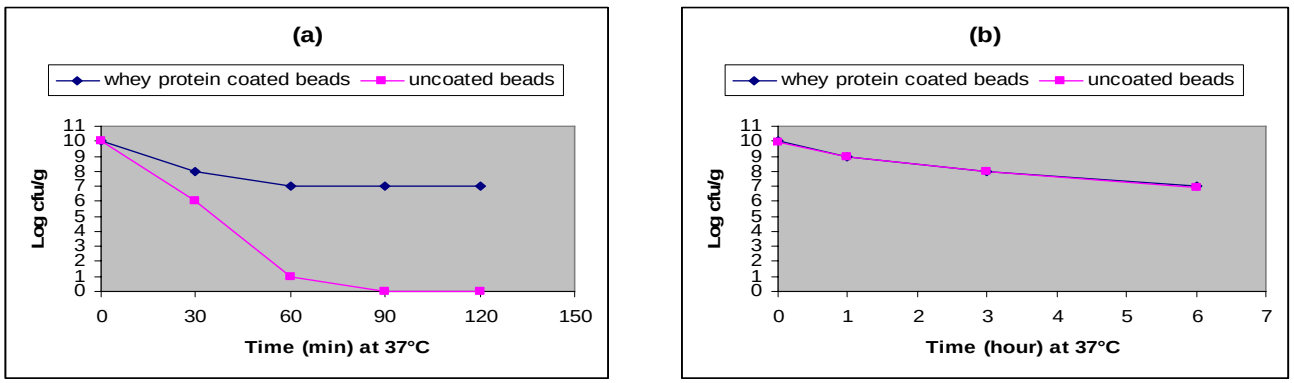

Figure 2. **Viability of immobilized cells in an alginate matrix with or without coating**
a) in simulated gastric juice pH 1.8 b) in 0.5% bile salts pH 7.5

Survival of microencapsulated cells after sequential incubation in SGJ and bile salts appears on figure 2. Initial cell numbers in the alginate microspheres ranged from 10^9 to 10^{10} cfu g^{-1} with the microencapsulation technique described. Microencapsulated probiotics values in both uncoated and coated beads in 0.5% bile solution (pH 7.5) at 37°C for 6h (Fig 2b) were similar, at this pH, a swelling of the beads is noted, and the matrix weakens. The survival of cells in both coated and uncoated beads (Fig 2a) after passage in acid medium was better than that of free cells (Fig 1a). Whey protein isolate coating provided the best protection compared to alginate coating which increases beads size and destabilizes initial matrix in simulated gastric juice. The pH of pure gastric juice ranges from 1.3 to 2.5, but the buffering capacity of ingested food may temporarily raise the stomach pH and protect the probiotics from exposure to extreme pH values.

4. Conclusion

Encapsulation only into alginate microspheres does not protect bacteria sufficiently but coating with whey protein isolate improves and maintains survival of *Lactobacillus plantarum 299V* after exposure to simulated gastric environment at pH 1.8. Further researches need to be carried out on other Lactobacilli strains, as well as studying initial cell numbers, acid tolerance and other parameters. Furthermore, food applications in cereals, biscuits, chocolate or jam with microencapsulated probiotic bacteria will be done.

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