

Original Research Article

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Microencapsulation of Probiotics by Calcium Alginate and Gelatin and Evaluation of its Survival in Simulated Human Gastro-Intestinal Condition

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ABSTRACT

Keywords

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In the recent past, there has been an explosion of probiotic health-based products. However, there are many reports indicated that there is poor survival of probiotic bacteria in these products. Further, the survival of these bacteria in the human gastro-intestinal system is questionable. Providing probiotic living cells with a physical barrier against adverse environmental conditions is therefore an approach currently receiving considerable interest. Microencapsulation being one of the most modern methods has considerable effects on probiotic survival. In this study *Lactobacillus acidophilus* (NCDC 014) and *Lactobacillus casei* (NCDC 018) were encapsulated using calcium alginate-gelatin and prebiotics (inulin and Lactulose) via extrusion technique, and were incubated in simulated gastric juice (along with pepsin, pH=1.5) for 2 hours and simulated intestinal juice (along with bile salts, pH = 8) for 4 hours at 37°C. . The results indicated that the survival of microencapsulated probiotic increased significantly in simulated gastro-intestinal condition. In general, this study indicated that microencapsulation with alginate-gelatin with prebiotic could successfully and significantly protect *L. acidophilus* and *L. casei* against adverse condition of simulated human gastro-intestinal condition and offers an effective means of delivery of viable bacterial cells to the colon.

Introduction

Probiotics are defined as live microorganisms which, when administrated in adequate amounts confer health benefit on humans (FAO, 2001). Bacteria belonging to genera *Bifidobacterium* and *Lactobacillus* are often used as probiotic supplements (Homayouni, 2008). With potential health advantages such as alleviation of symptoms of lactose malabsorption, cancer suppression, resistance to infectious gastro-intestinal disease, and improving digestion (Anal *et al.*, 2007; Aragon-Alegro *et al.*, 2007; Shah, 2007). The prebiotics are non-digestible food ingredients

that affect the host by selectively stimulating the growth and activity of bacteria in the colon. The most frequently studied examples are inulin and FOS (fructo-oligosaccharides) are known to have a positive effect on human health and promote the survival of probiotic bacteria (Capela *et al.*, 2006).

Microencapsulation with hydrocolloids as one of the most modern methods has remarkable effects on probiotic survival. Encapsulation process is a promising technique for probiotics protection against adverse

conditions to which probiotics can be exposed (Mokarram *et al.*, 2009). Several studies have been carried out investigating the protective role of this technique. Carbohydrate polymers such as alginate have been used in various microencapsulation procedures (Shu and Zhu 2002). Alginate is a natural heteropolysaccharide composed of D-mannuronic and L-glucuronic acid residues joined linearly by (1-4) glycosides linkages. Different studies have shown that calcium alginate microcapsules are better protected in the presence of coating polymers and prebiotics such as resistant starch, with the increase in survival of bacteria, under different conditions than when bacteria were tested in the free states (Donthidi *et al.*, 2010).

Gelatin is a protein derived from denatured collagen that contains high levels of hydroxyproline, proline and glycine and is useful as a thermally reversible gelling agent for encapsulation. Gelatin was selected here because of its excellent membrane-forming ability, biocompatibility and non-toxicity. The applicability of gelatine as a hydrogel matrix is limited because of its low network rigidity. However, its physical properties can be improved through the addition of cross-linking agents. Because of its amphoteric nature, it also is an excellent candidate for cooperation with anionic polysaccharides such as alginate and so on (Wenrong and Griffiths, 2000). However, alginate microcapsules (calcium alginate) are chemically susceptible to disintegration in the presence of excess monovalent ions, Ca^{2+} chelating agents such as phosphate and citrate and harsh chemical conditions such as those of low pH (Krasaekoopt *et al.*, 2004; Shu and Zhu, 2002; Lee *et al.*, 2004).

Furthermore, this study was undertaken on the alginate and alginate-gelatin encapsulation of probiotic bacteria with inulin and Lactulose as a prebiotic compound. The purpose of this

study was to enhance the effectiveness of alginate microencapsulation by gelatin and prebiotics (Inulin and Lactulose) and to evaluate their ability to improve the survival of *L. acidophilus* and *L. casei* during exposure to conditions simulated with that of human gastro intestinal environment human gastro-intestinal condition.

Materials and Methods

Experimental set up

Preparation of cell suspension

Lyophilized cultures of Probiotic bacteria, *Lactobacillus acidophilus* (NCDC 014) *Lactobacillus casei* (NCDC 018) were obtained from National Institute of Dairy, Karnal, Haryana. Lyophilized cells were inoculated in MRS broth (de Man-Rogosa-Sharpe) for 24 h under aerobic conditions at 37°C. Biomasses were then harvested by centrifuging at 5000 rpm for 10 min at 4 °C. The cultures were then washed twice by sterile saline solution (0.9%) and used in the microencapsulation process (Mokarram *et al.*, 2009).

Microencapsulation procedure

All glassware and solutions used in the protocols were sterilized at 121 °C for 15 min. The the preparation of encapsulated micro capsules was a modified version of methods basically reported by Donthidi *et al.*, in 2010 and Sultana *et al.*, in 2000. Briefly, 2 g sodium alginate was added to 100 mL distilled water and boiled until it formed a gel, then 2% gelatin was also added (Hi media, Mumbai) and required concentrations of inulin (1 %) and Lactulose (1%) were added separately and stirred until they were dissolved or dispersed. Then probiotic cultures of each bacterial species were transferred to the carrier solutions with

stirring under sterile conditions to ensure uniform distribution of the cells.

Preparation of alginate microspheres

The conditions used in the experimental work for the probiotic cells encapsulation were: a) 2% alginate; b) 2% alginate +1% Lactulose; c) 2% alginate + 1% inulin; d) 2% alginate + 2% gelatine; e) 2% alginate + 2% gelatine+1% Lactulose; f) 2% alginate + 2% gelatine+1% inulin. To form capsules, a cell suspension (equivalent of 10^8 CFU/g) was mixed with a 60 ml of 20 g/L alginate-gelatin with or without inulin or lactulose and the mixture was dripped into a solution containing CaCl_2 . The CaCl_2 concentration was at 0.1M and dripping was achieved with a sterile syringe. The distance between syringe and CaCl_2 solution was 10 cm. The droplets formed gel spheres instantaneously. Microspheres were hardened 30 min in CaCl_2 , and then rinsed with sterile NaCl (8.5 g/L).

Preparation of simulated gastric and intestinal juices and inoculation of cells

The simulated juices were prepared according to Brinques *et al.*, 2011 and Michida *et al.*, in 2006. Simulated gastric juices were prepared by dissolving pepsin (Himedia, Mumbai) in sterile sodium chloride solution (0.5%, w/v) to a final concentration of 3.0 g/L and adjusting the pH to 1.5 with hydrochloric acid. Simulated intestinal juices were prepared in sterile sodium chloride solution (0.5%, w/v), with 4.5% bile salts (Oxoid, Basingstoke, UK) and adjusting the pH to 8.0 with sterile NaOH (0.1 M). Both solutions were filtered for sterilization through a 0.22 μm membrane. The probiotic bacteria *L.acidophilus* and *L. casei* were inoculated to the simulated gastro-intestinal juice individually in six different forms, non-encapsulated, encapsulated with calcium alginate and calcium alginate-gelatin coated

with inulin or Lactulose as prebiotic. Further one gram of freshly encapsulated bacteria samples or 1 mL of cell suspensions (free cells) were gently mixed with 10 mL of sterile simulated gastric juice for 2 hours at 37⁰ C and followed by inoculation in sterile simulated intestinal juice and incubated at 37 °C for 4 hours.

In vitro release studies (GIT)

To examine the release behaviour of *L. acidophilus* and *L. casei* from microcapsules in GIT in vitro, 1 ml of free bacteria and 1 gram encapsulated samples (probiotic bacteria with or without prebiotic and alginate and or gelatin as encapsulating material) were added to 50 ml SGF (pH 1.5) and incubated at physiological temperature (37⁰ C) for 2 h and subsequently transferred into SIF (pH 8) for another 4 h. At specific time intervals (1 hour), 2.0 ml aliquots were removed and absorbance was measured at 600 nm in triplicate.

Release of entrapped bacteria

The capsules containing probiotic bacteria were released by citrate buffer (pH= 6.0, 1 %) reported by Mokarram *et al.*, in 2009. One gram of capsules was transferred to 9 mL buffer. The solution was stirred on a shaker (Remi Rotary Shaker, RS-12R) for 15 min vigorously until the bacteria from the capsules were released completely.. The counts (CFU/g) were determined by plating on MRS agar plates and incubating for 48 h at 37 °C. The free bacteria were treated similarly. All samples were counted in triplicates.

Encapsulation yield

Encapsulation yield (EY) *i.e.* the number of bacterial cells that survived the process and encapsulated inside the microcapsules was calculated as follows:

$$EY = (N/N_0) \times 100$$

Where N_0 is the number of viable bacteria in CFU/ml of culture and N is the number of viable bacteria in CFU/g of microcapsules.

Results and Discussion

In vitro release studies of *L. acidophilus* and *L. casei* from the microcapsules

Free cells and Microcapsule samples were treated with SGF and then with SIF to check the continuously release characteristics of *L. acidophilus* and *L. casei* in GIT and the results are shown in figure 1.

In SGJ (pH 1.5), the release amounts of cells were minor from each sample of the microspheres. Once the samples were transferred from SGF to SIF, the larger amounts and faster release rate of *L. acidophilus* (Fig. 1A) and *L. casei* (Fig. 1B) cells were found, indicated by increase in absorbance.

For *L. acidophilus* and *L. casei* faster release of cells occur when they are encapsulated in alginate- gelatin with inulin as prebiotic followed by lactulose. For both probiotics, alginate- gelatin along with prebiotic gave better protection in gastric juice and better release in intestinal fluid. Further free cells were very susceptible to SIF.

Survival of free and microencapsulated probiotics in simulated gastric juice

Figures II and III show the viability of free and encapsulated probiotic bacteria during incubation in the simulated gastro-intestinal condition, figure II illustrates that the survival of probiotics was lower in gastric juice and decreased further as the incubation period increased. Exposure to simulated gastric juice for 120 minutes resulted in a considerable decrease in the total number of free *Lactobacillus acidophilus* (Fig. 2A) and *L.*

casei (Fig. 2B) (only 25.6 % and 17.9 % viability respectively). However, the cell number of microencapsulated *Lactobacillus acidophilus* and *L. casei* decreased slightly after 120 min. Alginate –gelatin encapsulated *L. acidophilus* was observed to exhibit the highest viability (94.11%) when inulin was incorporated as prebiotic while greatest viability of 84.94 % was observed for alginate encapsulated *L. casei* without any prebiotic. In the case of both *L. acidophilus* and *L. casei*, the survival of cells in both alginate and alginate-gelatin were found to be higher when compared with free cells (Figure 2A,B). Chávarri *et al.*, in 2010 reported that encapsulation in chitosan-coated alginate microspheres significantly improved the survival of *Lactobacillus gasseri* and *Bifidobacterium bifidum* in simulated gastric juice along with pepsin. Many scientists have also reported that the survival rate of bifidobacteria in alginate microcapsules was higher than that of free cells (Hansen *et al.*, 2002; Yu *et al.*, 2001; Woo *et al.*, 1999). Many studies have shown coating the alginate matrix could increase the survival of bacteria due to curbing the diffusion of calcium ions outside of capsules (Chávarri *et al.*, 2010; Mokarram, 2009; Krasaekoopt *et al.*, 2004). Mokarram *et al.*, in 2009 showed that *L. acidophilus* and *L. rhamnosus* exposed to simulated gastric juice without pepsin had higher viability when encapsulated in calcium alginate with double coating sodium alginate.

They indicated that the reduction of pore size and distribution of gastric juice in double layer membrane lead to limitation of interaction between cells with the gastric juice. According to our study, microcapsules both alginate and gelatin coated along with prebiotic (lactulose or inulin) provided the best protection in simulated gastric juice. Furthermore, the increase in viable counts of bacteria could be attributed to the addition of prebiotic. Alginate and prebiotics such as

inulin or oligosaccharides tend to be synergistic in gelling and as a result may help maintain and improve the degree of protection to bacterial cells (Capela, 2006).

Survival of free and microencapsulated bacteria in simulated intestinal juice

The effect of the simulated intestinal juice on the viability of the microencapsulated and free probiotic bacteria after treatment with simulated gastric juice is presented in Figure III. The number of free probiotics decreased significantly as the incubation time increased.

Figure.1 O.D of free and microencapsulated *Lactobacillus acidophilus* (A) and *Lactobacillus casei* (B) in simulated gastro intestinal juice

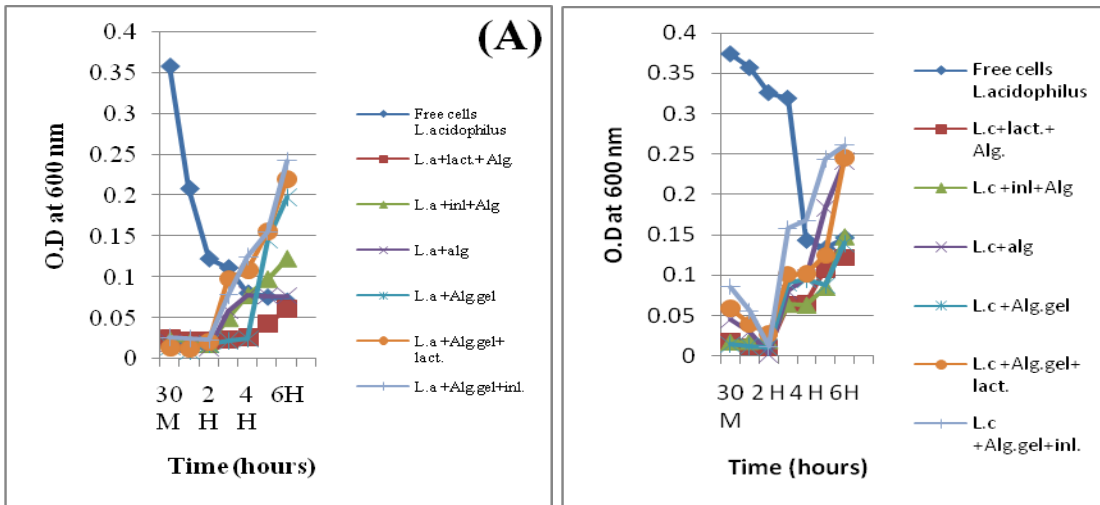


Figure.2 Survival of free and microencapsulated *Lactobacillus acidophilus* (A) and *Lactobacillus casei* (B) in simulated gastric juice

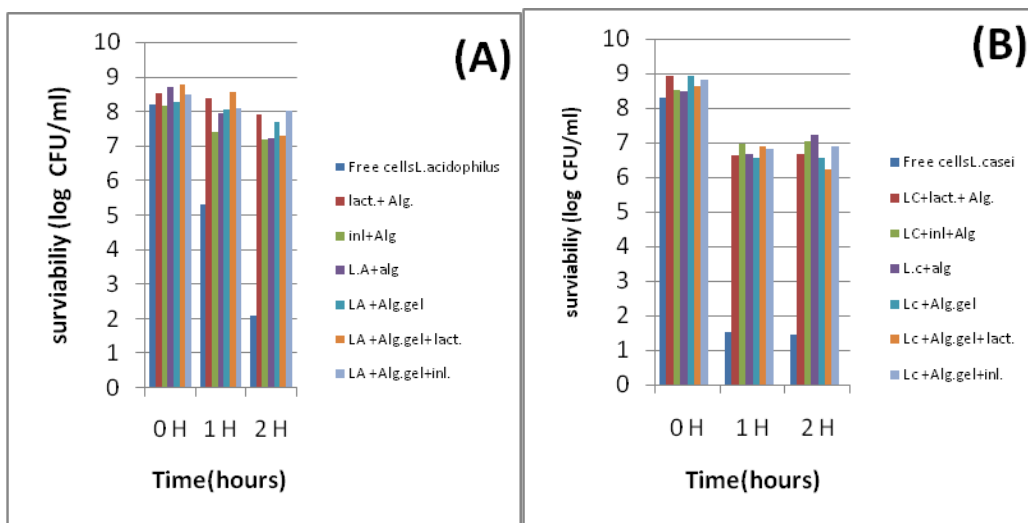
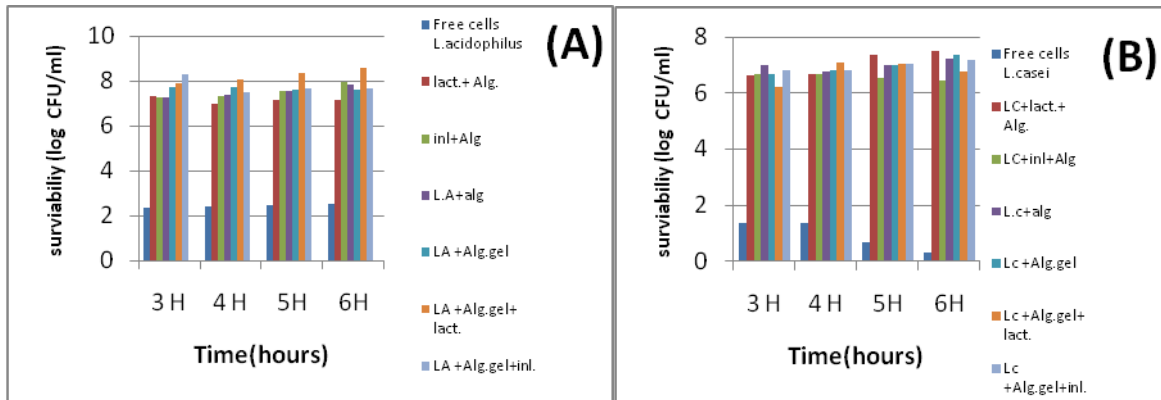


Figure.3 Survival of free and microencapsulated *Lactobacillus acidophilus* (A) and *Lactobacillus casei* (B) in simulated intestinal juice



Our result indicated that alginate-gelatin microcapsules with lactulose as prebiotic was most effective in protecting *L. acidophilus* [Fig. 3 (A)] from simulated intestinal juice with an encapsulation efficiency of 97.38 % followed by inulin encapsulated alginate beads (97.18%). This underlines the possibility that prebiotics have some effect on encapsulation efficiency. Similarly when *L.casei* (Fig.3B) after exposure to SGJ for 2 hours when introduced to SIJ, number of free cells decreased drastically with a viability of only 3.6 %, while, alginate encapsulated with lactulose showed more survival (85.9 %). This is in good agreement with the results of Krasaekoopt *et al.*, in 2004 who indicated that the survival of probiotic bacteria was highly enhanced in gastro-intestinal conditions when encapsulated with alginate-chitosan or poly-L-lysine. Different studies have shown that calcium alginate microcapsules are better protected in the presence of prebiotics (Sultana *et al.*, 2000, Chen *et al.*, 2005). For *L. acidophilus*, additional coating of sodium alginate with gelatin provided better survival in simulated gastrointestinal condition, but for *L.casei* gelatin confers any additional protection than alginate alone. For both probiotic lactulose provided more protection than inulin. All encapsulation method satisfy daily intake of probiotic bacteria above 10^6

living bacteria per milliliter or per gram of the product (Aragon-Alegro *et al.*, 2007).

In conclusion microencapsulation of *L. acidophilus* and *L. casei* in calcium alginate and calcium alginate-gelatin resulted in better *in vitro* release and survival of cells in simulated gastro-intestinal condition (along with pepsin and bile salt), compared to free cells. Introduction of prebiotic were found to provide better survival of both probiotic for gastro-intestinal condition. Therefore the applied approach in this study might prove beneficial for the delivery of probiotic cultures to the simulated human gastro-intestinal tract. Of the six types of microcapsules with prebiotic in this research, inulin gave better protection in SGJ but lactulose provided the best protection and survival of *L. acidophilus* and *L. casei* cells in simulated gastro-intestinal condition. Survival of free *L. acidophilus* and *L. casei* drastically decreased due to its low acid and bile resistance. In summary, the microencapsulation method reported in this paper under optimum conditions proved to be very efficient in increasing the viability of probiotic bacteria compared to non-encapsulated free cells. Alginate-gelatin microcapsules might be potentially used as a safe and protective delivery vehicle for administering viable probiotic bacteria. Future

researches in this realm may be focused on developing appropriate equipments which can provide potential improvements in the microencapsulation. Related researches are required to assess the efficacy of microencapsulation in the gastro-intestinal tract using animal models.

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