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Original Article

Evaluation of Viability of Probiotic Bacteria Encapsulated in Alginate/resistant Starch and Chitosan Beads at Bile Salts Solution and Simulated Gastrointestinal Juice Conditions

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ABSTRACT

Background: Microencapsulation of probiotics can be used to increase their viability during the process and delivery to target areas in the gut and intestinal tract. The aim of this study was to investigate the effect of microencapsulation on viability of probiotics bacteria (*Lactobacillus acidophilus* and *Bifidobactrium animalis subs lactis*) in bile salt solution and simulated gastrointestinal juice conditions.

Methods: First, 1 gram of probiotic bacteria was mixed in 100 ml of MRS broth and incubated at 37°C for 24 h until bacteria were activated. Microencapsulation of probiotics with sodium alginate/resistant starch and sodium alginate/chitosan were done by extrusion method. The number of viable bacteria was evaluated in bile salt solution (0.6%, w/v) and simulated gastric juice (0.08 mol/L HCl solution contained 0.2% NaCL and pH: 1.55 without pepsin), followed by incubation in simulated intestinal juice (0.05 mol/L KH_2PO_4 solution with 0.6 % bile salts and pH: 7.43).

Results: The microencapsulation could successfully and significantly protect probiotic bacteria against adverse condition of simulated human gastro-intestinal condition. Microcapsules containing sodium alginate/resistant starch had the highest survival rate at the end of the incubation time in bile salt solution ($6.3\pm0.2 \times 10^6$ and $4.6\pm0.3 \times 10^7$ for *Lactobacillus acidophilus* and *Bifidobacterium animalis*, respectively) and simulated gastrointestinal condition ($4.5\pm0.4 \times 10^7$ and $1.7\pm0.2 \times 10^6$ for *Lactobacillus acidophilus* and *Bifidobacterium animalis*, respectively).

Conclusion: Generally, the microencapsulation process improved the survival of probiotic bacteria under simulated gastrointestinal conditions and bile salts solution and in this case, sodium alginate / resistant starch coating was more effective than sodium alginate/ chitosan.

Keywords: Probiotic bacteria, Microencapsulation, Sodium alginate, Chitosan, Resistant starch, Gastrointestinal juice

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Introduction

The survival of probiotic cells is important because for their beneficial effects on the health of the host, they must survive to the point of operation. Many reports indicated that there is poor survival of probiotic bacteria in products containing free probiotic cells. Providing probiotic living cells with a physical barrier to resist against adverse environmental conditions is consequently an approach currently receiving considerable interest (1). Microencapsulation is a powerful technology which has been developed for use in the food industry and allows the protection of bacterial cells. Encapsulation has been investigated for improving the viability of microorganisms in both food products and the gastrointestinal (GI) tract (2, 3). The obtained micro particles have to be water-insoluble to maintain their integrity in the food matrix and in the upper part of GI tract and finally, particle properties should allow progressive release of the cells during the intestinal phase (3, 4). Different materials can be used for encapsulation wall. Alginate hydrogels are extensively used in cell encapsulation and sodium alginate is preferred for encapsulating probiotics because of its simplicity, non-toxicity, biocompatibility, and low cost (2). However, some disadvantages are attributed to the use of alginate. For example, alginate beads are sensitive to the acidic environment (5) which is not compatible for the resistance of the micro particles in the stomach conditions and its scaling-up of the process is very difficult (6). However, defects can be remedied by mixing alginates with other polymeric compounds, coating the capsules with another compound, or applying structural modifications to the alginate using various additives. (2). Resistant starch and chitosan are compounds that can be used with alginate in the microcapsules. Encapsulation of probiotic bacteria with alginate and chitosan or resistant starch coating provides protection in simulated gastrointestinal conditions and therefore, it is a good way of delivery of viable bacterial cells to the colon (7). However, chitosan has some disadvantages and it seems to have inhibitory effects on lactic acid bacteria (8). But it has been reported that the use of resistant starch as the second wall compared with the use of alginate alone improves the survival of probiotic bacteria because starch acts as a prebiotic (9).

The aim of this study was to investigate the viability of free and encapsulated (with alginate/resistant starch and alginate/chitosan) *Lactobacillus acidophilus* and *bifidobactrium animalis subs lactis* exposure to simulated gastrointestinal and bile salt solution.

Material and Methods

The materials included *Lactobacillus acidophilus* and *bifidobactrium animalis subs lactis* (CHR-Hansen, Denmark), Na–alginate (Sigma, USA), $CaCl_2$ (Merk, Germany), chitosan (low molecular weight, degree of acetylation more than 75%) (Sigma, USA), resistant starch (Merk, Germany), MRS agar and MRS broth (Merk, Germany), bile salts (Sigma, USA), Mupirocin, and salicin (Merk, Germany).

Preparation of encapsulated probiotics

Pure probiotic cultures of *Lactobacillus acidophilus* and *bifidobactrium animalis subs lactis* were inoculated into MRS-broth (de Man-Rogosa-Sharpe) and incubated at 37˚C for 24 h. The probiotic biomass in late-log phase was collected by centrifugation (Centrion Centrifuge, Model 2010, West Sussex, BNI8OHY, UK) at 10,000 rpm for 10 min. The sediments were washed twice by sterile saline. In this study, extrusion technique was performed for microencapsulation process described earlier by Mirzaei et al. (2012) (10). The mixture of cell suspension and Na–alginate solution (2% in distilled water) was injected into a $CaCl_2$ solution (0.1 M). The droplets formed gel spheres immediately. Then, beads were added to chitosan solution (1%) and resistant starch solution (1%) for formation of double-layer type of microcapsules. After 15 min mixing, beads were collected by filter. The distance between the syringe and $CaCl_2$ solution was 25 cm. Diameter of the resultant beads was 200–500 µm.

Bile salt solution tolerance of free and encapsulated bacteria

The stability of encapsulated and free *Lactobacillus acidophilus* and *bifidobactrium animalis subs lactis* was tested in bile salt solution. Suspensions of free probiotic bacteria (1 mL) or microspheres containing probiotic bacteria (1 g) were placed in a tube containing 9 mL bile salt solution (0.6%, w/v and pH: 8.25) and incubated at 37˚C for 2 h. Free and

encapsulated probiotic bacteria were collected at each 30 min time intervals (11).

Viable probiotic bacteria were measured by pour plate counting on MRS agar. Samples (1.0 ml) were added to 9.0 ml of sterile Ringers solution. Subsequently *Lactobacillus acidophilus* and *bifidobactrium animalis subs lactis* were plated onto MRS agar+ 10 % W/V salicin and MRS agar+5 ml/liter medium Cysteine HCl + 2.5 ml/liter Mupirocin, respectively. The colonies were counted after 72 h of incubation at 37°C. Colony forming units (CFU) were enumerated in plates containing 15 to 300 colonies and cell concentration was expressed as CFU/ml (12).

To count the microencapsulated bacteria, the entrapped bacteria were released from the beads according to the method of Mirzaei *et al.* (2012). Ten grams of sample were added to 100 ml of phosphate buffer (0.1 M, pH 7.0) followed by 15 min shaking on a shaker (IKA-Model Janke & Kunkel GMBH. Type VX5-Germany). The encapsulated bacteria were treated in a similar way as free bacteria conditions. All experiments were done in triplicate.

Survival of free and microencapsulated probiotic bacteria in simulated gastrointestinal juice

Suspensions of probiotic bacteria (1 mL) or microspheres containing probiotic bacteria (1 g) were placed in a tube containing 9 mL Simulated gastric solution (0.08 mol/L HCl solution contained 0.2% NaCL and pH: 1.55 without pepsin) and incubated at 37˚C for 0, 30, 60, 90 and 120 min. After incubation, 1.0 mL of these solutions was added to 9 mL of simulated intestine solution (0.05 mol/L KH_2PO_4 solution with 0.6 % bile salts and pH: 7.43) and incubated at 37˚C for 150 min. Assay of the viability of free and encapsulated probiotic bacteria was carried out as described above (11).

Statistical analysis

Results were expressed as mean ± SD values which were the average of triplicate experiments. Significant differences between the results were determined using the analysis of variance (ANOVA) method, and significant differences of means were compared using Duncan's test at $P<0.05$ significant level using the SAS software (2008).

Results

The initial cell count of probiotics before encapsulation was in the range of $9.5\pm3.1\times10^{11}$ cfu/ ml for *Lactobacillus acidophilus* and $8.1\pm1.6\times10^{12}$ cfu/ ml for *Bifidobacterium animalis*. High cell yield was achieved in the range of $6.5\pm1.2\times10^{10}$ and $7.4\pm2.1\times10^{11}$ cfu/ g in resistant starch-coated beads and $7.1\pm0.9\times10^{10}$ and $9.4\pm1.1\times10^{10}$ cfu/ g in chitosan-coated beads, which had an average diameter of 200–500 µm. The loss during encapsulation and coating was very low due to the gentle methods used.

Survival of free and encapsulated probiotic bacteria in bile salts solution (0.6 %, pH: 8.25)

The survival rates of probiotic bacteria of *Lactobacillus acidophilus* and *Bifidobacterium animalis* after 120 minutes of incubation in bile salts solution are shown in Table 1. According to the table, it can be seen that after 2 hours of incubation in bile salts solution, the reduction in the number of *Lactobacillus acidophilus* bacteria in all samples was higher than *Bifidobacterium aimalis*.

Table 1. Survival of free and encapsulated probiotic bacteria (*L. acidophilus and B. animalis subs lactis*) at bile salts solution (0.6 %, pH: 8.25)

Treatments		Incubation times				
		1	30	60	90	120
Free probiotic bacteria	*L. acidophilus*	$7.6\pm0.3\times10^{9}$	$5.2\pm0.4\times10^{7}$	$6.4\pm0.7\times10^{6}$	$<10^{6}$	$<10^{6}$
	B. animalis	$4.5\pm0.5\times10^{9}$	$3.6\pm0.7\times10^{8}$	$3.2\pm0.4\times10^{7}$	$2.5\pm0.6\times10^{6}$	$<10^{6}$
Encapsulated with resistant starch	*L. acidophilus*	$7.9\pm0.5\times10^{10}$	$6.1\pm0.3\times10^{9}$	$6.4\pm0.5\times10^{8}$	$1.7\pm0.4\times10^{7}$	$6.3\pm0.2\times10^{6}$
	B. animalis	$4.5\pm0.6\times10^{11}$	**$7.2\pm0.3\times10^{10}$**	$2.3\pm0.7\times10^{10}$	$5.5\pm0.4\times10^{9}$	$4.6\pm0.3\times10^{7}$
Encapsulated with chitosan	*L. acidophilus*	$5.3\pm0.2\times10^{8}$	$1.4\pm0.2\times10^{8}$	$7.3\pm0.5\times10^{7}$	$4.8\pm0.2\times10^{6}$	$3.6\pm0.3\times10^{6}$
	B. animalis	$1.1\pm0.5\times10^{9}$	$8.2\pm0.7\times10^{9}$	$9.4\pm0.3\times10^{8}$	$7.4\pm0.4\times10^{7}$	$5.1\pm0.5\times10^{6}$

As the incubation period increased, the survival of probiotic bacteria decreased. Microencapsulated bacteria with sodium alginate/resistant starch had the highest survival rate at the end of the incubation time ($6.3 \pm 0.2 \times 10^6$ and $4.6 \pm 0.3 \times 10^7$ for *Lactobacillus acidophilus* and *Bifidobacterium animalis*, respectively). Generally, microencapsulated bacteria showed a higher survival than non-microencapsulated bacteria.

Resistance of probiotic bacteria to simulated gastrointestinal fluids

In this study, the survival of *Lactobacillus acidophilus* and *Bifidobacterium animalis* in three different conditions of free (non-microencapsulated), microencapsulated with sodium alginate and resistant starch, and microencapsulated with sodium alginate and chitosan for 120 minutes under simulated gastrointestinal conditions were analyzed. The findings on the survival rate of these two bacteria are shown in Table 2.

Table 2. Survival of free and encapsulated probiotic bacteria (*L. acidophilus and B. animalis subs lactis*) in simulated gastrointestinal solution

Treatments		Incubation times				
		1	30	60	90	120
Free probiotic bacteria	*L. acidophilus*	$5.3\pm0.6\times10^9$	$4.2\pm0.2\times10^7$	$7.1\pm10.5\times10^6$	$<10^6$	$<10^6$
	B. animalis	$4.5\pm0.3\times10^9$	$3.6\pm0.7\times10^6$	$3.8\pm0.1\times10^6$	$<10^6$	$<10^6$
Encapsulated with resistant starch	*L. acidophilus*	$8.7\pm0.7\times10^{10}$	$7.1\pm0.4\times10^9$	$5.3\pm0.4\times10^9$	$5.7\pm0.1\times10^7$	$4.5\pm0.4\times10^7$
	B. animalis	$3.4\pm0.4\times10^{10}$	$7.3\pm0.5\times10^8$	$1.9\pm0.2\times10^7$	$1.5\pm0.4\times10^7$	$1.7\pm0.2\times10^6$
Encapsulated with chitosan	*L. acidophilus*	$5.4\pm0.6\times10^9$	$1.3\pm0.2\times10^8$	$7.9\pm0.5\times10^7$	$3.2\pm0.4\times10^7$	$2.5\pm0.5\times10^6$
	B. animalis	$1.9\pm0.5\times10^9$	$7.1\pm0.6\times10^7$	$1.3\pm0.2\times10^7$	$7.6\pm0.3\times10^6$	$1.1\pm0.6\times10^6$

The results of Table 2 illustrate the role of microencapsulation process during 120 minutes of incubation in simulated gastrointestinal conditions. Microencapsulation process improved the survival of two probiotic bacteria under simulated gastrointestinal conditions in several logarithmic cycles, and after 120 minutes, probiotic bacteria were more than 10^6 CFU/ml. According to the table, *Lactobacillus acidophilus* showed a higher survival rate compared to *Bifidobacterium animalis*. Microencapsulated bacteria with sodium alginate/resistant starch showed higher survival rates compared to free and microencapsulated bacteria with sodium alginate/chitosan.

Discussion

Results showed that after 2 hours of incubation in bile salts solution, the reduction in the number of *Lactobacillus acidophilus* bacteria in all samples was higher than that of *Bifidobacterium aimalis*. Generally, *Bifidobacterium* species are resistant to bile salts but are very sensitive to pH reduction (13). Free *Lactobacillus acidophilus* after one hour and free *Bifidobacterium animalis* disappeared after one and a half hours of incubation in bile salts solution, which could be due to the damage to the cell wall by bile salts activity (14).

Gallbladder plays an important role in the specific and non-specific defense mechanism of the intestine and its inhibitory effect is determined by the concentration of bile salts. In the human digestive system, the average concentration of bile salts is 0.3% w/v and this concentration is considered critical and enough for screening of bile salts-resistant strains. This concentration was selected in a study by Lotfi *et al.* (15) to evaluate the growth potential of 31 strains in which the strains were able to grow in 0.3% bile salts at different levels of resistance. Hassanzadazar *et al.* (16) investigated acid and bile salts tolerance properties of lactobacilli isolated from Koozeh cheese. Four strains isolated in their study can survive at 0.3 % Bile concentration for 4 hours.

Ziar *et al.* (17), reported that Calcium alginate-resistant starch mixed gel improved the survival of *Bifidobacterium animalis subsp lactis* and *Lactobacillus rhamnosus* in simulated gastrointestinal conditions. According to them, microencapsulation procedure consisting of mixing the sodium alginate gel with resistant starch confers stability to beads and leads to a

better protection against harmful environmental conditions. This could be explained by the fact that resistant starch and sodium alginate tend to be synergistic in gelling and as a result may help in providing additional protection to the entrapped bacterial cells. In addition, they postulated that the diffusion of bile salts into the beads may be limited by the sodium alginate gel matrix reinforced herein by incorporation of resistant starch.

Sultana *et al.* (9) investigated the viability of free and encapsulated *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in 1 and 2 % bile salts solution. They reported that the microencapsulation process can not increase the survival of these bacteria.

Many researchers have reported that chitosan coatings provide excellent protection against bile salts, because during the absorption of these salts by the microcapsules, an ion exchange reaction occurs, thereby increasing the permeability of the capsules are reduced and restricted to bile salts (7, 11). Etchepare *et al.* (18) observed that survival of the viable cells of *Lactobacillus acidophilus* in the simulated gastric environment and bile salts was higher in chitosan-coated alginate microparticles as compared to uncoated microparticles. The protection provided by the chitosan is due to strong bonding between chitosan and alginate by electrostatic interactions, leading to formation of a membrane on the surface of the granules, which reduces the probability of migration of coating materials.

In general, the comparison between the results of various studies is difficult due to the concentration and sources of bile salts, but Chandramouli *et al.* (19) and Kailasapathy (1) reported that microencapsulated probiotic bacteria have a higher viability than free bacteria in 1-3% bile salts solution. Of course, it should be noted that the resistance of bacteria against bile salts *in vitro* does not indicate their actual behavior in the gastrointestinal tract, because, like other physiological shocks. It is widely observed that environmental factors can enhance or weaken the behavior of microorganisms in specific conditions. In addition, unlike in laboratory conditions, the amount of bile salts in the intestine is not constant, and until the consumption of high-fat foods, the amount of these compounds in the intestine is very low, this is the factor that can be used to adapt the bacteria and increase their resistance to bile. In addition, the presence of food in the intestine can create a protective shield for microorganisms, and some probiotics act in the gut without contact with bile salts. The activity of bile salts in *in vitro* conditions may be much greater than the actual activity of them in the intestines, because it is possible in the intestine to combine these salts with phospholipids (16).

Microencapsulation process improved the survival of two probiotic bacteria under simulated gastrointestinal conditions. Mandal *et al.* (20) reported that microencapsulation increased the viability of bacteria in pH =1.5. Chandramouli *et al.* (19) also reported that microencapsulation of probiotics, improve their survival in simulated gastric acid conditions. The present study shows that the use of sodium alginate with chitosan also significantly increases the viability of both bacteria, although this level was lower than that of sodium alginate/resistant starch. Brinques and Ayub (21) improved the viability of *Lactobacillus plantarum* by microencapsulation with calcium alginate and chitosan. The present study showed that the initial population of free *Bifidobacterium animalis* and *Lactobacillus acidophilus* did not survive after 90 minutes of incubation in the same gastrointestinal conditions, although free *Lactobacillus acidophilus* showed more survival. Krasaekoopt *et al.* (22) reported that free *Bifidobacterium bifidum* has a very low survival in simulated gastric conditions. The findings of this study indicate that *Lactobacillus acidophilus* viability in similar gastrointestinal conditions is more than *Bifidobacterium animalis*, which may be due to the higher total resistance of *lactobacilli* to acidic conditions than *Bifidobacterium* (9, 22). Sabikhi *et al.* (23) also reported that the coating of calcium alginate and corn starch enhanced viability of probiotics in the intestinal condition. The researchers also stated that the presence of prebiotic compounds such as corn starch in calcium alginate capsules, delayed the penetration of gastric and intestinal juice into capsules and thus increased the viability of probiotics.

Chávarri *et al.* (7) showed that chitosan coating around calcium alginate capsules increases the probiotic survival in acidic conditions of the stomach and alkaline conditions of the intestine. Their research showed that microencapsulated bacteria with

chitosan coating had no reduction in acidic conditions in the stomach within 1 hour, while in the present study, both bacteria were reduced about 3 logarithmic cycles after 120 minutes of incubation.

Anal and Singh (24) reported that microencapsulation with sodium alginate effectively protects microorganisms from acidic and temperature treatments when transferred to the intestine, without affecting the probiotic function.

Shahdadi *et al.* (25) showed that encapsulation process with sodium alginate/chitosan improved stability of probiotic bacteria.

The strength of the present study was the use of extrusion as a new, gentle and effective process to keep probiotic bacteria in critical condition. An important limitation of this method is the high cost of materials used as a second wall on alginate.

Conclusion

In general, the results showed that among the microencapsulated and free cells, survival and tolerance at critical conditions were much higher in microencapsulated bacteria and among the two types of microencapsules, alginate coated starch and alginate coated chitosan, alginate coated starch was more resistant to the simulated conditions of gastrointestinal tract. The microencapsulation of *L. acidophilus* and *B. animalis subs lactis* cells with sodium alginate/chitosan and sodium alginate/resistant starch can successfully keep the count of this probiotic bacterium high enough for the therapeutic minimum (10^6 cfu/ml) in simulated gastrointestinal conditions. In the continuation of this research and to complete it, it is suggested to study the effect of other methods and different walls of microencapsulation on probiotic bacteria under both *in vivo* and *in vitro* conditions.

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Authors' contributions

All authors contributed with study design, data collection, data handling and manuscript preparation. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interests.

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