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The influence of multi stage alginate coating on survivability of potential probiotic bacteria in simulated gastric and intestinal juice ☆

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ABSTRACT

The probiotics, *Lactobacillus acidophilus* PTCC1643 and *Lactobacillus rhamnosus* PTCC1637, were encapsulated into uncoated calcium alginate beads and the same beads were coated with one or two layers of sodium alginate with the objective of enhancing survival during exposure to the adverse conditions of the gastro-intestinal tract. The survivability of the strains, was expressed as the destructive value (decimal reduction time). Particle size distribution was measured using laser diffraction technique. The thickness of the alginate beads increased with the addition of coating layers. No differences were detectable in the bead appearance by scanning electron microscopy (SEM). The alginate coat prevented acid-induced reduction of the strains in simulated gastric juice (pH 1.5, 2 h), resulting in significantly ($P < 0.05$) higher numbers of survivors. After incubation in simulated gastric (60 min) and intestinal juices (pH 7.25, 2 h), number of surviving cells were 6.5 log cfu mL^{-1} for *L. acidophilus* and 7.6 log cfu mL^{-1} for *L. rhamnosus* by double layer coated alginate microspheres, respectively, while 2.3 and 2.0 log cfu mL^{-1} were obtained for free cells, respectively.

1. Introduction

Probitics, defined as "living microorganisms which upon ingestion in certain numbers, exert health benefits beyond inherent general nutrition" (Guarner & Schaafsma, 1998) have become increasingly popular during the last decade. It has been recommended that food containing probiotic bacteria should contain at least 10^6 live microorganisms per g or mL, at the time of consumption in order to produce therapeutic benefits (Capela, Hay, & Shah, 2006; Picot & Lacroix, 2004; Shah, 2002). Several factors have been claimed to affect the viability of probiotic cultures including temperature (Ostlie Hilde, Treimo, Narvhus, & Judith, 2005; Vinderola, Bailo, & Reinheimer, 2000), pH (Corcoran, Stanton, Fitzgerald, & Ross, 2005). Concentration of lactic and acetic acids (Samona, Robinson, & Marakis, 1996) and processing conditions (Jaana, Alakomi, Vaari, Virkajärvi, & Saarela, 2006) .

Alginates are natural hetero polysaccharides made up of D-mannuronic and L-guluronic acid residues joined linearly by (1–4)-glycosidic linkages (Smidsrod, Haug, & Lian, 1972). Alginate beads can be formed by both extrusion and emulsion methods (Krasaekoopt, Bhesh, & Deeth, 2003). The use of alginate is favored because of its low cost, simplicity, and biocompatibility (Klein, Stock, & Vorlop, 1983; Martinsen, Skjak-Braek, & Smidsrod, 1989). Bacteria (0.5–4 μm in size) are well retained in the alginate gel matrix which is estimated to have a pore size of less than 17 nm (Klein et al., 1983). However, the gel is susceptible to disintegration in the presence of excess monovalent ions, Ca^{2+} chelating agents and harsh chemical environments (Smidsrod & Skjak-Braek, 1990). A cross-linked alginate matrix system at very low pH is reported to undergo a reduction in alginate molecular weight causing a faster degradation and release of active ingredients (Krasaekoopt, Bhesh, & Deeth, 2004; Gombotz & Wee, 1998). Cell entrapped in beads leak, escape from the gel matrix, and grow in the medium solution (Kuhn & Pfister, 1989). The maximum cell loading in the entrapped beads is limited to 25% by volume because of weak mechanical strength (Buchholz, Luttmann, Zakrzewski, & Schügerl, 1980). To overcome these problems soaking alginate beads in 0.1% alginate conforms second layer of alginate and increase bead strength. This paper reports a study on influence of multi stage coating on the properties of alginate beads (such as size distribution and external appearance by scanning electron microscopy (SEM) and the survivability in simulated digestive systems of microencapsulated probiotic bacteria in the beads coated with one or two layers of alginate.

2. Materials and methods

2.1. Preparation of microorganisms

Lactobacillus acidophilus PTCC1643 (Persian Type Collection Culture) and *Lactobacillus rhamnosus* PTCC1637 were purchased from

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Iran Scientific and Industrial Organization. A vial of freeze dried *L. acidophilus* and *L. rhamnosus* were individually inoculated into 5 mL MRS (de Man, Rogosa, Sharpe) broth (Merck, KGaA Germany) and incubated at 37 °C for 24 h under aerobic conditions. The cultures were then sub-cultured into 95 mL broth and incubated under the same conditions. The cells were harvested by centrifuging at 3000*g* for 5 min at 25 °C and washed twice with sterile 0.1% peptone (Merck KGaA Germany) solution.

2.2. Micro encapsulation of microorganisms

Calcium alginate microcapsules were made using an external gelation technique adapted from Sheu and Marshall (1993) as previously, described by Truelstrup Hansen, Allan-Wojtas, Jin, and Paulson (2002) and Allan-Wojtas, Truelstrup Hansen, and Paulson (2008). Briefly, calcium alginate microcapsules with average diameters of about 23 μm were produced by mixing 18 g alginate solution 10 g L^{-1}, medium viscosity and high mannuronic acid, (Sigma–Aldrich, Pool, UK) with 1 g washed bacteria suspension. The alginate bacterial mix was subsequently emulsified in 100 g vegetable oil containing 5 g L^{-1} Tween 80 (Sigma–Aldrich, Pool, UK) using a magnetic stirrer set at ca. 900 rpm for 20 min. Gelation was initiated by addition of 32 mL Ca^{2+} containing emulsion (60 g vegetable oil, 5 g L^{-1} Tween 80 and 62.5 mM $CaCl_2$). The alginate microcapsules were formed during continuous stirring for 20 min. The beads were allowed to stand for 30 min for gelification, and then rinsed with, and subsequently kept in, sterile 0.1% peptone solution at 4 °C.

2.3. Coating of alginate beads

Alginate beads with encapsulated *L. acidophilus* and *L. rhamnosus* were prepared as described above. Fifteen gram of the filtered (Whatman No. 4, filter paper, Fisher Scientific, Loughborough, UK) micro capsules were mixed to 100 mL of 0.5% (w/v) medium viscosity alginate solution and stirred at an initial speed of 500 rpm to disperse the beads. The microspheres were left to stir in alginate for 20 min before being filtered, collected and resuspended in 75 g of oil containing 5 g L^{-1} Tween 80 and 65.5 mmol L^{-1} $CaCl_2$ for 20 min to initiate the external Ca^{2+} cross-linking of the peripheral alginate layer. To break the emulsion, 45 mL of 0.05 mol L^{-1} $CaCl_2$ solution prepared in 0.1% peptone solution was added followed by transfer to a separatory funnel. The Ca^{2+} cross-linked alginate coated microspheres were collected and stored in 0.1% peptone solution at 4 °C until further analysis. For double layer coated beads, methods described in this section was repeated with mono layer coated beads.

2.4. Counts of entrapped cells

Freshly prepared beads (1 g), were liquefied in 99 mL of 1% (w/v) sterile sodium citrate (Merck, KGaA, Germany) solution at pH 6.0 by gently shaking at room temperature for 10 min. *L. acidophilus* and *L. rhamnosus* were enumerated in triplicates.

2.5. Physical examination of microcapsules

2.5.1. Particle size measurement

The size of the capsules was determined by using the laser diffraction particle size analyzer (SALD-2101 SHIMADZU, Japan). For the particle size analysis samples of individual treatments were dispersed in deionized water (purified by Milli Q Millipore USA, conductance 0.054 μs) and then directly placed into the module. The measurement of bead size was done immediately after sample preparation. The particle size distribution is calculated based on the light intensity distribution data of scattered light detected by a total of 81 sensor elements located behind the cuvette. It takes 0.145 s for a single detection cycle of light intensity signal from the all sensor elements. The particle diameter range were evaluated at room temperature. The particle diameter range was calculated as the best fit between the measured scattered pattern and that predicted by light scattering theory. All measurements were repeated in triplicate for each sample and results are reported as averages.

In all cases, mean value ± standard error was reported as microcapsules particle size. The mean value, was calculated from the following formula:

$$\text{Mean value} = 10^{\mu}$$

$$\mu = 1/100 \sum_{j=1}^{n} q_j \frac{\log_{10} x_j + \log_{10} x_{j+1}}{2}$$

$$\sigma = \sqrt{\left\{ \frac{1}{100} \sum_{j=1}^{n} q_j \left(\frac{\log_{10} x_j + \log_{10} x_{j+1}}{2} \right)^2 \right\} - \mu^2}$$

that x_j, q_j, and σ are particle diameter (μm), differential distribution (%), and standard error, respectively.

2.5.2. Morphology

The morphology of the microcapsules was observed using scanning electron microscope (SEM). The encapsulated samples were mounted on the stub with the aid of double side tape and coated by sputter coater (SC 7620, England) for 2 min using Au–Pd coat. Observations were made using the scanning electron microscope (LEO 1450 VP, Germany) at an accelerating voltage of 17.13 kV.

2.6. Viability of *L. acidophilus* PTCC1643 and *L. rhamnosus* PTCC1637 simulated gastric juice

The method described by Rao, Shiwnarain, and Maharaj (1989) was used. The freshly encapsulated (1 g) *L. acidophilus* and *L. rhamnosus* samples of individual treatments were completely dispersed in 10 mL of sterile simulated gastric juice (0.08 M HCl containing 0.2% NaCl, pH 1.5) without pepsin and incubated at 37 °C for 30, 60, 90, and 120 min. After incubation, the beads were removed and rinsed with 0.1% peptone solution at various time intervals. The viable cells of each bacterium were then enumerated in triplicates using method described in Section 2.4. and the counts were expressed as mean log cfu mL^{-1}.

For the free cells, 1.0 mL washed cell suspensions were inoculated into 10.0 mL of tempered 37 °C simulated gastric juice without pepsin, (Rao et al., 1989) with pH adjusted to 1.5 with HCl or NaOH. Triplicates samples were mixed well, incubated at 37 °C and sampled 30, 60, 90, and 120 min after addition of the bacteria. Surviving bacteria were enumerated by pour plate counts in MRS agar after incubation at 37 °C.

2.7. Survival of microencapsulated cells in simulated intestinal juice after incubation in simulated gastric juice

The method described by Krasaekoopt et al. (2004). The freshly encapsulated (1 g) *L. acidophilus* and *L. rhamnosus* samples of individual treatments were placed in 10 mL of simulated gastric juice (0.08 M HCl containing 0.2% NaCl, pH 1.5) without pepsin and incubated at 37 °C for 60 min. After incubation, the sampled capsules were neutralized with NaOH solution and then removed and placed in 9 mL of sterile simulated intestinal juice (0.05 M KH_2PO_4, pH 7.25) with 0.6% filter sterilized (0.2 μm, IWAKI, Japan) bile salt (ox gall; Sigma–Aldrich, Pool, UK). The tubes were then incubated at 37 °C for 30, 60, 90, and 120 min. After incubation, a 1.0 mL aliquot of dissolved beads of each bacterium was removed and enumerated in triplicates on MRS agar as described in Section 2.4.

2.8. Statistical analysis

A completely randomized factorial design was used for all analysis and all results were means of three replicates. MINITAB (Version 14, 2004) was used to conduct analysis of variance (ANOVA) to determine differences between treatments means. LSD tests method, at 5% level of significance was used to verify the significance of differences among treatments means.

3. Results and discussion

3.1. Number of cells entrapped and size of alginate beads

The initial cell population before encapsulation was in the range of 9.04–10.2 log cfu mL^{-1}. High cell entrapping in the range of 9.02–10.1 (log cfu g^{-1} beads) was achieved in both uncoated and coated beads. The results represented that there was no significant loss of viability for all strains and 99.8% of cells were successfully entrapped (Table 1,2). The beads were globular in shape (Fig. 1) and a thick skin around the strains was detectable. However, no significant difference in capsule shapes was observed with respect to the two different encapsulated probiotic strains and capsules produced with and without coating layers by a SEM.

The mean value of uncoated beads was 23.758 ± 0.161 μm, which was significantly ($P < 0.05$) lower than that of mono layer coated beads (47.543 ± 0.187 μm) and double layer coated beads (75.339 ± 0.209 μm) for all encapsulated probiotics. Furthermore, the uncoated capsules had the narrowest size distribution followed by mono and double layer coated capsules (Fig. 2). In the particle size distribution data graph, the horizontal axis represents particle diameter (μm) while the vertical axis represents the percent of the relative particle amount or volume fraction.

This is an improved technique to produce micron size beads rather than millimetre size produced by many researchers (Arnaud, Lacroix, & Choplin, 1992; Hyndman, Groboillot, & Poncelet, 1993) as it gives a smooth texture when the beads are incorporated into products. This agrees with Truelstrup Hansen et al. (2002) reported that very large calcium alginate beads (>1 mm) cause a coarseness of texture in live microbial feed supplements. Moreover, Koo, Cho, Huh, Baek, and Park (2001) and Annan, Borza, and Truelstrup Hansen (2008) reported that the shape and size of the beads were not changed when layer was added to alginate beads.

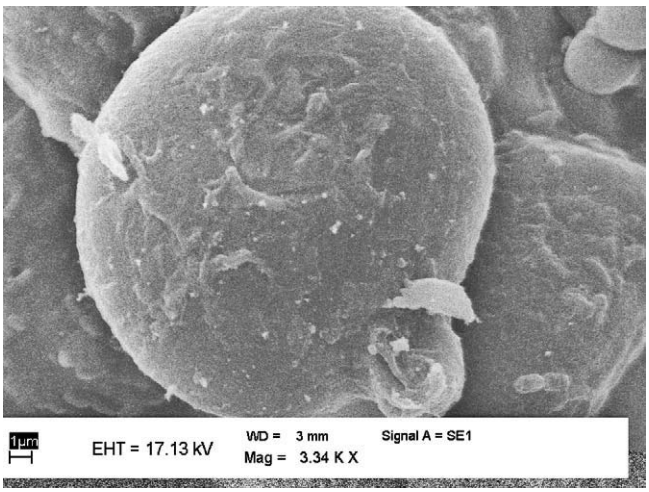

Fig. 1. Micrograph of the microcapsule containing *L. acidophilus PTCC1643* encapsulated in alginate.

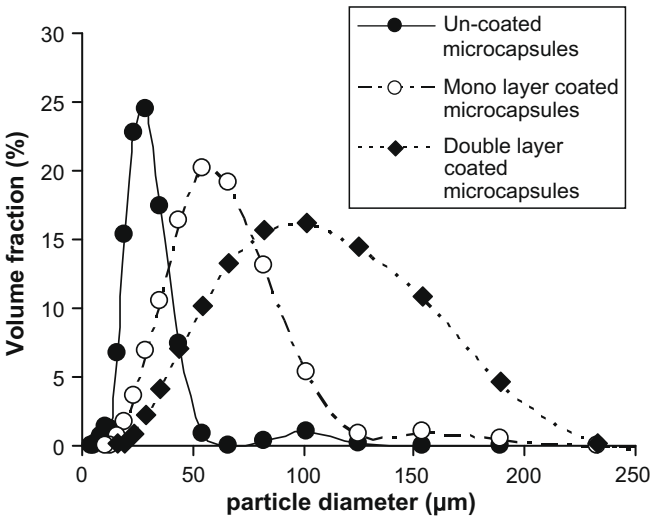

Fig. 2. Particle size analysis of microcapsules.

3.2. Survival of microencapsulated cells in simulated gastric juice

With the objective of improving viability of the strains during the exposure to the low pH of the stomach, HCl solution was used here to determine whether coating of the alginate beads would increase survival of cells in this environment, which is similar to that of the digestive system. The survivability of *L. acidophilus* and *L. rhamnosus* was expressed as the destructive value (*D*-value), which is the time required to destroy 90% or one log cycle of the organism (Table 1).

For *L. acidophilus*, with initial cell population in the range of $2.3 \pm 0.4 \times 10^9$–$3.6 \pm 0.1 \times 10^9$, the survival of cells in both coated and uncoated beads was significantly ($P < 0.05$) better than that of free cells. However, two layer coating (*D*-values 88.87 min) provided the best protection, followed by mono layer coating (*D*-values 67.79 min), uncoated (*D*-values 37.61 min), and free cells (*D*-values 23.66 min).

For *L. rhamnosus*, with initial cell counts in the range of $1.2 \pm 0.1 \times 10^9$–$8.2 \pm 0.1 \times 10^9$, double layer coating provided the best protection followed by mono layer coating and uncoated beads. The *D*-value of *L. rhamnosus* in double layer coated beads was lower than the *D*-value of mono layer beads. However, these two *D*-values were not significantly different. In all cases the uncoated alginate beads provided less protection than the coated beads (Table 1).

This result is in contrast to that of Sultana et al. (2000), who found that encapsulation of bacteria in alginate beads did not effectively protect the organisms from high acidity. But it is in agreement with (Kim et al., 2008) who reported that at pH 1.2, non-encapsulated *L. acidophilus* were completely destroyed after 1 h of incubation while encapsulated *L. acidophilus* maintained above 10^6 cfu mL^{-1} at pH 1.5 after 2 h. Favaro-Trindale and Grosso (2002) showed that none of *L. acidophilus* (La-05) survived in the artificial gastric environment of pH 1.0 after 1 h, but microencapsulated *L. acidophilus* (La-05) suffered a reduction of 1 log at pH 1.0 after 2 h incubation. Chandramouli, Kailasapathy, Peiris, and Jones (2004) reported a higher survival of *L. acidophilus* CSCC2400 and CSCC2409 immobilized in alginate bead in low pH environments. Moreover, Krasaekoopt et al. (2004) showed that for *L. acidophilus*, with initial cell counts in the range of 2.171–1.970×10^9, the survival of cells in coated beads was significantly ($P < 0.05$) better than that of uncoated beads.

Our results suggested that non-encapsulated *L. acidophilus* and *L. rhamnosus* were sensitive to the acidic environment (pH 1.5) and the ingestion of unprotected LAB might result in reduced via-

Table 1
Survived cells after exposure to pH 1.5 solution at different times (cfu g^{-1} bead).

Probiotics	Bead type	(min)					*D*-value (min)
		0	30	60	90	120	
Lactobacillus acidophilus	Free cell	$3.3 \pm 0.2 \times 10^9$	$1.6 \pm 0.2 \times 10^7$	$1.1 \pm 0.3 \times 10^6$	$1.1 \pm 0.05 \times 10^5$	$2.8 \pm 0.2 \times 10^4$	$23.66 \pm 2.47^{e\ A}$
	Uncoated	$3.6 \pm 0.3 \times 109$	$8.3 \pm 3.0 \times 10^8$	$4.1 \pm 0.2 \times 10^7$	$1.1 \pm 0.3 \times 10^6$	$2.3 \pm 0.2 \times 10^6$	37.61 ± 4.94^{d}
	Monolayer	$2.3 \pm 0.4 \times 10^9$	$1.8 \pm 0.3 \times 10^9$	$5.2 \pm 1.4 \times 10^8$	$1.3 \pm 0.6 \times 10^8$	$3.9 \pm 0.1 \times 10^7$	67.79 ± 17.52^{b}
	Double layer	$3.6 \pm 0.1 \times 10^9$	$3.2 \pm 0.1 \times 10^9$	$2.9 \pm 0.1 \times 10^9$	$9.7 \pm 0.2 \times 10^8$	$1.7 \pm 0.2 \times 10^8$	88.87 ± 1.34^{a}
Lactobacillus rhamnosus	Free cell	$1.5 \pm 0.3 \times 10^9$	$6.0 \pm 0.1 \times 10^7$	$1.8 \pm 0.7 \times 10^6$	$2.7 \pm 0.4 \times 10^4$	$1.6 \pm 0.4 \times 10^3$	20.1 ± 0.52^{e}
	Uncoated	$1.2 \pm 0.1 \times 10^9$	$2.4 \pm 0.3 \times 10^8$	$3.4 \pm 2.0 \times 10^7$	$4.4 \pm 0.4 \times 10^6$	$5.4 \pm 0.3 \times 10^5$	36.03 ± 0.36^{d}
	Monolayer	$7.3 \pm 0.4 \times 10^9$	$2.8 \pm 0.7 \times 10^9$	$7.0 \pm 1.0 \times 10^8$	$6.1 \pm 0.3 \times 10^7$	$2.9 \pm 2.0 \times 10^7$	50.1 ± 11.59^{bc}
	Double layer	$8.2 \pm 0.1 \times 10^9$	$5.4 \pm 0.8 \times 10^9$	$1.1 \pm 0.6 \times 10^9$	$4.7 \pm 3.3 \times 10^8$	$2.1 \pm 1.8 \times 10^7$	46.33 ± 7.35^{c}

Values are average ± standard error ($n = 3$).
[A] Values with the same letters are not significantly different.

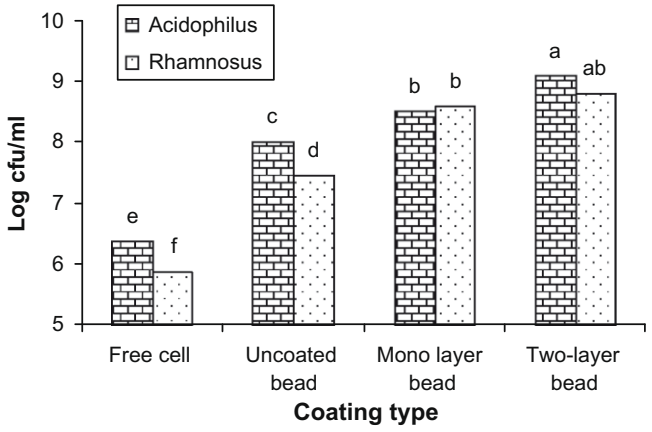

Fig. 3. Effect of coating on survival of strains under simulated gastric conditions. Values with the same letters are not significantly different ($P > 0.05$).

bility: 5 and 6 log reduction after 2 h for *L. acidophilus* and *L. rhamnosus*, respectively. According to this study the coating of beads provides the best protection in simulated gastric juice (Fig. 3) because a reduced pore size forms in the double layer membrane and as a result, the diffusion of gastric juice into the beads may be limited. This will protect encapsulated cells from interacting with the gastric juice (Murata, Toniwa, Miyamoto, & Kawashima, 1999).

3.3. Cells survival in simulated intestinal juice after incubation in simulated gastric juice

To determine the tolerance of the free and encapsulated strains to the acidic pH of the stomach, an *in vitro* system was utilized. The cultures were put into simulated gastric juice for 60 min, followed by a further incubation in intestinal juice with 0.6% bile salt for 30, 60, 90, and 120 min. The results are shown in Table 2.

The initial cell population of *L. acidophilus* was in the range of $4.6 \pm 0.5 \times 10^9$–$7.4 \pm 0.8 \times 10^9$ cfu mL^{-1}. As indicated by *D*-values microencapsulated cells in uncoated beads, mono layer beads and double layer coated survived better than free cells (Table 2).

However, the *D*-values of the cells in the mono layer coated, double layer coated and uncoated beads were not significantly different. These results indicate that alginate coating could not increase the survivability of encapsulated cells in such condition. In general the *D*-value of *L. acidophilus* incubated in simulated gastro-intestinal juice was lower than when it was incubated in simulated gastric juice without bile salt. This may be due to the fact that the environmental resistant of lactic acid bacteria is determined by many factors such as their medium and cytoplasmic membrane composition (Begley, Cormac, Gahan, & Hill, Colin, 2005). Therefore, the pH of the intestinal juice (7.25) may not be suitable for this organism as it grows and survives better in an acidic environment. Moreover, the use of different batches of encapsulated organisms for those two experiments may have contributed to the different tolerances observed. This concurs with the studies of Krasaekoopt et al. (2004) reported that microencapsulated cells survived better than free cells after sequential incubation in simulated gastric and intestinal juices, and alginate coating could not increase the survivability of encapsulated *L. acidophilus*. Kim et al. (2008) demonstrated micro encapsulation using alginate may be an effective way to increase the survival of *L. acidophilus* ATCC43121 in bile solution. Mituoka (1992) reported that *L. acidophilus* is most active in the small intestine and *B. bifidum* is most active in the large intestine of humans. Therefore, *L. acidophilus* should resist bile acid. It has been reported that *L. acidophilus*

Table 2
Average number (mean) cfu g^{-1} bead of survived cells and *D*-values of free and microencapsulated cells of *L. acidophilus* and *L. rhamnosus* after incubation in simulated gastric juice (60 min) and simulated intestinal juice (pH 7.25) at 37 °C for 2 h ($n = 3$).

Microorganism	Bead type	(min)					*D*-value (min)
		0	30	60	90	120	
Lactobacillus acidophilus	Free cell	$5.6 \pm 2.3 \times 10^9$	$3.3 \pm 2.2 \times 10^6$	$2.1 \pm 0.7 \times 10^5$	$3.4 \pm 1.1 \times 10^4$	$2.2 \pm 0.4 \times 10^2$	16.19 ± 0.43^{cA}
	Uncoated	$4.6 \pm 0.5 \times 10^9$	$2.3 \pm 0.6 \times 10^7$	$5.2 \pm 0.7 \times 10^6$	$5.6 \pm 0.4 \times 10^5$	$4.2 \pm 1.1 \times 10^5$	29.70 ± 3.56^{b}
	Monolayer	$5.7 \pm 0.8 \times 10^9$	$4.3 \pm 2.5 \times 10^8$	$3.0 \pm 1.3 \times 10^7$	$2.9 \pm 3.5 \times 10^6$	$7.3 \pm 0.7 \times 10^5$	30.84 ± 4.20^{b}
	Double layer	$7.4 \pm 0.8 \times 10^9$	$5.4 \pm 0.7 \times 10^9$	$3.5 \pm 1.0 \times 10^8$	$4.4 \pm 2.8 \times 10^7$	$3.4 \pm 0.9 \times 10^6$	36.03 ± 5.72^{b}
Lactobacillus rhamnosus	Free cell	$8.6 \pm 0.5 \times 10^9$	$7.1 \pm 2.1 \times 10^5$	$4.2 \pm 1.9 \times 10^4$	$7.5 \pm 0.7 \times 10^2$	$2.0 \pm 0.8 \times 10$	15.13 ± 1.24^{c}
	Uncoated	$7.9 \pm 0.5 \times 10^9$	$2.5 \pm 1.1 \times 10^8$	$3.4 \pm 0.9 \times 10^7$	$4.5 \pm 1.5 \times 10^6$	$5.4 \pm 0.9 \times 10^5$	28.77 ± 0.35^{c}
	Monolayer	$7.5 \pm 3.0 \times 10^9$	$3.8 \pm 0.5 \times 10^8$	$4.0 \pm 0.7 \times 10^7$	$3.1 \pm 1.7 \times 10^7$	$2.2 \pm 0.4 \times 10^5$	26.43 ± 6.25^{b}
	Double layer	$8.7 \pm 0.4 \times 10^9$	$5.3 \pm 1.9 \times 10^9$	$3.3 \pm 1.4 \times 10^8$	$2.5 \pm 0.5 \times 10^7$	$4.1 \pm 0.7 \times 10^7$	51.72 ± 9.65^{a}

Values are average ± standard error ($n = 3$).
[A] Values with the same letters are not significantly different.

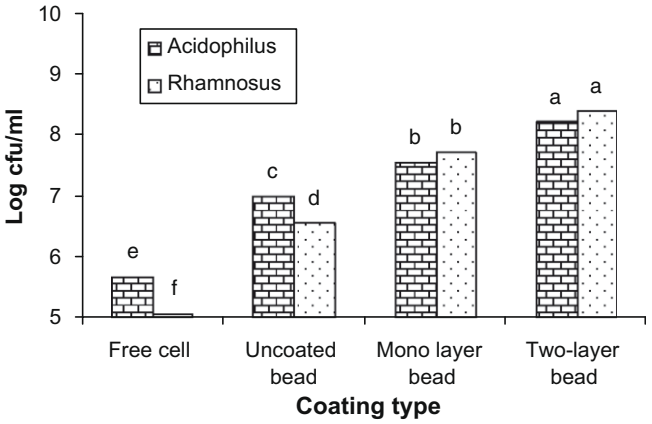

Fig. 4. Effect of coating on survival of strains after incubation in simulated gastric and intestinal juice. Values with the same letters are not significantly different ($P > 0.05$).

was significantly better with regard to bile tolerance than other cultures (Buck & Gilliland, 1994; Gilliland & Walker, 1990).

In the case of *L. rhamnosus*, the initial cell count was in the range of $7.5 \pm 3.0 \times 10^9$–$8.7 \pm 0.4 \times 10^9$. Double layer coated beads presented the best protection of cells (D-value = 51.72 min). There was very low survival of *L. rhamnosus* as free cells, after sequential incubation in simulated gastric juice and simulated intestinal juice due to its low acid and bile resistance. The cell numbers decreased to an undetectable level within 120 min. The cells in uncoated beads (D-value = 28.77 min) survived better than those in mono layer coated beads (D-value = 26.43 min) but not significantly.

For the survivability of *L. rhamnosus* encapsulated by double layer coated beads in gastric and intestinal juice, there was no significant differences from that of cells in simulated gastric juice. Thus, the presence of bile salt in the simulated intestinal juice affected the survival of this organism in all of the treatments except in double layer coated beads (data not reported).

However, our study indicated that these bacteria should be encapsulated within a particular size range (47.543–75.339 μm) (Fig. 4) and the encapsulated cells in mono layer and double layer coated beads were around 10^5–10^7-fold compared to free cells under gastro-intestinal conditions (Table 2).

These results are in agreement with Truelstrup Hansen et al. (2002) however, he reported that small beads of size less than 100 μm do not significantly protect the bacteria in simulated gastric fluid as compared to free cells. According to Anal and Singh (2007) the formation of a hydro gel barrier by the compacted sodium alginate layer was shown to retard the permeation of the gastric fluid into the cells. Many researchers have reported that HCl is more harmful to *L. rhamnosus*, than ox gall and exposing to acidic condition (pH 2.0 for 90 min) resulted in reduced viability of *L. rhamnosus*, by 2.0 to > 4.0 log cfu mL^{-1} depending on strain tested (Ding & Shah, 2007). On contrary, *L. rhamnosus*, showed to be very resistant to bile and its viability reduced by approximately 0.5 log cfu mL^{-1} after 90 min of exposure to ox gall (Kheadr, 2006).

It must be noted that the tolerance of strains in bile broth systems may not truly reflect their ability to tolerate bile *in vivo*. Like other physiological stresses, it is difficult to simulate exact *in vivo* conditions in a laboratory setting and all parameters that can affect survival are not taken into account. Conditions encountered in the external environment or in the host prior entry to the small intestine will determine the effects of bile on a strain. Exposure to various pHs, temperatures and growth atmospheres may either "harden" bacteria to the affects of bile or alternatively increase their susceptibility. Bile acid levels in the intestine are not constant and levels are relatively low until ingestion of a fatty meal. Pre-exposure of bacteria to these low levels may increase their tolerance to high levels. The presence of food in the intestine may also affect survival as bacteria may not be exposed to bile in certain microenvironments created by the food matrix or food constituents may even bind bile acids and prevent them from exerting toxicity (Begley et al., 2005). Finally, the *in vivo* antibacterial activity of bile may be lower than observed in broth systems as bile salts complexed in micelles with phospholipids may not be free to interact with bacterial cells.

4. Conclusions

This study compared the effect of different alginate beads type on the viability of *L. acidophilus* and *L. rhamnosus* during simulated gastro-intestinal transit. There appears to be much potential for using the prebiotic strains with alginate during encapsulation since it does enhance the survival of the probiotic bacteria. Our study has indicated that the survival of alginate immobilized bacteria may be dependent on the gel concentration and bead size. Negligible structural differences were detectable by SEM between the uncoated and coated beads with multi coating layers. Among three type of capsules applied in this research, double coating provided the best protection of cells. Further studies need to be carried out in order to monitor the effect of micro encapsulation on bacteria in the gut, using cells animal models, as well as studying other parameters such as the initial cell numbers and cell type.

Several parameters may determine the extent to which probiotic strains survive passage through the gastro-intestinal tract, *viz*., the degree of stomach acidity and the period of exposure, as well as the concentration and the period of exposure to bile salts. However, *in vivo* studies are still necessary to fully validate all previous *in vitro* studies (including our study), *viz*., microbiological analyzes of faecal samples after feeding of the inoculated products are required, as other factors (e.g. pancreatic juice) also play a role. Before reaching the intestine, probiotic bacteria must first survive the deleterious action of gastric juice during passage through the stomach. In general, the acid tolerance of lactic acid bacteria depends on the pH profile of H^+-ATPase and on the composition of the cytoplasmic membrane which is largely influenced by the type of bacterium, the type of growth medium and the incubation conditions.

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