Carbohydrate Polymers 90 (2012) 1281–1287

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Chitosan coated alginate beads for the survival of microencapsulated *Lactobacillus plantarum* in pomegranate juice

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ARTICLE INFO

Article history:
Received 4 May 2012
Received in revised form 15 June 2012
Accepted 23 June 2012
Available online 1 July 2012

Keywords:
Encapsulation
Alginate beads
Chitosan
Multi-layer coating
Pomegranate juice
Lactobacillus plantarum

ABSTRACT

This work studied the effect of multi-layer coating of alginate beads on the survival of encapsulated *Lactobacillus plantarum* in simulated gastric solution and during storage in pomegranate juice at 4 °C. Uncoated, single and double chitosan coated beads were examined. The survival of the cells in simulated gastric solution (pH 1.5) was improved in the case of the chitosan coated beads by 0.5–2 logs compared to the uncoated beads. The cell concentration in pomegranate juice after six weeks of storage was higher than 5.5 log CFU/mL for single and double coated beads, whereas for free cells and uncoated beads the cells died after 4 weeks of storage. In simulated gastric solution, the size of the beads decreased and their hardness increased with time; however, the opposite trend was observed for pomegranate juice, indicating that there is no correlation between cell survival and the hardness of the beads.

1. Introduction

The viability of probiotic bacteria in food products used as delivery vehicles is of major concern because a significant number of bacterial cells die during processing and storage of the food products, and subsequently during passage through the gastrointestinal tract (Champagne, Gardner, & Roy, 2005; Gueimonde et al., 2004; Shah, 2000; Vinderola, Bailo, & Reinhemier, 2000). In order to exert their health benefits, and taking into account the fact that the minimum effective dose is suggested to be 10^8–10^9 cells per day (Champagne et al., 2005; Kailasapathy & Chin, 2000), the minimum concentration of live probiotic should be at least 10^6–10^7 CFU/g at the time of consumption.

A methodology that has been used to improve probiotic survival in various food products, including dairy and non-dairy products, is encapsulation (Heidebach, Forst, & Kulozik, 2012; Homayouni, Azizi, Ehsani, Yarmand, & Razavi, 2008; Krasaekoopt, Bhandari, & Deeth, 2003; Ozer, Kirmaci, Senel, Atamer, & Hayaloglu, 2009). Various polymers can be used as encapsulation materials including alginate, pectin, k-carrageenan, xanthan gum, gellan gum, starch derivatives, cellulose acetate phthalate, casein, whey proteins and gelatin; among these alginate is the most studied encapsulation material. Alginate is an anionic copolymer of 1,4-linked-β-D-mannuronic acid and α-L-guluronic acid residues which forms a gel through the cross-linking of the guluronic acid blocks by the calcium ions, resulting in an a "egg-box" structure (Gombotz & Wee, 1998). The concentration of alginate commonly used to form the gel ranges from 0.6% to 3%, whereas that of calcium chloride from 0.05 to 1.5 M (Jankowski, Zielinska, & Wysakowska, 1997; Krasaekoopt et al., 2003; Sankalia, Mashru, Sankalia, & Sutariya, 2007; Takka & Gurel, 2010). The size of the produced beads depends mainly on the syringe size, the vibration of the system, the viscosity of the alginate solution and the distance between the syringe and the calcium chloride solution (Krasaekoopt et al., 2003).

Chitosan, the partly acetylated (1-4)-2-amino-2-deoxy-β-D-glucan obtained from chitin (Muzzarelli et al., 2012), has been used as a coating material for alginate beads, and has been shown to shown to increase the survival of probiotics in simulated gastric and intestinal juices compared to uncoated alginate beads (Chavarri et al., 2010; Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2011). The negatively charged alginate forms a semi-permeable membrane with the positively charged chitosan and as a result the capsule has a smoother surface and is less permeable to water soluble molecules (Koo, Cho, Huh, Baek, & Park, 2011; Krasaekoopt, Bhandari, & Deeth, 2004).

Although dairy products are the main types of foods used as probiotic carriers, fruit juices offer an alternative, as they have a large consumer market and are also suitable for consumers that

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http://dx.doi.org/10.1016/j.carbpol.2012.06.073

suffer from lactose intolerance (Ding & Shah, 2008). The survival of probiotics, such as lactobacilli and bifidobacteria, has already been studied in a variety of fruit juices during refrigerated storage. It was shown that the free cells survived well during storage for up to 6 weeks (cell decrease <1 log) in certain fruit juices, such as orange, apple, grapefruit, blackcurrant, pineapple and lemon juices (Champagne, Raymond, & Gagnon, 2008; Ding & Shah, 2008; Nualkaekul & Charalampopoulos, 2011; Nualkaekul, Salmeron, & Charalampopoulos, 2011, Saarela, Virkajarvi, Nohynek, Vaari, & Matto, 2006; Sheehan, Ross, & Fitzgerald, 2007). In other juices however, such as pomegranate, strawberry and cranberry, the probiotic cells died very quickly, within 1–4 weeks. The reason for this was most likely the very low pH of these juices ($pH \leq 3$) and the high total phenol concentration (Nualkaekul & Charalampopoulos, 2011; Nualkaekul et al., 2011).

The aim of this study was to investigate the potential protective effect of uncoated as well as single and double chitosan coated alginate beads on the survival of a model *Lactobacillus plantarum* strain in simulated gastric solution and in pomegranate juice, a highly acidic juice. The objectives were to study the effect of coating on the size and hardness of the beads and understand the behaviour of the beads in the two systems and how physical characteristics relate to the cell survival data. This is to our knowledge is the first study investigating multi-layer coating of alginate beads applied in probiotic containing acidic foods.

2. Materials and methods

2.1. Materials

L. plantarum NCIMB 8826 isolated from human saliva was purchased from the National Collection of Industrial and Marine Bacteria (NCIMB, UK). Sodium alginate (viscosity: 15–20 cP, 1% in H_2O [L]; ratio of mannuronic acid:guluronic acid: 3.3 ± 0.3 (Wright et al., 2012), low molecular weight chitosan (103 kDa), calcium chloride dihydrate and hydrochloric acid (37%) were obtained from Sigma, UK. Pomegranate juice was purchased from a local supermarket (PomeGreat PurePlus™, total sugar: 11.1 g/100 mL; protein: 0.1 g/100 mL, according to label). The total phenol concentration was 4.3 g/L and the citric acid concentration was 6.7 g/L, measured previously (Nualkaekul & Charalampopoulos, 2011). MRS broth, MRS agar and phosphate buffer saline (PBS) was purchased from Oxoid, UK. Concentrated HNO_3 was obtained from BDH, UK. Lanthanum chloride solution (10%, w/v) and calcium standard solution (1000 ppm in 1 M nitric acid) were obtained from Fisher Scientific, UK.

2.2. Preparation of culture

The *L. plantarum* strain was preserved in 2 mL-cryovials containing 40% glycerol, stored at −80 °C. The cells were cultivated at 200 rpm, 37 °C, in 100 mL of MRS medium for 16 h. The cells were harvested by centrifugation at $3200 \times g$ for 15 min. The pellets were washed once with 0.1 M phosphate buffer saline (PBS) and were resuspended in 10 mL of PBS. The cell concentration in the final PBS/cell suspension was approximately 3×10^{10} CFU/mL.

2.3. Preparation of alginate beads

The extrusion technique was used for the preparation of the alginate beads as described previously (Cook et al., 2011). Briefly, 1 mL of cell suspension was mixed with 9 mL of sterile (121 °C, 15 min) 3% (w/v) sodium alginate solution. The cell suspension/alginate mixture (1 mL) was extruded through a 0.8 mm diameter needle into sterile 0.15 M $CaCl_2$ (10 mL). Using this procedure, 66–68 beads were produced each time, corresponding to 35–42 mg (dried weight). The amount of cells per bead ranged between 7 and 9×10^{10} cells/g of dried bead. The beads were allowed to stand for 30 min to harden and were then harvested using a sieve.

2.4. Coating of beads with chitosan

The chitosan solution was prepared by dissolving 4 g of chitosan in 950 mL of 0.1 M acetic acid; the pH was adjusted to 6.0 with 1 M NaOH. The mixture was adjusted to 1 L by distilled water and filtered through a Whatman #4 filter paper. The suspension was then heated at 72 °C for 30 s on a hot plate and immediately put on ice to cool down; it was subsequently used for coating.

Single coated beads were prepared by adding the beads produced as described in Section 2.3 into 7 mL of chitosan solution; the suspension was mixed at 100 rpm for 10 min using an orbital shaker to speed up the coating process. The single coated beads were harvested and washed with PBS before use.

For double coated beads (alginate–chitosan–alginate–chitosan beads), after coating the alginate beads with the first layer of chitosan, they were harvested and added into 10 mL of sterile sodium alginate solution (0.17%, w/v) and mixed for 10 min at 100 rpm. The beads were then harvested and were added into 7 mL of chitosan solution. The suspension was once more mixed at 100 rpm for 10 min using an orbital shaker, and the beads harvested and washed with PBS before use.

2.5. Size and texture of beads

The diameters of 10 randomly selected beads from each experiment were measured using a micrometre (Fowler IP54, USA). A texture analyser (Brookfield CT3, UK) was used to measure the hardness of the beads using a 35 mm diameter cylindrical clear acrylic probe and a speed of 0.1 mm s^{-1}; the analysis was conducted in compression mode, with the distance set at 50% of the diameter of the bead. The peak force was measured in grams. Nine beads were used for each measurement, and five replicates were carried out per sample.

2.6. Cell survival in simulated gastric solution

The simulated gastric solution consisted of distilled water with its pH adjusted to 1.5 by 5 M HCl; the solution was filter-sterilised using a 0.2 μm filter. The assay was initiated by transferring the produced beads (uncoated, single and double coated) to 9 mL of simulated gastric solution; the initial cell concentration was approximately 3×10^8 CFU/mL. The suspension was incubated at 37 °C; samples were collected at 0, 1, 2, 3 and 4 h and analysed for viable cell counts as described in Section 2.8. The survival experiments were conducted in duplicate for each condition using different inoculums.

2.7. Cell survival in pomegranate juice

The beads (uncoated, single and double coated beads), produced as described in Section 2.4, were added into 9 mL of pomegranate juice; the initial cell concentration in the juice was approximately 3×10^8 CFU/mL. The juices were stored at 4 °C for 6 weeks. The survival experiments were conducted in duplicate for each condition using different inoculums.

2.8. Bacterial enumeration

The plate count method was used to determine the number of viable bacterial cells. The 9 mL of simulated gastric solution or pomegranate juice containing either uncoated or coated beads were blended with 90 mL of PBS in a Seward stomacher, model 400C

Table 1
Survival of *L. plantarum* (cell concentration is expressed as log CFU/mL) encapsulated within uncoated and single and double chitosan coated alginate beads in simulated gastric solution (pH 1.5) during incubation at 37 °C for up to 4 h. Results are presented as mean ± standard deviation ($n = 6$).

Type of beads	0 h	1 h	2 h	3 h	4 h
Free cells (control)	8.3 ± 0.0	X	X	X	X
Uncoated	8.3 ± 0.1	6.3 ± 0.2	4.5 ± 0.1	3.4 ± 0.2	3.8 ± 0.2
Single coated	8.3 ± 0.1	7.1 ± 0.0	5.8 ± 0.3	5.1 ± 0.1	4.0 ± 0.1
Double coated	8.4 ± 0.1	7.1 ± 0.1	6.1 ± 0.1	5.8 ± 0.4	5.2 ± 0.4

X indicates cell concentration < 100 CFU/mL (i.e. 2 logs).

(Seward, UK) at 300 rpm, for 10 min. The suspension was then serially diluted in PBS; 100 μL of the appropriate dilution was spread onto MRS agar in triplicate. The plates were incubated aerobically at 37 °C for about 3 days, after which they were counted and expressed as CFU/mL.

2.9. Microscopic analysis of the beads

The beads were separated from the juice by sieving and were placed on a glass slide intact or a cross-section of them. A Leica EZ4 stereo microscope (Leica, UK) was used for observing the beads; a 35X magnification was used for visualising the cross-section of the beads and a 10× magnification for the whole bead.

2.10. Calcium analysis

The calcium content of coated and uncoated beads and pomegranate juice samples was analysed by atomic absorption spectrophotometry, according to the AOAC 991.25 method. More specifically, the beads or 9 mL of pomegranate juice were placed on a silica dish and dried in an oven (Memmert, UK) at 105 °C overnight, and then burned in a furnace (Carbolite, UK) at 525 °C overnight; the samples were subsequently cooled in a dessicator. The ash was dissolved by adding 1 mL concentrated HNO_3 into the silica dish, and then the dissolved ash was diluted up to 100 mL with distilled water and filtered through a no. 1 filter paper. Forty millilitres of this solution were then transferred to a 100 mL volumetric flask and 1 mL lanthanum chloride solution was added; the solution was then diluted to 100 mL with distilled water. The absorbance of the unknown samples was measured by an atomic absorption spectrophotometer novAA® 350 (Analytik Jena, UK) at 422.7 nm, and the calcium concentration in the samples was determined using a calibration curve prepared using a calcium standard metal solution in the range of 0–10 ppm. The R^2 value of the calibration curve was 0.999.

2.11. Statistical analysis

The results are presented as mean ± standard deviation. Comparisons between the various sets of data were carried by ANOVA and *t*-test using the SPSS statistics program (Ver. 16, USA). A *P*-value below 0.05 (presented as $P < 0.05$) was considered statistically significant.

3. Results and discussion

3.1. Cell survival in simulated gastric solution

The cell concentration of *L. plantarum* over a 4 h incubation period in simulated gastric solution (pH 1.5) is presented in Table 1. It can be observed that the encapsulation of the cells in alginate improved considerably cell viability compared to the free cells; the latter decreased to less than 2 log CFU/mL within 1 h. This is in accordance with several studies on probiotics, although the level of protection usually depends on the species and strain too (Brinques & Ayub, 2010; Ding & Shah, 2009). The alginate used comprised a high mannuronic acid percentage (77%) and a low guluronic acid percentage (23%). The M:G acid ratio can potentially be manipulated to increase the permeability of gels as necessary to improve the viability of encapsulated cells. High mannuronic acid alginates are however more biocompatible and clinically useful than high guluronic acid alginates.

Compared to the alginate encapsulated cells, single chitosan coating improved cell survival by about 0.5–1 log, whereas double chitosan coating by about 1.5 log. The protection offered by chitosan is most likely a result of the strong binding of chitosan to alginate beads through electrostatic interactions. This leads to the formation of a strong membrane on the surface of the beads, which reduces the likelihood of leakage of the entrapped materials (Gaserod, Smidsrod, & Skjak-Braek, 1998). It was previously shown that at pH ~ 2, similar to the pH that the assay was performed at in this study, the stability of the alginate–chitosan beads during short storage was at its maximum (Cook et al., 2011; Huguet, Neufeld, & Dellacherie, 1996). Moreover, the protective effect could be due to the fact that the polysaccharides might be acting as a buffer against acids as they penetrate into the bead by binding to the protons, thus raising the pH inside the matrix to a level which is less harmful for the bacteria. The level of protection identified in this study with the single chitosan coat was similar to that observed in previous studies with *L. acidophilus* and *L. casei* in a simulated gastric solution at pH 1.55 (Krasaekoopt et al., 2004), with *L. bulgaricus* at pH 2 (Lee, Cha, & Park, 2004), and with *Bifidobacterium breve* using the same gastric model solution (Cook et al., 2011). In terms of the double chitosan-coated alginate beads, this is the first study that uses this approach for the protection of probiotics with the view to enhance their survival, and demonstrates a significant improvement compared to the single coated beads. In the only similar study found, double layer alginate chitosan beads were produced to entrap *Saccharomyces* and study their fermentation activity; however the system used was different as it involved adding the chitosan into the gelling solution (Liouni, Drichoutis, & Nerantzis, 2008). A recent study also demonstrated the effectiveness of multiple coating (up to six layers) for the protection of probiotics (*L. acidophilus*), although the approach involved the direct adsorption of polyelectolytes (chitosan and carboxymethyl cellulose) on the surface of the cells (Priya, Vijayalakshmi, & Raichui, 2011) rather than the formation of a gel, which is subsequently coated.

3.2. Physical characteristics of beads in simulated gastric solution

The size of the produced single coated and double coated beads were slightly higher compared to the uncoated beads (between 0.1 and 0.2 mm) (Table 2), which is in agreement with our previous report, in which the chitosan coat thickness was found to be <10 μm, measure using fluorescently labelled chitosan and confocal microscopy (Cook et al., 2011). Fig. 1, which depicts cross section images of the beads, demonstrates that in the case of the coated beads, a much thicker and denser outer membrane was produced, most likely representing the alginate/chitosan layers. Upon

Table 2
Changes in the size and hardness of uncoated and single and double chitosan coated alginate beads during exposure to simulated gastric solution for 4 h (pH 1.5). Results are expressed as mean ± standard deviation ($n = 20$, size), ($n = 90$, hardness).

Type of beads	Control[a]	0 h	2 h	4 h
Size of beads (mm)				
Uncoated	2.9 ± 0.1	2.7 ± 0.1	2.6 ± 0.1*	2.6 ± 0.1*
Single coated	3.0 ± 0.1	3.0 ± 0.1	2.9 ± 0.1*	2.8 ± 0.1*
Double coated	3.1 ± 0.1	3.1 ± 0.1	3.1 ± 0.1	3.0 ± 0.1
Texture of beads – hardness (g)				
Uncoated	169.6 ± 3.6	195.7 ± 14.4	201.0 ± 16.4	212.3 ± 17.2*
Single coated	156.7 ± 6.4	173.9 ± 18.7	187.2 ± 12.4*	189.3 ± 15.0*
Double coated	107.1 ± 11.9	121.9 ± 18.4	146.1 ± 16.3*	148.2 ± 16.1*

* Significantly different to the 0 h sample at $P < 0.05$.
[a] The control beads were measured immediately after production.

introduction of the uncoated beads to the simulated gastric solution, their size significantly ($P < 0.05$) decreased by about 0.2 mm, and continued to decrease slightly for up to 4 h. In the case of the single coated beads, their size decreased gradually with the time of exposure whereas the size of the double coated beads was not significantly ($P > 0.05$) affected.

The hardness of the beads is an important physical property as it is likely to influence the stability of the beads during short and long term storage (Gaserod, Sannes, & Skjak-Braek, 1999) and potentially within a food product. No significant differences were observed between the uncoated alginate beads and the single chitosan coated beads, which is in accordance with the study of Krasaekoopt et al. (2004). However, the double chitosan coated beads were significantly ($P < 0.05$) less hard than the single coated beads indicating that the polymer matrix was less compact in the former case. This can be attributed to a decrease in the internal Ca^{2+} cross-linking, as indicated also by the lower calcium concentration of the coated beads (Table 6), and to the loose interactions between alginate and chitosan. Upon introduction of the beads to the simulated gastric solution and during the 4 h exposure the hardness increased significantly ($P < 0.05$) with time, in all cases. This was most likely due to the fact that alginate has an acid gel character below the pK_a of the acidic saccharide residues (~3.5) comprising it; this was most likely affecting the hardness of both uncoated and coated alginate beads. Due to this, in previous studies very little swelling of dried alginate beads was seen at pH below 2 (Cook et al., 2011), whereas the levels of alginate leaching out of the gel beads were very low, indicating an increased stability for these particular beads (Draget, SkjakBraek, Christensen, Gaserod, & Smidsrod, 1996).

In order to understand the effect of pH on the size and hardness, the beads were suspended in solutions of different pH (from pH 1 to 8) for 1 h and the size and hardness of the beads measured after exposure (Fig. 2). For all three types of beads a considerable decrease in size and hardness was observed as the pH increased from 1 to 3, whereas the rate of decrease was lower as the pH increased from 3 to 8. These observations further support the acid gel hypothesis. However, it is important to note that the survival data from the experiment with the simulated gastric juice demonstrated that the softer beads gave nevertheless better protection to the cells, highlighting the importance of the chitosan coat in providing additional protection compared to the uncoated beads.

3.3. Cell survival in pomegranate juice during refrigerated storage

Table 3 shows the cell survival data during storage of the free and encapsulated cells in pomegranate juice. The cell concentration decreased with a similar rate for the free cells and the uncoated beads; in both cases after 4 weeks all cells died. In contrast, the rate of decrease in cell concentration was much lower in the case of the single and double coated beads. In both cases, after six weeks of storage the cell concentration in the juice was higher than 5.5 log CFU/mL, with the double coated beads being slightly more protective. The significant decrease in the viability of free cells that was observed during their storage in pomegranate juice was most likely associated with the very low pH of the juice (pH ~ 3.2) and its high content in total phenol (~4.3 g/L), which was much higher than the concentrations reported for other juices, such as orange, grapefruit and blackcurrant (Nualkaekul et al., 2011). Pomegranate juice contains considerable amounts of polyphenols, including punicalagins, ellagic acid and punicalins, which have been shown to inhibit the growth of pathogenic clostridia and *Staphyloccocus aureus*; however, lactobacilli and bifidobacteria were generally not affected by ellagitannins, although this was species and tannin dependent (Bialonska, Kasimsetty, Schrader, & Ferreira, 2009).

The increased protection by chitosan coating was initially considered to be associated with the increase in the pH of the juice in the case of the coated beads; as the pH of the double coated and single coated alginate beads increased from 3.2 to 3.6 and 3.4, respectively (Table 5). The increases in pH were most likely

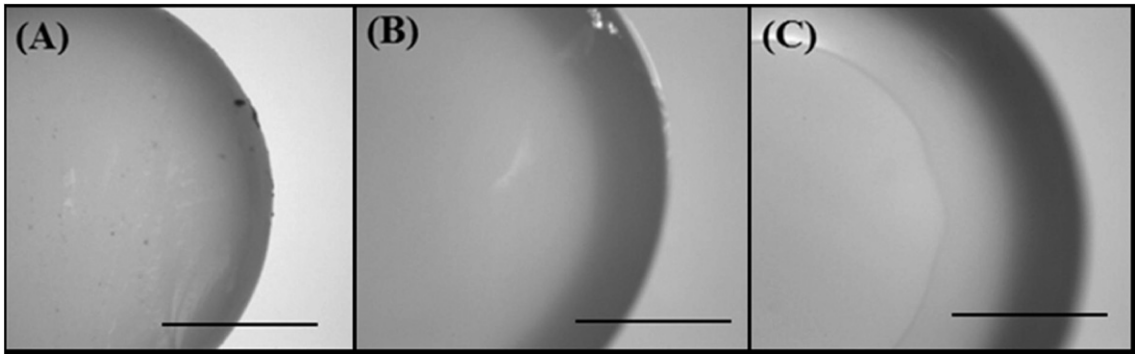

Fig. 1. Cross-section of uncoated (A), single chitosan coated (B) and double chitosan coated (C) alginate beads. A 35× magnification was used for visualising the cross-sections, using a Leica EZ4 stereomicroscope. The scale bar represents 1 mm.

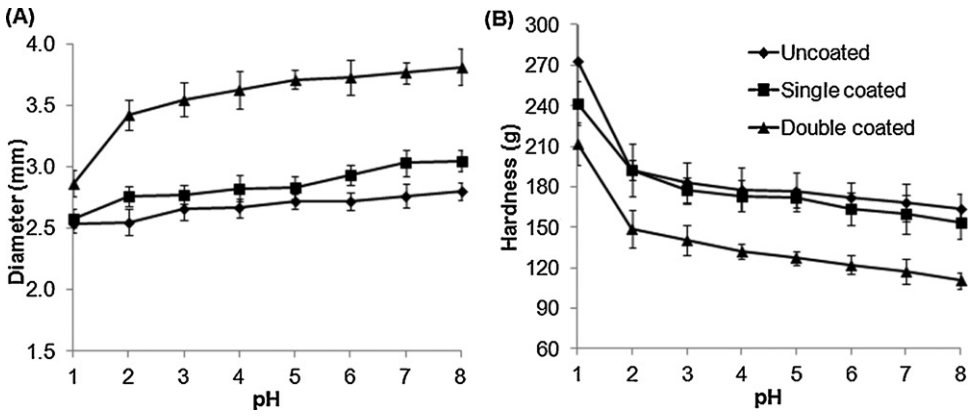

Fig. 2. Size (A) and hardness (B) of uncoated, single chitosan coated and double chitosan coated beads after incubation for 1 h (37 °C) in water solutions adjusted to different pH (pH 1–8). The error bars represent standard deviation (n = 20, size), (n = 90, hardness).

Table 3
Survival of *L. plantarum* (cell concentration is expressed as log CFU/mL) encapsulated within uncoated and single and double chitosan coated alginate beads during 6 weeks of storage in pomegranate juice at 4 °C. Results are expressed as mean ± standard deviation (n = 6).

Sample	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	$[\log_{10}N_{0\,week} - \log_{10}N_{6\,week}]$
Free cells	8.6 ± 0.1	8.3 ± 0.1*	7.9 ± 0.2*	6.5 ± 0.1*	X	X	X	8.6
Uncoated	8.6 ± 0.1	8.3 ± 0.1*	8.0 ± 0.2*	6.5 ± 0.5*	X	X	X	8.6
Single coated	8.6 ± 0.2	8.6 ± 0.1	8.3 ± 0.1	7.9 ± 0.3*	6.1 ± 0.2*	6.0 ± 0.1*	5.7 ± 0.1*	2.9
Double coated	8.6 ± 0.1	8.6 ± 0.1	8.4 ± 0.1	8.3 ± 0.2	7.2 ± 0.1*	6.7 ± 0.1*	6.6 ± 0.1*	2

X indicates cell concentration < 100 CFU/mL (i.e. 2 logs).
* Significantly different to Week 0 sample at $P < 0.05$.

Table 4
Changes in the size and hardness of uncoated and single and double chitosan coated alginate beads during storage for 6 weeks in pomegranate juice at 4 °C. Results are expressed as mean ± standard deviation (n = 20, size), (n = 90, hardness).

Type of beads	Control[a]	Week 0	Week 3	Week 6
Size of beads (mm)				
Uncoated	2.9 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.6 ± 0.2
Single coated	3.0 ± 0.1	2.7 ± 0.1	2.9 ± 0.1*	3.1 ± 0.1*
Double coated	3.1 ± 0.1	3.0 ± 0.1	3.1 ± 0.1	3.5 ± 0.2*
Texture of beads – hardness (g)				
Uncoated	168.8 ± 4.1	198.4 ± 2.9	173.6 ± 5.0*	141.4 ± 5.6*
Single coated	155.0 ± 8.2	180.8 ± 9.2	91.6 ± 1.5*	65.4 ± 3.2*
Double coated	96.6 ± 3.6	94.4 ± 8.3	45.2 ± 2.4*	36.0 ± 1.3*

* Significantly different to the week 0 sample at $P < 0.05$.
[a] The control beads were measured immediately after production.

due to the fact that chitosan is soluble in various organic acids, such as citric, malic and succinic (Romanazzi, Gabler, Margosan, Mackey, & Smilanick, 2009), which were all shown to be present in pomegranate (Hasnaoui et al., 2011; Poyrazoglu, Gokmen, & Artik, 2002). In order to elucidate the pH effect, an experiment was conducted whereby the pH of the pomegranate juice was adjusted to 3.4 and 3.6, and the cell viability monitored for up to 6 weeks. In all cases the cells died within 4 weeks of storage suggesting that the raised pH was not the reason for the increased protection by the single and double chitosan coated beads (data not shown). The enhanced protection therefore in the case of the single and double coated beads compared to the uncoated beads was most likely due to the increased ability of the polyelectrolyte matrix to buffer acidic compounds as they penetrate into the beads, which was probably reflected by the thicker membrane observed in the images of the beads (Fig. 1).

3.4. Physical characteristics of beads in pomegranate juice during storage

The size of the different type of beads and their hardness during storage in pomegranate juice is shown in Table 4. The size of uncoated and single coated beads significantly ($P < 0.05$) decreased by approximately 0.3 mm, whereas the hardness increased upon introduction into the juice. This could be due to acid gel formation at the low pH of the juice, as in the case of the beads in the simulated

Table 5
Changes in the pH of the simulated gastric solution and pomegranate juice upon introduction of uncoated and single and double chitosan coated alginate beads during storage for up to 4 h (37 °C) and 6 weeks (4 °C), respectively.

Type of beads	pH of simulated gastric solution			pH of pomegranate juice		
	0 h	2 h	4 h	Week 0	Week 3	Week 6
Uncoated	1.59 ± 0.02	1.57 ± 0.01	1.58 ± 0.01	3.21 ± 0.02	3.19 ± 0.01	3.20 ± 0.01
Single coated	1.67 ± 0.01	1.67 ± 0.01	1.68 ± 0.01	3.46 ± 0.03	3.43 ± 0.01	3.45 ± 0.01
Double coated	1.78 ± 0.01	1.78 ± 0.01	1.78 ± 0.01	3.62 ± 0.02	3.64 ± 0.01	3.63 ± 0.01

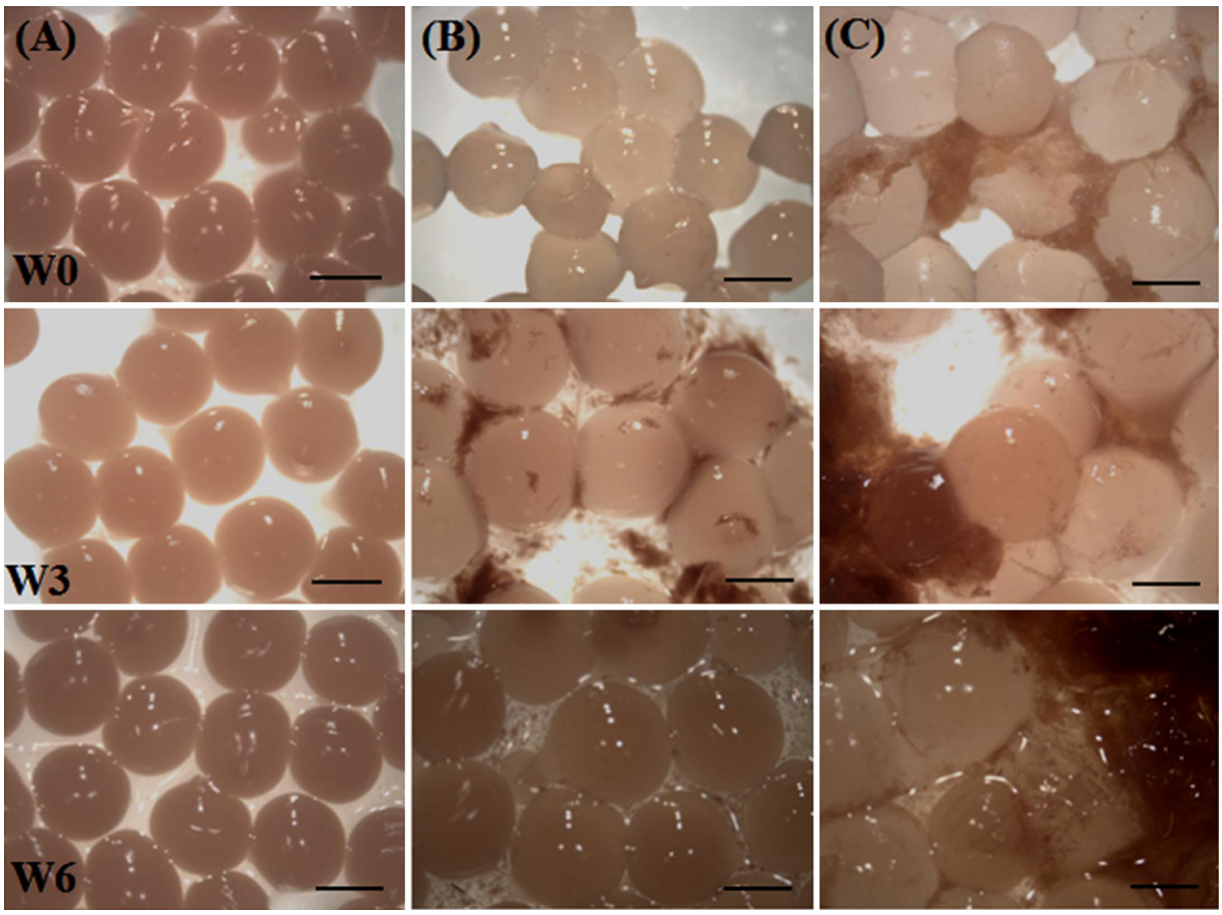

Fig. 3. Images of uncoated (A), single chitosan coated (B) and double chitosan coated (C) alginate beads harvested from pomegranate juice at week 0 (W0), week 3 (W3) and week 6 (W6) of storage at 4 °C. A 10× magnification was used for visualising the beads, using a Leica EZ4 stereomicroscope. The scale bar represents 2 mm.

gastric solution, and/or due to the high osmotic pressure resulting from the high concentration of total sugar (111 g/L; according to the label); this could have led to the expulsion of water from the beads, causing them to shrink gradually.

In contrast to the trend observed in the experiment with the simulated gastric solution (Table 2), storage of single coated and double coated beads in pomegranate juice resulted in a significant ($P < 0.05$) increase in size and a decrease in hardness, from week 0 to week 6 (Table 4); especially the double coated beads were very soft. The most likely reason for these observations was that a chelating agent sequestered the calcium ions resulting in their exchange with non-gelling monovalent ions, such as sodium and potassium, which were present in the juice. This was also indicated by the results of the Ca^{2+} analyses in the beads and pomegranate juice, which are shown in Table 6. The uncoated, single coated and double coated beads had a calcium concentration of approximately 57, 53 and 25 mg/100 mg of dried beads, respectively. After adding the beads to the juice, the calcium concentration of the three beads ranged from 18.5 to 22.6 mg/100 mg, indicating that the calcium from the beads was released into the juice. This consequently led to increased swelling inside the beads and to the progressive increase in bead size with time, and probably to the destabilisation of the capsule membranes and the presence of deposit on the surface (most likely alginate). Such deposits were seen in the images of

Table 6
Concentration of calcium in the produced uncoated and single and double chitosan coated alginate beads (control beads) and in the beads harvested from pomegranate juice at week 0 and at week 6 of storage. Results are expressed as mean ± standard deviation ($n = 12$).

Sample	Concentration of calcium in samples (mg/100 mg dried beads)	
Control beads		
Uncoated alginate beads	57.0 ± 3.4	
Single chitosan coated alginate beads	53.1 ± 1.0	
Double chitosan coated alginate beads	24.5 ± 4.5	
Sample	**Concentration of calcium in samples (mg/100 mg dried beads)**	
	Week 0	**Week 6**
In juice		
Uncoated beads in pomegranate juice	22.6 ± 5.5	15.2 ± 1.0
Single coated beads in pomegranate juice	23.6 ± 5.3	22.6 ± 4.2
Double coated beads in pomegranate juice	18.5 ± 2.5	13.2 ± 1.9
Pomegranate juice	15.9 ± 0.3 mg/100 mL of juice	

the double chitosan coated beads after 3 and 6 weeks of storage (Fig. 3B and C, respectively). This mechanism has been demonstrated to take place in phosphate solutions for alginate–polycation microcapsules (Thu et al., 1996) and alginate–chitosan microcapsules (Gaserod et al., 1999). Compositional analysis indicated that pomegranate contained about 6.7 g/L of citrate (Nualkaekul & Charalampopoulos, 2011) as well as small amounts of sodium and potassium (data not shown), and therefore it is likely that such a mechanism took place in pomegranate juice, thus explaining the differences observed compared to the experiment with the simulated gastric solution.

4. Conclusions

Overall, this study indicated that multi-layer chitosan coating increased the protection provided by alginate beads and is an approach that can be applied for increasing the survival of probiotic cells in highly acidic food systems, such as pomegranate juice. The higher protective effect of the single and double coated beads was most likely due to the thick alginate–chitosan and alginate–chitosan–alginate–chitosan membranes that were formed, which most likely buffered the acids as they penetrated into the beads, increasing the pH. It is interesting to note though that there was no correlation between cell survival and the hardness of the beads and that the latter depended on the content of the carrier solution in sequestering agents (e.g. citrate, malate) and monovalent ions. However, the physical characteristics of the beads, such as hardness, size and stability are important aspects from a food product development point as they affect the organoleptic properties of the product and its acceptability.

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