Viability of calcium-alginate-microencapsulated probiotic bacteria in Iranian yogurt drink (Doogh) during refrigerated storage and under simulated gastrointestinal conditions

Introduction

Improving the poor viability of probiotic bacteria in different food products, especially fermented milks, as well as under gastrointestinal tract conditions after ingestion, has been by far the most significant concern in the field of probiotics during recent years. The therapeutic value of the product at the point of consumption (defined as the minimum count of each probiotic micro-organism in the consumed product needed for pharmaceutical effectiveness) is the most important qualitative parameter in probiotic products. While there is no universal rule in this regard, a count of 10^7 cfu/mL has been accepted in the case of yogurt by the vast majority of dairy companies, starter culture-producing companies and national regulatory organisations, as well as the International Dairy Federation (IDF) (Mortazavian and Sohrabvandi 2006). In fermented milks, bacteriostatic and/or bactericidical factors such as low pH, organic acids, high redox potential, hydrogen peroxide, molecular oxygen, bacterial competition, relatively high temperatures during storage and duration of storage cause loss of viability of probiotic micro-organisms. Moreover, a high viable population of probiotic bacteria in food products at the point of consumption does not guarantee the same survival after arrival of the cells in the intestine. The very low pH of the stomach, along with the presence of bile salts in the small intestine, are the main reasons for the dramatic decline in viability of delivered cells (Mortazavian and Sohrabvandi 2006).

Immobilisation of living probiotic bacterial cells by microencapsulation using hydrocolloid materials provides one of the best possible means of protecting them against detrimental conditions in fermented milk products (Krasaekoopt *et al.* 2003; Mortazavian *et al.* 2007b). Microencapsulation is a process in which the bacterial cells are entrapped within the coatings of hydrocolloidal materials in order to be segregated from adverse environmental conditions (Mortazavian *et al.* 2007b). This process has been recently used as an efficient means of improving the viability of probiotic bacteria in fermented milks (Adhikari *et al.* 2000; Krasaekoopt *et al.* 2005; Sultana *et al.* 2000), as well as in the gastrointestinal tract (Hansen *et al.* 2002; Krasaekoopt *et al.* 2004; Lee *et al.* 2003; Rao *et al.* 1989; Wenrong and Griffiths 2000). Alginate is the most widely used encapsulating material because of its biocompatibility, cheapness, simplicity and good intestinal digestibility (Krasaekoopt *et al.* 2003; Mortazavian *et al.* 2007b).

Doogh is a popular yogurt-based drink produced in Iran. Recently, it has been formally identified as the 'Iranian national

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Abstract

The effects of microencapsulation of AB-type culture (*Lactobacillus acidophilus* LA-5 and *Bifidobacterium lactis* Bb-12) with calcium alginate on cell survival in Iranian yogurt drink (Doogh) during storage at 4°C for 42 days, as well as under simulated gastrointestinal conditions, were studied. The pH of the product at the beginning of storage was 4.53 and the final pH at the end of storage were 4.52 and 3.78 for the samples containing encapsulated and free cells, respectively. The acetic acid content in the encapsulated-cell-containing Doogh increased by 0.01% (from 0.05 to 0.06%) during the storage period, whereas for free-cell-containing Doogh the increase was 0.04% (from 0.05 to 0.09%). At day 42, the viable counts of *L. acidophilus* and bifidobacteria in the samples containing encapsulated cells were 5.5 and 4.0 log cycles higher than those containing free cells, respectively. To evaluate the protective impact of encapsulation on cell survival in *in vivo* situations, the product was subjected to three simulated gastrointestinal conditions, including extreme conditions (pH 1.5, 90 min/2% bile, 90 min), intermediate conditions (pH 1.5, 90 min/1% bile, 90 min) and normal conditions, i.e. the situation in the gastrointestinal tract of a normal healthy person after the consumption of a probiotic-containing dairy drink, when the stomach has not been free for a relatively long time (pH 2.0, 30 min/0.6% bile, 60 min). The viability of the probiotic cells increased from 0.6% and 0.2% (*L. acidophilus* and bifidobacteria, respectively) as free cells to 18.0% and 9.5% under the extreme gastrointestinal conditions, after encapsulation. Under normal gastrointestinal conditions, the cell survival rates were 16.1% for *L. acidophilus* and 21% for bifidobacteria before encapsulation, and 26.3 and 34.0% (*L. acidophilus* and bifidobacteria, respectively) after encapsulation.

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drink'. Surveys show that this product may gain a good share of market in European countries because of its characteristic sensory properties. Doogh consists of non-fat milk solids (min 3.2% W/W), milk fat (max 25% of total dry non-fat in final product), salt (0.2-1.0% W/W) and flavouring (normally mint or ziziphore essences) with a maximum pH of 4.50. Carbon dioxide (min 0.4% W/W) may be artificially or naturally (through fermentation) added to the product. Thickening agents and/or anti-whey separation compounds can also be added (max 10% of total dry non-fat matter in the final product) (Anon 2008).

Although the effect of calcium alginate on the survival of probiotic bacteria in fermented milks during storage has been the subject of several studies (Hansen *et al.* 2002; Krasaekoopt *et al.* 2004; Sultana *et al.* 2000), no study has been carried out specifically for a yogurt drink such as the Iranian Doogh. Similarly, a microcapsule-containing product (rather than a cell suspension) has not been sequentially subjected to gastric and intestinal conditions. Furthermore, there have been some conflicting reports about the impact of encapsulating materials on the survival of probiotic cells in simulated *in vivo* conditions. The objective of this work was to investigate the protective effects of microencapsulation with calcium alginate on AB-type probiotic cells in Iranian Doogh, over 42 days of refrigerated storage (at 4°C), as well as under simulated gastrointestinal conditions**.**

Materials and methods

Starter cultures

DVS pouches of commercial lyophilised cultures, including Y-type (mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*), A-type (single culture of *Lactobacillus acidophilus* LA-5) and B-type (single culture of *Bifidobacterium lactis* Bb-12, were supplied by Chr. Hansen (Horsholm, Denmark). The cultures were maintained at -18°C, according to the manufacturer's instructions, until used.

Enumeration of probiotic bacteria and definition of growth indexes

L. acidophilus and bifidobacteria were enumerated selectively using MRS-bile agar (MRS agar: Merck, Darmstadp, Germany and Bile: Sigma, Reyde, US) by the Subtractive Enumeration Method according to Mortazavian *et al*. (2007c). Anaerobic conditions were generated by the GasPak system (Merck, Darmstadt, Germany).

Encapsulated bacteria were enumerated after the capsules were digested in 0.1 M phosphate buffer, pH 7.0, containing 0.04% NaCl (Merck, Darmstadt, Germany) and incubated at 37°C for 90 min (Dimantov *et al.* 2004).

Microencapsulation procedure

Powders of lyophilised cultures of L. acidophilus LA-5 and B. lactis Bb-12 were gently dispersed in a defined volume of sterilised phosphate buffer saline solution (pH 7.0). Cells were harvested by centrifugation at 3000 g for 10 min at 5ºC and washed and re-centrifuged twice with the same solution and under the same conditions (Mortazavian 2007). The viable cell counts of both probiotics in the resulting cell concentration/cell suspension were determined before the encapsulation procedure. Microencapsulation was accomplished using a modified method of Capela et al. (2006). Briefly, 150 mL of sterile 2% W/V sodium alginate (Provisco, Swiss) was mixed with 30 mL of concentrated cell culture. The mixture was dispensed by pipette into a beaker containing 600 mL of pure corn oil and 1 g of Tween 80 while stirring at 180 rpm. Calcium chloride (0.1 M) was gently added at the side of the beaker until the emulsion was broken. After 1 hour, gel beads were separated using a decanter and centrifuged at 300 g for 5 min at 5ºC. After washing the beads with calcium chloride solution and recentrifugation under the same conditions, the total harvested beads obtained from a beaker batch containing a mixture of L. acidophilus and bifidobacteria cells (from single cultures of A-type and B-type) were incorporated into the defined volume of thermised Doogh (pH 4.5) produced by Y-type starter culture. By sampling and enumerating the viable counts of unencapsulated probiotic bacteria in the calcium chloride phase after decantation and washing, the total encapsulated population, i.e. the incorporation level of each probiotic was determined. The cell coating yields for L. acidophilus and bifidobacteria in the alginate beads were 99.7% and 99.9%, respectively.

Particle size distribution analysis

The particle size distribution was analysed using the light scattering method and Mastersizer instrument (MAL 101594, Malvern, UK). The assessed indexes were minimum diameter (μm), maximum diameter (μm), peak diameter (μm; the highest percentage in diameter distribution of particles) and specific surface area (the surface area of mass unit of particles: m2/g).

Sample preparation

Doogh milk with 4% dry matter was prepared by reconstitution of skim milk powder. The mixture also contained 0.5% sodium chloride. After the heat treatment (90ºC for 15 min), cooling of samples to the incubation temperature (40ºC) and inoculating lyophilised powder of the traditional yogurt starters (0.0048% according to Chr. Hansen's instruction), fermentation was carried out until a pH of 4.50±0.02 was reached. The samples were kept at 4ºC.

Exposure of microencapsulated-cell-containing product to gastrointestinal conditions

The total sample of Doogh (500 mL) containing either microencapsulated or free cells was added into solutions representing different gastric conditions with pH 1.5 or pH 2 and 0.2% of sodium chloride with careful stirring. The solutions were incubated at 37ºC for 90 min or 30 min and then transferred into different solutions representing intestinal conditions consisting of phosphate buffer pH 7.0 (Merck, Darmstadt, Germany) and 2%, 1% or 0.6% bile (bovine, Oxgall, Sigma, Reyde, US) solutions after their pHs were adjusted to 7.0±0.2 by adding a few drops of concentrated sodium hydroxide solution. Incubation was carried out at 37ºC for 90 or 60 min. The samples were gently shaken during the gastric and intestinal incubations.

Determination of acetic acid concentration

Quantification of acetic acid was carried out by high performance liquid chromatography (Varian Analytical

Instruments, CA, US) according to the method described by Alkam *et al*. (2004). Briefly, for extraction of acids, 4.0 g of Doogh was diluted to 25 mL with 0.1 N H_2SO_4, homogenised and centrifuged at 5000 g for 10 min. The supernatant was filtered through Whatman #1 filter paper and through a 0.2 µm membrane filter, and 2 mL aliquots were stored in HPLC vials at -20ºC until HPLC analysis. A Jasco UV-980 detector and a Nucleosil 120-5C_{18} column (Macherey Nagel, Duren, Germany) were used. The mobile phase was 0.009 N H_2SO_4 at a flow rate of 0.5 mL/min. The wavelength of detection was 210 nm. A standard solution of acetic acid (Sigma, US) was prepared in distilled water. The retention time for acetic acid was 7.15 min and the standard curve regression coefficient was 0.995.

Statistical analysis

were performed in quadruplicate and the significant differences among the means were analysed using the ANOVA test from Minitab software (version 13, 2002).

Results and discussion

Cell stability during refrigerated storage period

The effect of encapsulation with alginate on the viability of probiotic micro-organisms during 42 days of refrigerated storage (4°C) in 7-day intervals is presented in Table 1. Table 2 indicates the percentage decrease/increase in viability of probiotic bacteria over this period. According to Table 1, viable free cells of *L. acidophilus* were not detected at d 42, while the encapsulated cells had a significantly higher viability of 5.5 logs. Also, viable counts of encapsulated bifidobacteria were higher by 3.96 logs compared with unencapsulated cells at the end of storage. The protective effect of microcapsulation on probiotic bacteria arises from their barrier properties against the adverse conditions of the Doogh environment such as low pH, presence of organic acids, hydrogen peroxide produced by *L. delbrueckii ssp. bulgaricus* during the fermentation, molecular oxygen and high redox potential (about 176 mV immediately after fermentation). Increasing cell stability of encapsulated probiotic bacteria with calcium alginate during refrigerated storage has been reported in several investigations (Sultana *et al.* 2000; Hansen *et al.* 2002; Krasaekoopt *et al.* 2003), which is in agreement with the results obtained from the present research. However, the rate of improvement was much more evident from our observation. This could be primarily attributed to the physical and microstructural characteristics of the micro-beads formed during the encapsulation process and secondarily to the strain characteristics of the probiotic bacteria used. In the investigations made by Chandramouli *et al.* (2004) and Sheu and Marshall (1993), the higher protective effectiveness of micro-capsules resulted when the capsules' size and the cell load within the capsules increased. In our study, the size distribution of the capsules was 20-1096 µm with a peak size of 340 µm. The size of the probiotic cells used was about 1.5-6.0 µm (for single or attached cells) with a peak size of 1.36 µm. Hence, most of the capsules generated in the present study should have been loaded with several cells and presumably a significant aqueous portion. It has been reported that the survival of probiotics in alginate-starch microspheres in the size range of 0.5-1.0 mm was improved during refrigerated storage in yogurt (Sheu and

Table 1: Viability of probiotic bacteria (log cfu/mL) in encapsulated and free cell forms over 42 days' storage at 4ºC, per seven-day intervals. *

Treatment	Probiotic	Storage time (d)						
		0**	7	14	21	28	35	42
Encapsulated	A	5.90	6.01a	5.83a	5.72a	5.62a	5.54a	5.50
	B	6.54	6.62A	6.53A	6.52A	6.47A	6.42A	6.32A
Nil; free cells	A	5.90	5.64b	4.70b	4.00b	3.08b	1.95b	-
	B	6.54	6.49B	5.33B	4.78B	4.00B	3.40B	2.36B

Notes:
** Means in the same column with different small and capital English letters imply significant differences among L. acidophilus (A) and bifidobacteria (B) viable cell counts, respectively.*
***Immediately after fermentation*

Table 2: Loss/increase percentage in viability of probiotic micro-organisms during 42 days' storage at 4ºC, per seven-day intervals (compared to the initial viable cell counts immediately after fermentation or the viable cell counts at the last days of each 7-day storage interval).*

Treatment	Probiotics	Storage time (d)										
		0-7	7-14		14-21		21-28		28-35		35-42	
		LP0/IP0**	LP0	LP7	LP0	LP14	LP0	LP21	LP0	LP28	LP0	LP35
Encapsulated	A	$+29^{a\,I}$	$-15^{a\,I}$	$-34^{a\,I}$	$-34^{a\,I}$	$-23^{a\,I}$	$-48^{a\,I}$	$-21^{a\,I}$	$-56^{a\,I}$	$-17^{a\,I}$	-60^{I}	-10^{II}
	B	$+20^{A\,II}$	$-2^{A\,II}$	$-19^{A\,II}$	$-4^{A\,II}$	$-2^{A\,II}$	$-15^{A\,II}$	$-11^{A\,II}$	$-24^{A\,II}$	$-11^{A\,II}$	$-40^{A\,II}$	$-21^{A\,I}$
Nil (free cells)	A	$-45^{b\,I}$	$-94^{b\,I}$	$-89^{b\,I\text{-}II}$	$-99^{b\,I}$	$-80^{b\,I}$	-99^{b}	$-88^{b\,I}$	$\sim$$-100^{b\,I}$	$-92^{b\,I}$	-	-
	B	$-11^{B\,II}$	$-94^{B\,I}$	$-93^{B\,I}$	$-98^{B\,I}$	$-73^{B\,II}$	-99^{B}	$-83^{B\,I}$	$\sim$$-100^{B\,I}$	$-90^{B\,I}$	$\approx$$-100^{B}$	-77^{B}

Notes:
** Means in the same column with different small and capital English letters indicate significant differences among L. acidophilus (A) and bifidobacteria (B) viable cell counts, respectively. Latin letters represent significant differences among the two probiotics in encapsulated or free cell form.*
***LP/IP = loss or increase percentages; little numbers (0, 7, 14, 21, 28, 35) represent reference points for cell count comparisons.*

Marshall 1993). However, very large beads (about 1000 μm or larger) cause a coarseness of texture in mouthfeel and weakness in the coated structure (Hansen *et al.* 2002; Krasaekoopt *et al.* 2003). According to sensory analysis performed in the present study, no coarseness was identified (data not shown). From the results obtained from this study, it seems that capsules in the intermediate range (about 300-500 μm) provide good protection of the encapsulated cells during refrigerated storage of Doogh; smaller capsules provide less protection and have insufficient cell loads (Sheu and Marshall 1993; Chandramouli *et al.* 2004). This diameter range comprised 42.0% of the total size distribution area, while the diameter range of 500-1000 μm accounted for only 8.13% of the area, only. Complementary experiments revealed that the capsules adequately maintained their integrity in Doogh throughout the 42-day refrigerated storage without significant degradation (details not presented).

As shown in Table 2, over the first 7 days of storage, the viability of unencapsulated L. acidophilus cells decreased by 45%. The corresponding viability loss for bifidobacteria was 11%. Greater viability loss of the former bacteria is presumably largely due to the presence of hydrogen peroxide generated by *L. delbrueckii. ssp. bulgaricus* during the fermentation. This fact has been previously reported as the main reason for dramatic loss of viability of *L. acidophilus* during storage in ABY-type fermented milks (Dave and Shah, 1997; Mortazavian *et al.* 2006a, b, 2007a). In the case of encapsulated cells, by day 7, the counts of the probiotics increased by 29% and 20% for *L. acidophilus* and *bifidobacteria*, respectively. This indicates cell multiplication of entrapped probiotic cells in the capsules. It could be assumed that in sufficiently large capsules (as was the case for capsules formed in this study) containing several cells, as well as enough nutrient-containing aqueous portion, the situation is suitable for cell growth. This increase is consistent with the results reported by Adhikari *et al.* (2000). The higher growth rate for *L. acidophilus* compared with bifidobacteria supported the idea that hydrogen peroxide damages the cells of the former bacteria, because capsules physically protect the entrapped cells against this compound. From day 7 onwards, the encapsulated cells of both probiotics lost viability, but the counts for *L. acidophilus* were significantly higher (15% compared to day 0 or 34% compared to day 7 versus 2% compared to day 0 or 19% compared to day 7) than those of the bifidobacteria. The overall viability decline of probiotic bacteria can be attributed to the adverse effects of accumulated organic acids and hydrogen ions within the capsules after the cells grew inside the beads until day 7; however the greater decline in survivability of *L. acidophilus* cells may be due to the gradual increase in the amount of hydrogen peroxide inside the capsules after slow diffusion through the capsules pores from the Doogh environment. Also, the capsules may have provided bifidobacteria cells with protection against molecular oxygen. The great difference in redox potential changes during the refrigerated storage period for encapsulated and free cells supports this idea (Table 3). Subsequent to the sharp decline in the viability of *L. acidophilus* after day 7, further loss in viability occurred at a fairly constant rate. This was also observed in the case of bifidobacteria, with the exception of a rapid decline in their viability after day 21. This could be due to the greater tolerance of *B. lactis* Bb-12 of the conditions in the product compared to *L. acidophilus*. According to Table 2, the viability loss of the free cells of both probiotics reached 99% at day 21, close to 100% at day 28 and very close to 100% at day 35 compared with their initial cell counts. Conversely, the total viability loss of encapsulated cells was markedly lower, i.e. 60% and 40% at day 42 for *L. acidophilus* and *bifidobacteria*, respectively.

Table 3 shows the final pH, titrable acidity and redox potential of the treatments containing free and encapsulated cells after a 42-day refrigerated storage period at 4ºC, as well as the pH decrease and acidity increase over this period. As shown in Table 3, the pH of Doogh at the end of refrigerated storage was 3.78 in the sample containing free cells compared to 4.51 for that containing encapsulated cells. The acidity and redox potential were significantly higher in the former. Also, there were significant and marked differences between the two treatments in the case of the pH decrease and acidity increase rates. On the other hand, the concentration of acetic acid in the samples containing encapsulated cells increased from 0.05% at day 0 to 0.06% at day 42, while the final amount in the samples with free cells was 0.09%. Therefore, microencapsulation of free cells greatly, but not completely, restricted their metabolic activity. This has been observed by other researchers (Adhikari *et al.* 2000; Sultana *et al.* 2000; Hansen *et al.* 2002; Krasaekoopt *et al.* 2004; Capela *et al.* 2006). Apart from direct contact of free cells (compared to encapsulated cells) with adverse environmental factors in Doogh during storage, the higher rates of pH decrease, and acidity and redox potential increase in the product also directly correlate with the significantly sharper viability loss of probiotic cells.

Table 3: Final pH, titrable acidity and redox potential of Doogh containing free and encapsulated cells after 42 days' storage at 4ºC as well as the pH decrease and acidity increase rates over this period.*

Treatment	pH		Acidity (ºD)		Redox potential (mV)		pH decrease rate (pH value/week)	Acidity increase rate (Dornic degree/day)	Acetic acid (%)	
	0**	42	0	42	0	42			0	42
Encapsulated	4.53[a]	4.51[a]	37.8[a]	43.2[b]	146.0[a]	150.0[b]	0.003[b]	0.13[b]	0.05	0.09
Nil (free cells)	4.53[a]	3.78[b]	38.1[a]	97.7[a]	147.1[a]	204.3[a]	0.125[a]	1.42[a]	0.05	0.06

Notes:
** Means in the same column with different letters are significantly different.*
** 0 = immediately after fermentation, 42=at the end of storage.*

Cell viability after the exposure of product to simulated gastrointestinal conditions

The survival rates of free and encapsulated probiotic cells in Doogh after their successive exposure to different simulated conditions of the stomach and small intestine are presented in Table 4. At extreme conditions (pH 1.5, 90 min/2% bile, 90 min), the survival of probiotic cells were 0.6% and 0.2% for *L. acidophilus* and bifidobacteria, respectively. These percentages increased at intermediate conditions (pH 1.5, 90 min/1% bile, 90 min) to 11.0% and 1.5% and at normal conditions (pH 2.0, 30 min/0.6% bile, 60 min) to 16% and 21% for *L. acidophilus* and bifidobacteria, respectively. Therefore, bifidobacteria cells were primarily susceptible to the very low pH of the stomach rather than the bile concentration, whereas *L. acidophilus* cells were substantially sensitive to the bile concentration. The same observation was reported by Krasaekoopt *et al.* (2004). As can be seen in Table 4, alginate micro-beads increased the survival of free cells of *L. acidophilus* by 17.4% and bifidobacteria by 9.3% under extreme gastrointestinal conditions and by 10.2% and 13.0% under normal conditions. Complementary experiments revealed that alginate beads were degraded by 27.2% and 28.4% (details not shown; data obtained by applying light scattering method) after 30 min and 90 min of exposure to gastric conditions (pH 1.5). Therefore, hydrogen ions may partially affect the integrity of the beads, probably by acting as partial imperfect substitutes for calcium ion bridges within the capsule matrix. Partial digestion of the beads leads to higher exposure of the bacterial cells to the high concentration of hydrogen ions in the gastric juice and, as a result, a sharp loss in viability of entrapped cells. Apart from this, natural micro-pores present in a gel-bead matrix permit the diffusion of hydrogen ions inwards, which would be magnified in the case of cracked beads. This explains why, for example, the viability of free cells of bifidobacteria, which are hydrogen ion-sensitive, was 9.3% higher in extreme gastrointestinal conditions after encapsulation compared with 13.0% higher in normal conditions; the higher survival may have been due to the alleviated environmental conditions rather than the greater protection of alginate capsules. Higher pH along with shorter exposure time in the latter situations causes less diffusivity of hydrogen ions into the capsules and subsequently greater viability of the cells. As mentioned previously, the size distribution of the beads, as well as the cell load within them, significantly influence the survivability of encapsulated cells. Also, as pointed out above, the micro-beads formed in this study were loaded with several cells and presumably a significant aqueous portion. Such conditions inside the beads slow down the micro-flux of hydrogen ions into the capsules, at least for the central cells, which results in considerably higher survival of the cells particularly for the shorter exposure time (30 min compared to 90 min). It seems that the bead size range in this study, which maintained the cell stability over the refrigerated storage period, could be also successfully applied *in vivo*, because the rates of survival improvement in the present study were much more evident than in previous reports. Regardless of the beads' characteristics, interactive effects of various factors such as strain characteristics of starters, simulated conditions of the gastrointestinal tract, type and specification of the encapsulating material(s) and the methodology of encapsulation, as well as viability assessment procedures (both testing and enumeration), should not be understated. These factors may explain why there is a variation in the reports of the impact of encapsulation on the survival of probiotic cells. For instance, Hansen *et al.* (2002) reported that microencapsulation of bifidobacteria with Ca alginate did not significantly improve their survival when they were exposed to simulated gastric juice. According to Sultana *et al.* (2000), encapsulation of *L. acidophilus* and *Bifidobacterium* spp. with alginate-starch did not demonstrate a significant increase in survival when subjected to *in vivo* high acid and bile salt conditions. In contrast, the results of other research (including the present work) indicate a significant impact of encapsulation on the survival of the entrapped cells. As mentioned, it seems that the protection efficiency of encapsulation would be more marked if the severity of gastrointestinal stress factors was alleviated. For example, according to Krasaekoopt *et al.* (2004), encapsulated cells of *L. acidophilus* were more viable (0.62% versus 0.15%) compared

Table 4: Survival percentage of free and encapsulated probiotic cells in Doogh after exposure to different gastrointestinal conditions.*

Gastrointestinal conditions	Encapsulated state	Probiotic	Viability before and after exposure (log cfu/mL)	Percentage survival
Extreme (pH 1.5, 90 min/2% bile, 90 min)	Free	*L. acidophilus*	6.54-4.32	0.6^{h}
		bifidobacteria	6.90-4.20	0.2^{hi}
	Encapsulated	*L. acidophilus*	6.54-5.79	18.0^{d}
		bifidobacteria	6.90-5.88	9.5^{f}
Intermediate (pH 1.5, 90 min/1% bile, 90 min)	Free	*L. acidophilus*	6.54-5.58	11.0^{f}
		bifidobacteria	6.90-5.08	1.5^{g}
	Encapsulated	*L. acidophilus*	6.54	nT**
		bifidobacteria	6.90	nT
Normal (pH 2.0, 30 min/0.6% bile, 60 min)	Free	*L. acidophilus*	6.54-5.75	16.1^{de}
		bifidobacteria	6.90-6.22	21.0^{c}
	Encapsulated	*L. acidophilus*	6.54-5.96	26.3^{b}
		bifidobacteria	6.90-6.43	34.0^{a}

Notes:
** Means in the same column with different letters are significantly different ($p<0.05$).*
***Not tested*

to free cells when exposed to gastric juice of pH 1.55 for 90 min. Reducing the exposure time by 30 min caused a considerably greater difference between the viability of encapsulated and free cells (10.47% versus 1.15%). Similar results were observed in the present research.

Conclusions

This work demonstrated that microencapsulation of probiotic bacteria with calcium alginate significantly improves their viability in Iranian yogurt drink (Doogh), during storage at 4°C for 42 days. Also, the survival of the encapsulated probiotic bacteria was significantly higher than that of free cells when subjected to simulated gastrointestinal conditions, especially normal conditions (e.g. pH 2.0, 30 min/0.6% bile, 60 min). The characteristics of the beads produced in this study appeared to be suitable for providing adequate protection to the encapsulated probiotics in Doogh, for both product and simulated gastrointestinal conditions. The beads maintained their integrity in the product throughout the storage period without significant degradation.

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