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SURVIVABILITY OF ENTRAPPED *Lactobacillus rhamnosus* IN LIQUID- AND GEL-CORE ALGINATE BEADS DURING STORAGE AND SIMULATED GASTROINTESTINAL CONDITIONS

SOBREVIVENCIA DE *Lactobacillus rhamnosus* ENTRAMPADOS EN CÁPSULAS DE ALGINATO CON CENTRO LÍQUIDO Y GELIFICADO DURANTE EL ALMACENAMIENTO Y EN CONDICIONES GASTROINTESTINALES SIMULADAS

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Abstract

L. rhamnosus cells were encapsulated in liquid-core (LCBR) and gel-core (GCBR) calcium alginate beads, and cell survivability under storage conditions and simulated gastrointestinal conditions were evaluated, and compared with that of non-encapsulated cells. The average external diameters of both beads (1.37 ± 0.25 mm) were non-significantly different, and the average thickness of alginate gelled layer in LCBR was of 0.27 ± 0.01 mm. The bacteria entrapped into LCBR tended to gather together forming clusters in the bulk of the liquid phase of the bead, whereas the bacteria entrapped into GCBR were compartmentalized in the gelled bead biopolymer matrix. LCBR showed significant lower hardness and chewiness, higher cohesiveness, and comparable springiness values than GCBR. Cells survivability under storage and simulated gastrointestinal conditions was significantly higher in LCBR than in GCBR and for the non-encapsulated free cells.

Keywords: encapsulation, gelation, sequestration, *Lactobacillus rhamnosus*, liquid core beads, survivability.

Resumen

Células de *L. rhamnosus* fueron entrampadas en cápsulas de alginato de calcio con centro líquido (LCBR) y centro gelificado (GCBR), evaluando la sobrevivencia de las células bajo condiciones de almacenamiento y condiciones gastrointestinales simuladas en comparación con las de células sin entrampar. No hubo diferencias significativas entre los diámetros externos promedio (1.37 ± 0.25 mm) de ambas cápsulas, mientras que el grosor promedio de la capa gelificada en LCBR fue de 0.27 ± 0.01 mm. Las bacterias entrampadas en LCBR tendieron a agregarse en el seno de la fase líquida de la cápsula, mientras que las bacterias entrampadas en GCBR se distribuyeron homogéneamente en la matriz biopolimérica de la cápsula. LCBR mostró valores de dureza y masticabilidad significativamente menores, una cohesividad significativamente mayor y una elasticidad comparable a los exhibidos por GCBR. La sobrevivencia de las células bajo condiciones de almacenamiento y condiciones gastrointestinales simuladas fue significativamente mayor en LCBR que en GCBR y para las células libres sin encapsular.

Palabras clave: entrampamiento, gelación, agente quelante, *Lactobacillus rhamnosus*, cápsula de centro líquido, sobrevivencia.

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1 Introduction

The lactic acid bacteria (LAB) *Lactobacillus rhamnosus* is considered as probiotic microorganism as it inhibits pathogens growth and has the capability to adhere to intestine cells (Figueroa-González *et al.*, 2010). However, probiotic bacteria can only exert positive health effects if they reach their site of action in adequate numbers (Sultana *et al.*, 2000). Many studies have shown that probiotics viable cells number is considerably reduced when they are incorporated into dairy products, during storage, and in transit through the gastrointestinal tract (Rao *et al.*, 1989). Thus, an ongoing research topic is to develop inexpensive and readily available methods that may help improve the survivability and achieve high viable cell numbers of probiotic bacteria. Microencapsulation techniques have been successfully used to improve the survivability of probiotic microorganisms in dairy products (Picot and Lacroix, 2004) or/and under artificial gastrointestinal juice (Chandramouli *et al.*, 2004). Food-grade polymers such as alginate, chitosan, carboxymethylcellulose, carrageenan, gelatin and pectin have been used as microcapsules forming materials using various microencapsulation technologies (Anal and Singh, 2007). In particular, calcium alginate gel is widely used to encapsulate probiotic bacteria because of its biocompatibility and because the gelling procedure is very simple and occurs under mild conditions (Wang *et al.*, 1995). However, probiotic cells entrapped in alginate beads tend to grow at the periphery of the calcium alginate beads, resulting in an increased cells leakage release caused by gel surface breakage (Seki *et al.*, 1990). Moreover, as cell density increases in alginate gels, the strength of polymeric matrices decreases (Nussinovitch *et al.*, 1996b); an expansion of alginate beads may occur with cell growth (Koyama and Seki, 2004); and apparent diffusivities of substrates and products within the gel decrease. Diffusion of substrates and products through beads occurs and is determined by pore size of the gel, reflected by the viscosity of the carrier, due to the size of the molecule, and/or its concentration can affect the diffusion of the substrates or products and limit the reaction rates of the encapsulated cells (Tanaka *et al.*, 1984).

Probiotic cell entrapment in liquid-core capsules constitutes an alternative cell immobilization procedure that may overcome drawbacks encountered in gel-core capsules entrapment. In liquid core beads, the probiotic cells are suspended in a liquid

medium, but on turn surrounded by a thin layer of alginate gel, which allows for a uniform cell growth and reduces mass transfer limitations. At the same time, the semi-permeable membrane layer acts as a barrier to cell release and minimizes phage contamination (Dembczynski and Jankowski, 2002). In order to obtain a liquid-core capsule, a calcium sequestering agent must be used for controlling the calcium alginate gelling rate (Bennett, 1989). The calcium binding competition between the sequestering agent and sodium alginate results in a slowing down of the rate of alginate gelling, and thereby leading to a successful liquid-core calcium alginate bead formation (Monshipouri and Price, 1995).

While a lot of information is available regarding the obtention and properties of gel-core beads, little is known about liquid-core beads including their mechanical and diffusive properties (Nussinovitch *et al.*, 1996a).

The aim of this work was to compare the survivability of entrapped *L. rhamnosus* in liquid-core and gel-core alginate beads, during storage and after exposure to simulated gastrointestinal conditions.

2 Materials and methods

2.1 Materials

Low molecular weight sodium alginate (SA), with 60.5% guluronic acid content was purchased from Danisco Mexico, S.A. of C.V. (Grindsted alginate FD 175). Sodium citrate (SC), calcium chloride (CaCl_2), and analytical grade methanol (MeOH) were obtained from J.T. Baker Chemical Co., Phillipsburg, NJ. Bacto peptone, de Man Rogosa Sharpe (MRS) lactobacilli broth and agar were purchased from BD DIFCO Becton Dickinson and Company (Detroit, Mi).

2.2 Bacterial strain and culture

A lyophilized culture of *L. rhamnosus* LC705 (DANISCO, Niebüll, Germany) was activated by inoculating twice in MRS broth ($1 \text{ g } 100 \text{ ml}^{-1}$) for 18 h at 37°C (Pimentel-González *et al.*, 2009). The cells were harvested in the late exponential growth phase (18 h, 37°C) by centrifugation at 15800 g for 10 min using a minispin plus centrifuge (Eppendorf Centrifuge Type 22331, Eppendorf AG, Hamburg, Germany). The supernatant was discarded and the cells were re-suspended in fresh and sterile MRS broth in order to obtain a cell concentrate (CC) containing a load of $11.34 \log_{10} \text{ cfu ml}^{-1}$.

2.3 Beads forming procedure

A preliminary study was carried out in order to formulate and prepare mechanically stable liquid-core beads (LCB) using an aqueous dispersion of SA (Fizman *et al.*, 2002). To this end, the initial attempt was to determine the concentration of SC that would slow down the crosslinking between SA and CaCl₂ in aqueous dispersion, but at the same time would permit the formation of stable beads with a liquid-core by the extrusion technique used in this work. Beads with liquid-core volumes larger than 58% presented a weak structure prone to rupture and were discarded. It was found that an aqueous dispersion formed by 1g 100 ml⁻¹ of SA and 0.2 g 100 ml⁻¹ SC (SASC), and an aqueous dispersion containing 0.08 g 100 ml⁻¹ CaCl₂ yielded mechanically stable beads having a liquid-core that represented from 40-57 % of the total volume of the beads, and these conditions were used for obtaining the LCB containing *L. rhamnosus* (LCBR). Thus, the detailed method for obtaining the LCBR beads was as follows: CC containing a load of 11.34 log₁₀ cfu ml⁻¹ was blended in a volume ratio of 1:9 with SASC aqueous dispersion (sterilized at 121 °C, 15 min) obtaining CC-SASC suspension. The beads were formed by Muthukumarasamy *et al.* (2006) modified method. Fifty milliliters of CC-SASC suspension were dripped into one hundred milliliters of sterile CaCl₂ solution (0.08 g 100 ml⁻¹) using a peristaltic pump with a tubing discharge of 1 mm of diameter and a flow of 3 ml min⁻¹ (Cole-Parmer, Chicago Il) applying continuous agitation at 100 rpm. After the CC-SASC suspension addition to CaCl₂ solution was ended, the formed capsules were left to stand in this calcium chloride solution for 10 min, then they were filtered through a stainless steel mesh of 0.19 mm, rinsed with sterile physiological saline solution (0.08 g of NaCl 100 ml⁻¹ for 3 min) and subsequently kept in sterile physiological saline solution at 4 °C.

The same general procedure was followed for obtaining the gel core beads (GCB) containing *L. rhamnosus* (GCBR), 50 ml of CC-SA suspension were dripped into 100 ml of a CaCl₂ solution (0.08 g CaCl₂ 100 ml⁻¹), but the GCBR were left to rest in the calcium chloride solution for 1 h for complete hardening.

2.4 Entrapped *L. rhamnosus* enumeration

In order to release the entrapped *L. rhamnosus* cells, 1 g of the beads variations were suspended in 9 ml

of a SC solution (0.5 g 100 ml⁻¹), followed by gentle shaking at room temperature (20 ± 2 °C) for 1 min (Nedović *et al.*, 2001). Numbers of viable cells in the suspensions were determined by plate counting using MRS agar with incubation at 37 °C for 48 h (Pimentel-González *et al.*, 2009), and reported as log₁₀ cfu ml⁻¹.

2.5 Beads diameter and volume

The external diameter (D_e) of 30 freshly prepared LCBR and GCBR was measured with a digital micrometer caliper (Starrett model 436-1 IN, Leon Weill, Mexico City, Mexico), and the radius (R_e) was calculated. The thickness of alginate gelled layer (GL_r) of LCBR was estimated as follows: 30 beads were cut in half, the liquid core drained, and the thickness of the remaining bead shell was measured at least five times at different locations with the micrometer caliper. Liquid-core volumes (V_{lc}) of the LCBR were calculated by subtracting GL_r from R_e and considering a spherical morphology.

2.6 Beads microstructure

LCBR and GCBR were prepared for observing their structure under low and high vacuum conditions with a JEOL Scanning Electron Microscope JMS-035 (Jeol Ltd., Akishima, Japan), operated at 20 kV. The beads variations were cut in half, and for low vacuum observation, samples were mounted on scanning electron microscopy (SEM) stubs with the inner surface up, and observed at a magnification of 200×. For high vacuum inspection, the samples were fixed in 2 % v/v buffered glutaraldehyde (0.1 M phosphate buffer, pH 7.2) at room temperature for 6 h, washed with phosphate buffer, dehydrated by immersion in aqueous ethanol solutions of increasing concentration (50, 60, 70, 80, 90 and 100 % v/v, 30 min in each), and placed in acetone for 1 h (Lobato-Calleros *et al.*, 2006). Samples were then critical point dried in a CPA II Techniques Critical Point Dryer (Tousimis, Rockville, MD), coated with a thin layer of gold in a Fine Coat Ion Sputter JFC 1100 (Jeol Ltd., Akishima, Japan), mounted on SEM stubs with their inner surface up, and observed at a magnification of 5000×. Representative micrographs were selected for presentation.

2.7 Textural properties of the capsules

Textural properties of the LCBR and GCBR were determined by Texture Profile Analysis (TPA)

(Bourne, 2002) using a Stable Micro Systems Texturometer model TA-XT2i (Texture Technologies Corp., White Plains, NY) equipped with a 25 kg load cell, and a cylindrical steel probe with a large contact area (35 mm in diameter). Each measurement was carried out at room temperature on 30 g of beads placed on a fixed bottom glass plate under the probe. The automatic detection of the contact by the probe with the beads was carried out with a contact force of 0.005 N (Artignan *et al.*, 1997). In all cases the samples were compressed 30%, using two compression cycles at a constant crosshead velocity of 0.5 mm s⁻¹ (Sandoval-Castilla *et al.*, 2010). The primary textural properties of hardness (maximum force of the first peak), cohesiveness (ratio of the positive force areas under the second and first peaks), springiness (length of the second compression, from its beginning to its peak/ length of the first compression, from its beginning to its peak) (Artignan *et al.*, 1997), and chewiness (hardness × cohesiveness × springiness) (Lobato-Calleros *et al.*, 2007) were obtained using the equipment's Texture Expert Software for Windows Version 3.2.

2.8 Survivability of entrapped *L. rhamnosus* during storage

LCBR (8.85 ± 0.09 log₁₀ cfu ml⁻¹), GCBR (8.85 ± 0.13 log₁₀ cfu ml⁻¹), and non-encapsulated *L. rhamnosus* cells (8.64 ± 0.33 log₁₀ cfu ml⁻¹), the latter used as control, were poured into 50 ml of sterile physiological solution, followed by gentle stirring. All the samples were stored at 4 °C, and the viability of *L. rhamnosus* was determined during 25 days, at intervals of 5 days, as indicated above. The experimental data (log₁₀ cfu ml⁻¹) were converted into survival percentage by dividing the final viable population between the initial viable population, and multiplying by 100.

2.9 Tolerance of *L. rhamnosus* in simulated gastrointestinal conditions

LCBR (8.34 ± 0.21 log₁₀ cfu ml⁻¹) and GCBR (8.54 ± 0.11 log₁₀ cfu ml⁻¹), and non-encapsulated *L. rhamnosus* cells (8.47 ± 0.03 log₁₀ cfu ml⁻¹) were placed separately into 9 ml of 0.05 M sodium phosphate buffer solution adjusted to pH 2.3 with 0.1 N HCl (Pimentel-González *et al.*, 2009). Samples were incubated at 37 °C for 2 h with agitation at 100 rpm. Afterwards, the beads exposed to acid solution

were recovered by filtration, washed with sterile physiological solution and put into 9 ml of fresh MRS broth with 0.3 g of bile salts (B8631, Sigma-Aldrich Chemical Co., Steinheim, Switzerland) per 100 ml. On the other hand, 1 ml of the non-encapsulated *L. rhamnosus* cells acid suspension was placed into the same bile solution as mentioned above. Samples were incubated at 37 °C for 24 h with continuous stirring at 100 rpm. At the end of acid and bile exposure periods, *L. rhamnosus* viable cells were enumerated.

2.10 Statistical analysis

All the assays were independently carried out by triplicate. The experimental data were subjected to Simple Classification Variance Analysis and whenever it was adequate to Tukey's test. The significance was established at $P < 0.05$. All data were analyzed using the NCSS 2000, version 5, software (Wireframe Graphics, Kaysville, UT, USA).

3 Results and discussion

3.1 Characterization of LCBR and GCBR

3.1.1 Beads diameter and volume

Both the LCBR and GCBR displayed spherical morphologies, with average external diameters (D_e) of 1.37 ± 0.25 mm, which were non-significantly different. The average thickness of alginate gelled layer (GL_r) was 0.27 ± 0.01 mm and the liquid alginate core volume (V_{lc}) fell in-between 45-56 % for LCBR. All the beads obtained exhibited an adequate mechanical stability against rupture under manipulation.

3.1.2 Textural properties of capsules

The extent of the crosslinking reaction between calcium-alginate polymeric networks influenced the mechanical responses to deformation of the beads. LCBR showed significant lower hardness and chewiness, higher cohesiveness, and comparable springiness values than GCBR (Table 1). Hardness, considered as the force required for compressing the sample, and chewiness, which grades the resistance and energy when chewing the sample (Segnini and Dejmek, 1999), was directly proportional to the thickness of the biopolymer gelled fraction in the bead, which on turn was related to extent of the reaction time, so that these parameters were higher for GCBR.

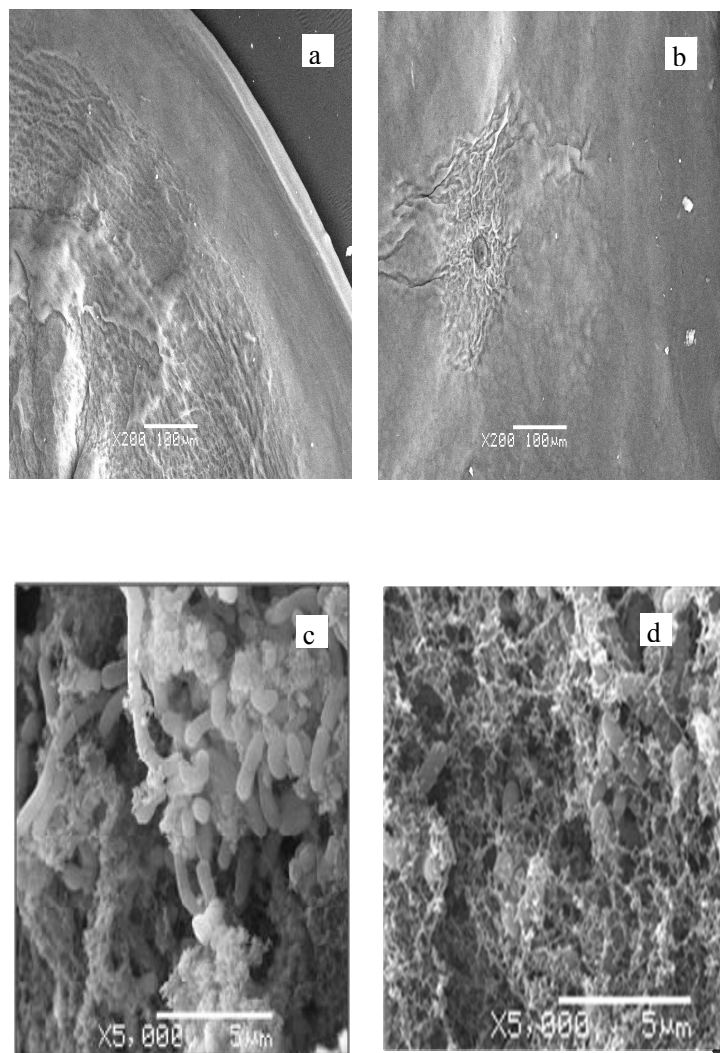


Fig. 1. Low vacuum SEM micrographs of: (a) liquid-core and (b) gel-core calcium alginate beads. High vacuum SEM micrographs of: (c) liquid-core and (d) gel-core calcium alginate beads.

Table 1. Textural properties of liquid-core and gel-core beads variations (means \pm SD) containing *L. rhamnosus*

Bead code	Hardness (N)	Cohesiveness (Ratio)	Springiness (mm)	Chewiness (N mm)
GCBR	0.49 \pm 0.04 ^a	0.65 \pm 0.05 ^a	0.93 \pm 0.12 ^a	0.30 \pm 0.05 ^a
LCBR	0.21 \pm 0.00 ^b	0.78 \pm 0.05 ^b	0.99 \pm 0.00 ^a	0.16 \pm 0.01 ^b

^{a,b} Different superscripts within the same column indicate that the means differ significantly ($p < 0.05$).

GCBR= Gel core beads with *Lactobacillus rhamnosus*.

LCBR= Liquid core beads with *Lactobacillus rhamnosus*.

Cohesiveness, considered as the amount of sample that deforms rather than splits apart (Segnini and Dejmek, 1999), may be considered as directly proportional to the liquid core fraction in the bead, and this parameter was higher for LCBR. Springiness, is the rate at which the sample returns to its original shape (Moyny, 2002), and both beads possessed an equivalent overall ability to reform broken linkages when stress application was released.

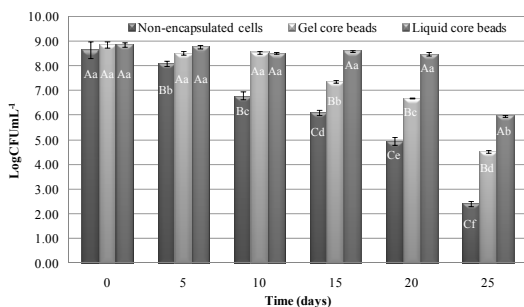


Fig. 2. Survivability of *L. rhamnosus* in liquid-core beads, gel-core beads, and non-encapsulated cells under storage conditions of 4 °C for 25 days. Different lower case letters (a, b, c) indicate significant differences for the same bead variation. Different upper case letters (A, B, C) indicate significant differences among the bead variations.

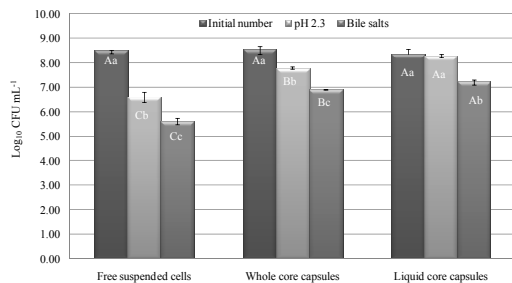


Fig. 3. Survivability of *L. rhamnosus* in liquid-core beads, gel-core beads, and non-encapsulated cells under simulated gastrointestinal conditions. Different lower case letters indicate significant differences for the same bead variation. Different upper case letters indicate significant differences among the bead variations.

3.2 Survivability of LCBR and GCBR

3.2.1 Survivability of *L. rhamnosus* during storage

Bacteria survivability data during storage are presented in Fig. 2. The GCBR, LCBR and non-encapsulated free cells initial counts ($t = 0$) were non-

significantly different. However, the non-encapsulated free cells showed a significant decay in cell numbers as storage time increased, experiencing a loss of about 6.24 log cycles after 25 days storage. In contrast, GCBR showed non-significant differences in cell numbers during the first 10 days of storage, but at longer storage showed a progressively significant decrease in cell numbers, accounting for about 4.34 log cycles at the end of storage time. Likewise, LCBR showed a remarkably high survivability that was non-significantly different at day 20 from the initial count, but a significant diminution in cell count of about 2.9 log cycles was shown at the end of storage time. These results clearly indicate that the survivability of LCBR was significantly higher than for GCBR, which on turn was significantly higher than for the non-encapsulated free cells. One of the main expected properties of probiotic microorganisms is that they possess the capability to survive refrigerated storage (4 °C) as formulated products (Brusch-Brinques and Záchia-Ayub, 2011). The results of this work suggest that immobilization of probiotics in liquid center beads constitutes a promising technology for improving the survivability conditions of the microorganisms than those achievable in gel-core beads, and of course, those obtainable with non-encapsulated cells. While for GCBR, active cell growth took place with colonies occupying gel cavities close to the gel surface (Arnaud et al., 1992), in LCBR it occurred at the bead liquid core, where cell mobility was considerably less restricted, and allowed a greater survivability.

3.2.2 Survivability under simulated gastrointestinal conditions

Data of survivability of non-encapsulated free cells, and GCBR and LCBR exposed to sequential simulated gastrointestinal conditions (pH 2.3 and bile salts) are given in Fig. 3. Exposure to pH 2.3 conditions did not affect significantly the viability of LCBR, but did affect significantly that of GCBR and the non-encapsulated cells, producing cell count reductions of about 0.76 log and 1.87 log cycles, respectively. Further exposure to bile salts, resulted in a significant reduction of the cells viability in the three treatments, but LCBR showed a significantly lower cell count reduction (~ 0.77 log cycles) than GCBR (~ 0.88 log cycles), and the latter lower than the non-encapsulated cells (~ 1.00 log cycle). The survivability percentage was of 89.9 % for LCBR, 80.8 % for GCBR, and 66.1% for non-encapsulated cells. From these results, it may be inferred, that the protective

biopolymer coating formed around LCBR and GCBR, effectively isolated *L. rhamnosus* from the simulated gastrointestinal conditions, hindering the interaction between cells with this harsh environment. Moreover, the protection provided by the biopolymer shell in LCBR was superior to that provided by the completely gelled bead (GCBR).

The highest survival of cell culture encapsulated on liquid core beads observed in these work, can be supported in studies that have demonstrated that cells entrapped in gel core beads, tend to grow at the periphery of the calcium alginate beads (Seki et al., 1990). The better performance of the liquid core beads versus gel core beads might partially be attributed to the prevention of cell release from the liquid core capsules. As the results of extensive surface growth, cells are released from the gel core beads into the fermentation medium, thus decreasing cell population recorded in the beads (Morin et al., 1992). In gel core beads, active cell growth takes place with microcolonies occupying gel cavities close to the gel surface (Arnaud et al., 1992). Therefore, cells exposed in gel surface are in direct contact with adverse conditions and survival can decrease in comparison with liquid core bead.

Conclusions

Microencapsulation in liquid-core calcium alginate beads by the extrusion technique provided better protection to *L. rhamnosus* cells during storage and from simulated gastrointestinal conditions than when microencapsulation was done in extruded gel-core calcium alginate beads or when cells were non-encapsulated. Thus, it is implied that liquid-core calcium alginate beads enhance the protection of the sensitive cells from degradation by reducing the reactivity from outside detrimental environmental factors.

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