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Lactobacillus plantarum G1 Microencapsulation enhanced its Viability during Storage and Gastrointestinal Transit

(Pemikrokapsulan *Lactobacillus plantarum* G1 Mempertingkatkan Kebolehhidupannya semasa Storan dan Transit Gastrousus)

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ABSTRACT

A Lactobacillus plantarum *strain G1 was previously isolated from chicken crop and it was found to show interesting probiotic properties. In this study, several microencapsulation combined materials were used to test their ability to protect cells from gastrointestinal harsh conditions. The results on kinetics of cell release after exposition to acidic pH (2.0) indicated that the efficiency of cell entrapment of the gels was as follows starting from the highest capacity to the lowest one: sodium alginate, alginate-agar (AA), alginate-starch (AS) and κ-carrageenan. In addition, effect of bile salts on cell release was also tested; the results showed that the efficiency of entrapment of the gels was different and as follows starting from the highest capacity to the lowest one: AA, sodium alginate, κ-carrageenan, and AS. Moreover, viability of free and alginate-microencapsulated* Lactobacillus plantarum *after exposure to acid, bile and pancreatic enzymes was investigated; the results showed that microencapsulation was capable of protecting cells against the harsh conditions of the gastrointestinal tract. In addition, microencapsulation enhanced the viability of* Lb. plantarum *during cold storage for 2 weeks and during a range of heat treatments.*

Keywords: Gastrointestinal conditions; Lactobacillus plantarum*; microencapsulation; probiotics*

ABSTRAK

Strain G1 Lactobacillus plantarum *diasingkan daripada tembolok ayam dan ia didapati menunjukkan sifat probiotik yang menarik. Dalam kajian ini, beberapa bahan gabungan pemikrokapsulan telah digunakan untuk menguji keupayaan mereka untuk melindungi sel daripada keadaan yang teruk dalam gastrousus. Keputusan pembebasan sel kinetik selepas pendedahan kepada pH berasid (2.0) menunjukkan bahawa kecekapan pemerangkapan sel oleh gel adalah seperti berikut; bermula daripada kapasiti tertinggi ke terendah: natrium alginat, alginat-agar (AA), alginat-kanji (AS) dan κ-carrageenan. Di samping itu, kesan garam hempedu ke atas pembebasan sel juga telah diuji; keputusan menunjukkan bahawa kecekapan pemerangkapan gel adalah berbeza dan seperti berikut bermula daripada kapasiti tertinggi ke terendah: AA, natrium alginat, κ-carrageenan dan AS. Selain itu, kebolehhidupan* Lactobacillus plantarum *yang bebas dan alginat-pemikrokapsulan selepas terdedah kepada asid, hempedu dan enzim pankreas dikaji; hasil kajian menunjukkan bahawa pemikrokapsulan mampu melindungi sel terhadap keadaan yang teruk dalam saluran gastrousus. Di samping itu, pemikrokapsulan meningkatkan kebolehhidupan* Lb. plantarum *semasa penyimpanan sejuk selama 2 minggu dan semasa pelbagai rawatan haba.*

Kata kunci: Keadaan gastrousus; Lactobacillus plantarum*; pemikrokapsulan; probiotik*

INTRODUCTION

In the early 1900s, the discovery of Elie Metchnikoff on the unique benefits of fermented cow's milk has led to a Nobel Prize in Medicine in 1908. The reason for these nutritional benefits is the presence of probiotic lactic acid bacteria (LAB) in milk (Akhiar 2010). Probiotics have been defined in several ways, according to the understanding of the mechanisms of their action on health and well-being of man. The most used is that of Fuller (1989): Probiotics are live microbial food supplements, which display beneficial effects on the host by improving its intestinal balance (Anal & Singh 2007).

However, probiotics must meet several criteria before being applied, including the activity and viability in products; adherence to epithelial cells, tolerance to low pH, gastric juice, bile salts, pancreatic juice and survival *in vivo*. The viability of probiotics is a key parameter for the development of probiotics in food (Soccol et al. 2010). Obstacles encountered in the various compartments of the gastrointestinal tract (GIT) (i.e. acidity, bile acids) and the long retention period in food (heat treatment and long period of storage), limit the survival and stability of viable bacterial cells before reaching their target site. Thus, for an efficient protection of live probiotic bacteria, an effective carrier system is required to protect them; the technology of microencapsulation (ME) appears to be a support system suitable for this purpose. Various food matrixes composed of polysaccharides such as alginate,

gellan gum, carrageenan and starch are the materials most commonly used for the ME of probiotics bacteria (Ariful et al. 2010; Nazzaro et al. 2009; Sifour et al. 2014). The techniques applied for the ME of probiotics were the emulsion, extrusion and spray drying. The bead stability can be improved by using different coating materials, for example, chitosan and starch (Rokka & Rantamaki 2010). Future challenges in this area are the development of powerful new technologies as well as the selection of techniques, materials and bacterial strains to minimize the additional costs of microencapsulation (Ariful et al. 2010).

The aimed of this work was to compare the use of different polysaccharide matrixes for the microencapsulation of the probiotic *Lactobacillus plantarum* G1 and to evaluate their effect on some probiotic properties and technological skills.

Materials and Methods

Bacterial strain and culture conditions

Lactobacillus plantarum G1 was previously isolated from chicken crop and identified by 16S rRNA sequencing (Accession number: KC965107). The strain was grown on De Man, Rogosa and Sharpe (MRS) medium at 37°C.

Microencapsulation of Lb. plantarum G1 strain in sodium alginate-based capsules

Alginate (2% w/v) capsules containing the *Lb. plantarum* cells were prepared by dissolving 2 g of sodium alginate (Louis Francois) in 80 mL distilled water under constant mechanical stirring and heating at 80ºC. The solution was autoclaved and cooled to 40ºC to which 20 mL of a freshly prepared cell suspension was added and mixed. The final solution contained approximately 88.10^{11} CFU/mL. The mixture was injected through a needle into 100 mL of autoclaved and pre-cooled 0.05M $CaCl_2$ crosslinking bath. The resulting capsules were allowed to harden in the cross-linking solution for 30 min and then washed three times with sterile distilled water (Boyaval et al. 1985).

Lactobacillus plantarum cells were also encapsulated in 2% sodium alginate supplemented with 0.5% modified starch (E 1442, Danone) as described by Boyaval et al. (1985) with some modifications. The resulting capsules were called AS. Furthermore, *Lb. plantarum* cells were encapsulated in 2% sodium alginate supplemented with 2% agar-agar (Institut Pasteur-Alger) by extrusion according to a modified method of Morgan and Yarmush (1999). The obtained capsules were called AA.

Microencapsulation of LAB in K-carrageenan

Microencapsulation of *Lb. plantarum* in 2% κ-carrageenan (Sigma) was carried out by thermal gelation according to Wijffels (2000).

Kinetics of cell release

The rate of cell release from the microcapsules was monitored as function of incubation time in GIT-like conditions by measuring OD 660_{nm} of the culture as described by Klinkenberg et al. (2001). The kinetics of cell release was first tested by incubating 15 microcapsules of different materials in MRS broth (pH2.0) for 3 h at 37ºC; OD 660_{nm} was recorded at 1 h intervals over the assay period. Cell release was also studied when microcapsules were incubated in MRS supplemented with 0.3% bile salts for 7 h.

Survival of LAB in acidic conditions

The viability of free and microencapsulated cells of *Lb. plantarum* in acidic conditions was tested by incubating MRS broth (pH2.0) inoculated with approximately 10^{10} CFU/mL (free or encapsulated cells) for 2 h at 37°C. A viable count on MRS agar was carried out at 1 h intervals over the assay period after appropriate serial dilution in normal saline. The plates were incubated at 37ºC for 48 h. For microencapsulated cells, the count was determined after lysis of the capsules in 2 M phosphate buffer (pH7.0). The results were expressed in percent viability.

Tolerance to bile

The viability of free and microencapsulated cells of *Lb. plantarum* in bile conditions was studied by incubating MRS broth supplemented with 0.3% bile salts (Institut Pasteur, Alger) with approximately 10^{10} CFU/mL (free or encapsulated cells) for 8 h at 37°C. A viable count on MRS agar was carried out at 1 h intervals over the assay period after appropriate serial dilution in normal saline. The plates were incubated at 37ºC for 48 h. For microencapsulated cells, the count was determined as described before.

Survival of LAB in pancreatic enzymes by simulating the intestinal fluid

Simulation of intestinal fluid was realized according to Woraharn et al. (2010). The simulated intestinal fluid (SIF) was prepared by NaCl 9 g/L, pancreatin 10 g/L, trypsin 10 g/L and bile salts 3 g/L; pH was adjusted to 6.5 with NaOH. An appropriate number of free and microencapsulated cells (approximately 10^{10} CFU/mL) was used to inoculate the SIF, which was then incubated for 3 h at 37ºC; a viable count on MRS agar was carried out at 1.5 h intervals over the assay period after appropriate serial dilution.

Tolerance to different temperatures and cold storage conditions

The tolerance of free and microencapsulated *Lb. plantarum* to different temperatures (25, 40, 50 & 60°C) was tested by incubating the bacterium in both forms at the different temperatures for 20 min. A viable count was carried out before and after the incubation period (Mandal et al. 2006). For cold storage tolerance, the method of Brinques and Ayub (2011) with some modifications was used. The cells

were conserved in a refrigerator at 4±1°C for 15 days. A viable count was carried out each week.

Results and Discussion

The probiotic *Lb. plantarum* cells were encapsulated using different combinations of gels, consequently, different physical characteristics of the beads were obtained (Table 1). The differences were not of great importance, since the number of viable cells was not variable, the slight variation in physical characteristics were due to technical factors like the distance between the origin of extruding needle and the gelation bath, or to some gel-associated factors, like concentration, viscosity, density, elasticity and the degree of homogeneity (Mortazavian et al. 2007; Wijffels 2000). The use of automated extrusion systems will undoubtedly improve and standardize the size as well as the shape of beads.

Kinetics of Cell Release

The cell release test provides information about the capacity of a gel to retain cells within the beads. The results in Figure 1 shows that, at acidic pH, the release of cells vary from a gel to another. Thus, after 3 h of incubation, the release rate of cells from alginate beads was not detectable, while the release of cells from beads of A-A and A-S were approximately equals (0.34 & 0.3, respectively). However, carrageenan beads easily released their content when exposed to acidic pH (OD=0.254). The results indicated that the capacity of the studied gels to retain cells within the beads was different; alginate was the strongest gel, while carragenan was the weakest. Actually a progressive dissolution of carrageenan beads was observed leading to a logical increase in cell release, while alginate-based beads, shrunk and became rigid.

Figure 2 represents the effect of bile salts on *Lb. plantarum* cell release from different gels. Cell release from alginate beads increased gradually during the incubation period, it reached 0.15 after 5 h and remained constant hereafter. A-A beads displayed better results, since no released cells were observed in presence of bile salts until 3 h of incubation, where it increased gradually to reach approximatly 0.14 and remained constant after.

Like A-S beads, carrageenan beads, lost easily their content even in the first 3 h of incubation. As observed with acidic pH, bile salts influenced the physical appearance of beads, alginate-based beads were subjected to a swelling followed by a late dissolution, while carrageenan beads were dissolved completely after 3 h. The different cell-release rates obtained with the different combinations of gels could be attributed to many factors, some of them were related to the polymer itself, for example the composition, the texture, the viscosity and the degree of porosity (Mortazavian et al. 2007).

Factors that were related to bacterial cells include biomass distribution inside the bead, cell density as well as biomass distribution near the surface of the beads.

TABLE 1. Physical characteristics of the obtained microcapsules

Characteristics / Matrixes	Diameter (mm)	Aspect	Weight (g)	Number of cells/ bead	Number of beads/mL of gel
Alginate (A)	2.5	Spherical	0.0123	16×10^{10}	70
Alginate-agar (A-A)	2/3	Elliptical	0.008	15×10^{10}	70
Alginate-Starch (A-S)	2	Spherical	0.0129	22×10^{10}	50
ϰ-carrageenan (C)	2/3	Cylindrical	0.0135	n.d	n.d

n.d. not determined

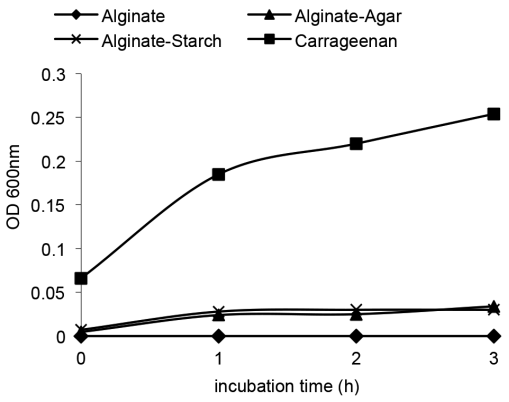

FIGURE 1. Effect of microencapsulation material on the release of *Lb. plantarum* cells from the capsules when exposed to acidic pH2.0

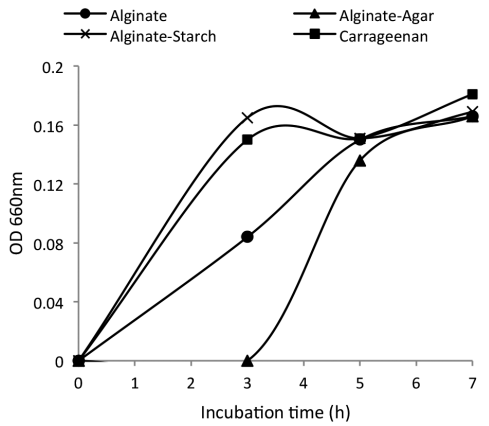

FIGURE 2. Effect of microencapsulation material on the release of *Lb. plantarum* cells when exposed to 0.3% bile salts

Furthermore, interactions between bacterial cells and the polymers were not to be excluded, since they affect the cell release rate (Anal & Singh 2007; Klinkenberg et al. 2001). Other factors with great influence were the interactions between the polymer and the external environment, in our case, bile salts, which were toxic as they display detergent properties could be the main significant factor influencing cell release after gel dissolution.

Based on the previous results, sodium alginate (2%) was selected to carry out further studies on probiotic characteristics, since it displayed a low or acceptable cell release rate in both acid and bile-containing media.

SURVIVAL OF *LB. PLANTARUM* CELLS IN GIT-LIKE CONDITIONS

Free and alginate microencapsulated *Lb. plantarum* cells were subjected to acidic conditions in order to simulate the gastric juice effect. The viable count of cells showed that free cells lost slightly less than 50% of their initial count (only 23.10^6 CFU/mL survived the acidic stress) after 1 h of incubation, the survival of cells decreased again after 2 h to reach 30% (6.10^3 CFU/mL). On the other hand, the number of microencapsulated cells that survived the acid conditions decreased too, but, it reached 23.10^7 CFU/mL after 1 h of incubation which was equal to approximately 65 %, however, slightly less than 50% of cells also survived after 2 h of incubation (Figure 3(a)).

These results indicated that the microencapsulation using sodium alginate (2%) protected efficiently the probiotic cells in conditions similar to those of stomach, since an improvement of about 18% was recorded after 2 h of incubation.

Similarly, Ding and Shah (2009) found that microencapsulation of *Lb. plantarum* and *Bifidobacterium* in sodium alginate beads (2%) leads to a percent viability of 63 and 40.55%, respectively. Other researchers also reported an interesting enhancement of cell viability of probiotic bacteria under stomachal conditions *in vitro*. The viability of microencapsulated *Lb. acidophilus* reached 60.1% as reported by Krasaekoopt et al. (2004). Furthermore, 57% of *Lb. casei* NCDC-298 cells survived gastric conditions when microencapsulated within alginate beads at 2% after 3 h of incubation (Mandal et al. 2006). In a similar work, the survival of alginate-entrapped *Lb. curvatus* G7 cells, isolated from chicken crop, in acidic conditions was higher than that of free ones (Ouled-Haddar et al. 2012).

The second barrier to overcome by probiotic bacteria to reach their site of action is bile salts, they display a detergent action (emulsification and degradation of lipids) as a result they show a considerable antibacterial effect, essentially due to cell membrane dissolution (Bergley et al. 2006; Ding & Shah 2009). Based on this, the survival of *Lb. plantarum* in 0.3% bile salts was evaluated to simulate the duodenal conditions.

According to the data presented in Figure 3(b), the number of free cells of *Lb. plantarum* decreased from 8.10^{10} to 5.10^7 CFU/mL after 4 h of incubation and continues to decrease to reach 9.10^2 CFU/mL after 8 h of incubation in the presence of bile salts. In other words, the viability reached 70% after 4 h and attained only 35% after 8 h. However, the viability of microencapsulated cells of *Lb. plantarum* was higher, since 82% (10^9 CFU/mL) of the cells survived the treatment after 4 h and 62% (65.10^5 CFU/mL) survived the bile salts treatment after 8 h.

As a result, an improvement of cell viability of about 27% was obtained using sodium alginate microcapsules. Our results are comparable to other researches, for example, 64 and 65% of the microencapsulated cells of *Lb. plantarum* and *Lb. salivarius* were capable to survive bile treatment, respectively (Ding & Shah 2007). Similar results were reported for *Lb. plantarum* and *Lb. rhamnosus*, microencapsulated in sodium alginate, the results were 68.6 and 65.4% of cell viability, respectively (Ding & Shah 2009). Better results were obtained with microencapsulated *Lb. acidophilus* CSCC2400 treated with 0.5 and 1% bile salts for 6 h, the percent of survival was high: 93 and 97%, respectively. The use of 0.1 M $CaCl_2$ as a hardening solution for alginate beads was to be mentioned in this case (Chandramouli et al. 2004).

In a more reliable experiment, simulation of the intestinal conditions was carried out by incubating both free and microencapsulated *Lb. plantarum* cells in a fluid containing pancreatic enzymes, bile salts, trypsine and NaCl for 3 h. The results shown in Figure 3(c) showed that the viability of free cells decreased to 81% (22.10^5 CFU/mL) after 1.5 h of incubation and reached 47% after 3 h of incubation. Moreover, 94.6% (95.10^5 CFU/mL) and 54% of the microencapsulated cells were capable of surviving the SIF after 1.5 and 3 h, respectively. Based on these last results, it can be concluded that the improvement in cell viability using microencapsulation was moderate and equal to 7%. It should be mentioned that an incomplete dissolution of alginate beads was observed and this could be attributed to the amylolytic activity of the pancreatic enzymes.

Microencapsulation using sodium alginate also improved the viability of *Lb. plantarum* CMU-FP002 after 3 h of incubation in a SIF having the same composition of our medium. The research was previously reported by Woraharn et al. (2010) and they found that 60% of the cells were capable of surviving. On the other hand, a *Lb. acidophilus* was encapsulated using a combination of gels (alginate 2%, xanthan 0.15% and inuline 1%) and incubated in a SIF containing only MRS broth, pancreatin (1g/L) and bile salts (4.5 g/L) in the presence of carrot juice. The percent of viability was equal to 80% (Nazzaro et al. 2009).

EFFECT OF HEAT TREATMENT

The effect of heat treatment at different temperatures (40, 50 & 60°C) on the viability of free and microencapsulated *Lb. plantarum* was studied since heat treatments (like pasteurization) are universally employed for the destruction of non-desired microorganisms found in food.

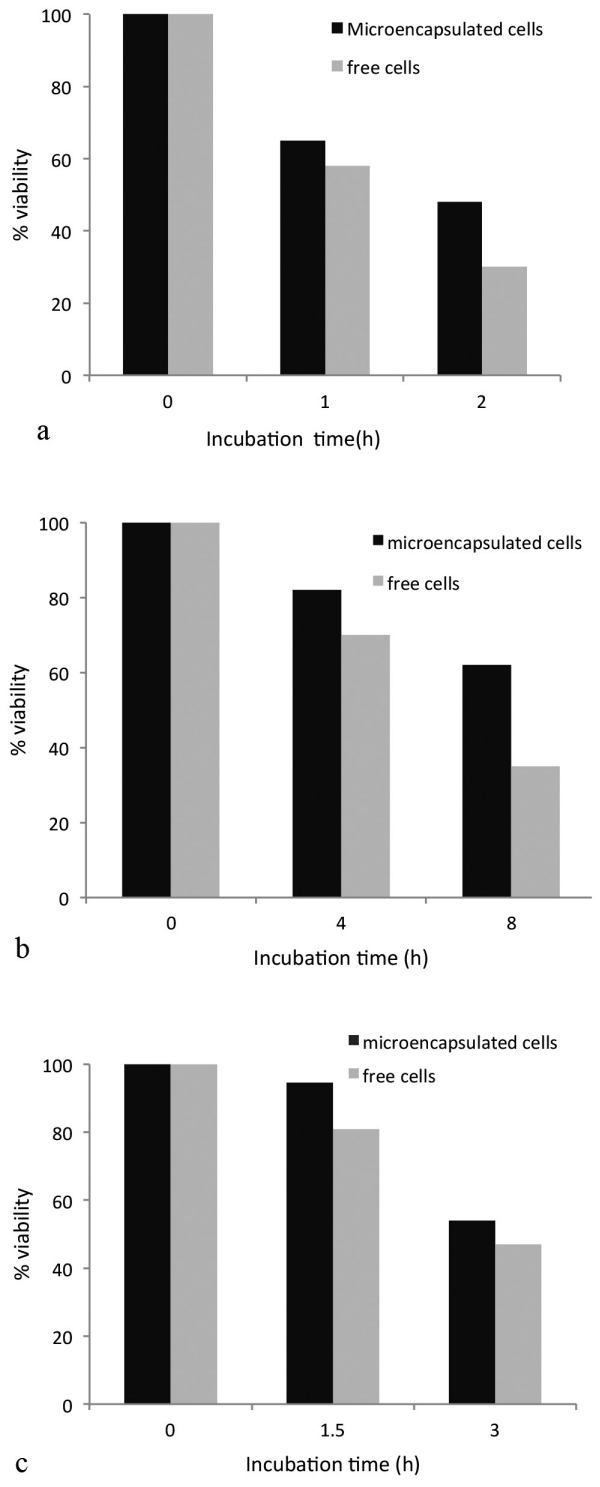

FIGURE 3. Viability of free and alginate-microencapsulated *Lb. plantarum* in GIT-like conditions a) Cells are exposed to acidic pH2.0, b) Cells are exposed to 0.3% bile salts and c) Cells are exposed to a simulated intestinal fluid containing pancreatin

The results of the experiment are presented in Figure 4. Approximately, the same initial count of cells was used for both free and microencapsulated forms (9.10^{10} CFU/mL). As shown in Figure 4, the number of coated cells did not decrease when they were exposed to 40°C, however, a slight decrease was observed with free cells (91.32% of survival). On the other hand for higher temperatures, free cells were subsequently lost, only 40.5% survived after exposure to 60°C. In contrast, the decrease in cell viability for microencapsulated cells was less important since 90% of cells survived at 50°C and 67.94% at 60°C.

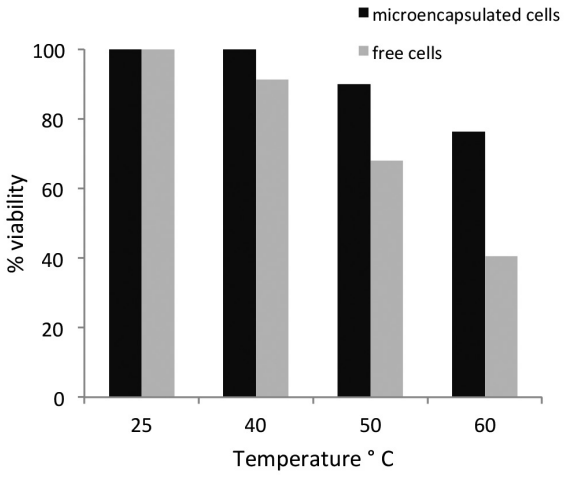

FIGURE 4. Effect of different temperature treatments on the viability of free and alginate-microencapsulated *Lb. plantrum* cells. Cells were subjected to temperature treatments for 20 min

Based on these results, cells of *Lb. plantarum* were protected against heat treatment by microencapsulation using sodium alginate at 2%. Several researchers reported the protective role of alginate microcapsules, for example Mandal et al. (2006), studied the effect of temperature (55, 60 or 65°C for 20 min) on cell survival of *Lb. casei* NCDC-296 encapsulated within different concentrations of sodium alginate (2, 3 and 4%), they found that the survival of encapsulated *L. casei* was better during heat treatment as compared to free cells. In addition, Kim et al. (2008) tested the viability of microencapsulated *Lb. acidophilus* ATCC 43121 when exposed to 65°C for 30 min; the survival percent was high and equal to 90%.

EFFECT OF COLD TREATMENT

Usually, food storage exerts a negative effect on LAB viability, particularly prolonged storage. For this, the effect of 15 days storage at 4°C on *Lb. plantarum* was carried out. As seen in Figure 5, the results showed an important reduction in free cells number (63% survived after 15 days), however, microencapsulated cells, resisted well and kept their viability to reach 93.4%. Like the heat treatment, it can be suggested that microencapsulation within alginate beads (2%) confers a protection to LAB cells against cold storage.

According to Brinques et al. (2011), the microencapsulation of *Lb. plantarum* BL011 using alginate reduced the effect of cold prolonged storage, when introducing the microcapsules in yoghurt, 27% of the cells survived after 36 days of conservation. Better results were

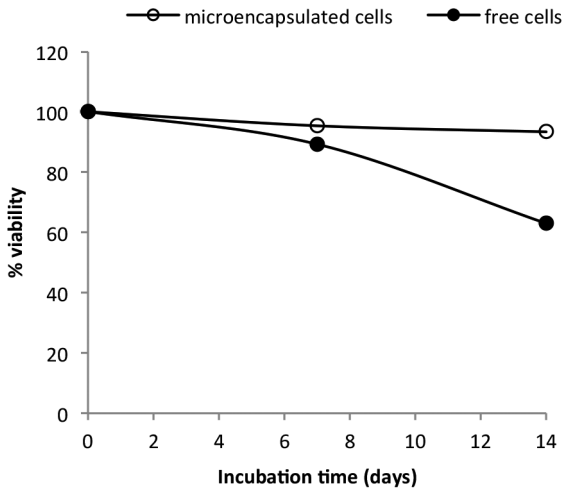

FIGURE 5. Effect of cold storage (4 °C) on the viability of free and alginate-microencapsulated *Lb. plantarum* cells

obtained with *Lb. plantarum* CMUF-002, since 75% of the cells survived after 8 weeks of cold storage (Woraharn et al. 2010). Moreover, a study on the viability of *L. acidophilus* and Bifidobacteria during a combination of heat-related treatments in yoghurt showed that storage at 2°C for 20 days leads to the highest viability of *L. acidophilus*, whereas for bifidobacteria the highest viability was obtained when yogurt was stored at 8°C (Mortazavian et al. 2006).

CONCLUSION

The results indicated that kinetics of cell release of the studied gels were different; alginate seems to be a relatively strong gel. Moreover, the microencapsulation within alginate beads improved the viability of the probiotic cells in the presence of acid, bile and pancreatin when compared to free cells. Heat and cold treatments affected less the viability of the alginate-protected cells. The above findings gave an additional evidence that microencapsulation within sodium alginate (2%) is the best choice for functional food production. Moreover, further investigations on the delivery of microencapsulated probiotic ingredients using an *in vitro* system are required.

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