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EFFECT OF DIFFERENT FOOD STRESS CONDITIONS ON THE VIABILITY OF ENCAPSULATED *Lactobacillus plantarum* AND *Lactobacillus casei* ISOLATED FROM KLILA (AN ALGERIAN TRADITIONAL FERMENTED CHEESE)

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ABSTRACT

Klila is one of a variety of Algerian traditional cheeses. The present work aimed to study the viability of two lactobacilli: *Lactobacillus plantarum* and *Lactobacillus casei* isolated from Klila and immobilized by extrusion in 2% sodium alginate under different stress conditions (different NaCl concentrations, sugar stress, different pH values and simulated gastrointestinal conditions) during storage at 4°C.
Results showed that viability of encapsulated cells was enhanced at high salt concentration; at 9%, viability of free *Lb. casei* cells decreased by 9 Log CFU/ml 14 days, and by only 0.2 Log CFU/ml for encapsulated cells. Encapsulated *Lb. plantarum* resisted up to 28 days while viability of free cells decreased by 9 Log CFU/ml in the 7th day at 9%.
pH 2 showed the lowest viability which is decreased as the time of storage increased. After 14 days of storage, *Lb. casei* free cells decreased by 9 Log CFU/ml, encapsulated ones by only 2.8 Log CFU/ml, *Lb. plantarum* free cells by 9 Log CFU/ml and by 2.6 Log CFU/ml for encapsulated cells. Exposure to simulated gastrointestinal conditions showed that *Lb. casei* resisted such conditions compared to non-encapsulated cells which was not the case with *Lb. plantarum.*
Storage in a commercial strawberry beverage showed that free cells did not resist more than the7th day while coated cells resisted till the 14th day. We conclude that encapsulation enhance the viability of bacteria in harsh conditions.

Keywords: Klila, lactobacilli, encapsulated, extrusion, viability, stress conditions

INTRODUCTION

Probiotics are live microorganisms which when administered in adequate quantities confer beneficial effects on the health of the host **(FAO/WHO, 2002)**. To be considered as potentially probiotic, microbial cells should be able to stay viable and stable in front of storage conditions, they should also overcome the harsh conditions of the human or animal gastrointestinal tract. Probiotics are able to improve the intestinal microbial balance through various mechanisms **(Krasaekoopt *et al.*, 2003; Vasiljevic and Shah, 2008; Vrese and Schrezenmeir, 2008; Bron *et al.*, 2012; Amine *et al.*, 2014),** in addition, they possess antimutagenic and anticarcinogenic properties. Even though they are regarded as safe, probiotics should not carry transferable antibiotic resistance genes **(Havennar and Huisint't Veld, 1992; Holzapfel and Schillinger, 2002).** On the other hand, viability loss of probiotic cells during their storage within a food matrix is a commonly encountered problem **(Brinques and Ayub, 2011; Amine *et al.*, 2014)**. One of the solutions is microencapsulation, known to be suitable for the oral delivery of living probiotic bacteria making it a powerful and a promising food technology **(Heidebach *et al.*, 2009; Malmo *et al.*, 2013; Damodharan *et al.*, 2017)**. It lowers cell loss and consequently increases cell viability, which is usually altered during storage, industrial processing, or throughout the food digestion process, because of the high resistance conferred by the immobilzation **(Chavarri *et al.*, 2010; Chen *et al.*, 2017)**.
Basically, using different cell encapsulation techniques, such as direct gelation, emulsification or complex coacervation, the probiotic bacteria are entrapped into polymeric microbeads. The matrix itself is made of one or more types of bio-polymers, commonly used as food additives, all originating from nature, like alginate and carrageenan from algae, starch, arabic gum, soy and pea protein from plants, gellan and xanthan from bacteria and milk, gelatin and whey protein from animal origin. These polymers were reported to be biologically compatible and safe for both bacteria and consumer. **(Dong *et al.*, 2013; Bosnea *et al.*, 2014; Wang *et al.*, 2014; Eratte *et al.*, 2015)**.
Alginate, known to be regarded as safe, bioavailable, biocaompatible and cost effective as well as easily prepared is widely used to immobilize probiotic microorganisms. This anionic polymer is obtained from brown seaweed, and is composed of a succession of 1,4-linked β -D-mannuronic acid and α-L-guluronic acid monomers **(Mokarram *et al.*, 2009)**.
According to **FAO/WHO (2001)** guidelines, and prior to be used as food added probiotics, the new candidate bacterial strains should be necessarily evaluated for their survival rate in the following conditions: gastrointestinal, processing as well as storage conditions. In addition, lactic acid bacteria are evidently the most important group of bacteria comprising powerful probiotics, as their beneficial role in food, agriculture and medicine is very well documented **(Bintsis, 2018)**.
In the present work, the principal aim is to study the viability of two probiotic lactobacilli strains (*Lb. plantarum* and *Lb. casei*) isolated from a traditional Algerian fermented cheese "Klila" immobilized in sodium alginate beads after exposure to different stress conditions (different NaCl concentrations, strawberry juice, different pH values and simulated gastrointestinal conditions).

MATERIALS AND METHODS

Isolation and identification of lactobacilli

After serial dilution of "Klila" in normal saline, and seeding on MRS agar plates, the incubation was performed at 37 °C for 48 h. Separated colonies were taken and inoculated again on MRS agar till obtaining pure cultures with the same shape, same color, and same size. Morphology, Gram staining, catalase test, growth at various temperatures, and the carbohydrates fermentation test were performed to identify the bacterial isolates (the used carbohydrates were: sucrose, mannose, glucose, galactose, xylose, sorbose, maltose, dextrine, raffinose, starch, glycerol, salicine and adonitol) **(Schillinger and Lücke, 1987; Stiles and Holzapfel, 1997)**. The identification was confirmed by 16S rDNA technique.
Stock cultures were preserved in MRS broth (CONDA, pronadisa, Madrid, Spain) with glycerol (30%) at −20 °C and were subcultured twice in MRS broth

(pH 6.2) for activation prior to be used. The purity was confirmed by culture on MRS agar and by Gram staining; cultures were incubated at 37 °C for 48 h.

Encapsulation of bacterial cells

The described method by **Sheu *et al.* (1993)** with some modifications was used. An overnight culture of bacteria on MRS broth was centrifuged for 10 min at 6,000 rpm. The obtained cell pellets were then washed with 10 ml of normal saline (0.9%) and were resuspended in the same volume of distilled water. From a stock culture, fresh cells were prepared for each one of the experiments carried out in duplicate (n=2). Cell concentration was adjusted to approxiametely ~10^{10}–10^{11} CFU/ml. This bacterial suspension was mixed with autoclaved sodium alginate (SIGMA, Milan, Italy) solution at 2% then let droplet through a syringe of 2.5 ml in a cooled solution of $CaCl_2$ under slight agitation.
The amount of beads corresponding to 1 ml of the bacterial suspension was quantified, the cell suspension was mixed with a solution of 2% (w/v) sodium alginate, and the number of beads corresponding to 1 ml was counted. This procedure was repeated 5 times. The average obtained number was 50 beads per 1 ml.

Tolerance to acid pH

One ml of the bacterial suspension mentioned above (free cells) and 50 beads of encapsulated bacteria (immobilized cells) were added to 9 ml of normal saline (0.9% NaCl) separately at three pH values, 2, 4 and 7 according to the method described by **Bosnea *et al.* (2014)**. The incubation was carried out at 4 °C for 3 h, 7 and 14 days for evaluation of the applied stress and 1 ml of the previous solutions was transferred to 9 ml of phosphate buffered saline PBS and after serial dilutions viable cell count was determined on MRS agar. This experiment was carried out in duplicate.

Tolerance to NaCl

Three saline solutions with different salt concentrations were used (3%, 6% and 9%), and the same procedure used for the pH stress was followed. The mixture was incubated at 4 °C for 3 h, 7, 14, 21 and 28 days **(Bosnea *et al.*, 2014)**. Cell count was determined as previously described.

Storage in strawberry juice

To evaluate the effect of strawberry beverage composition on the viability of both strains, one ml of free and encapsulated cells was introduced separately in tubes containing 9 ml of a commercial strawberry beverage (TOUDJA, Bejaia, Algeria). The tubes were stored at 4 °C, and viable count on MRS agar was evaluated at 0, 3 h, 7 and 14 days. The initial cell number in the beverage was approximately 2×10^{10} CFU/ml for both strains **(Nualkaekul *et al.*, 2013)**.

Viability under simulated gastrointestinal (GI) conditions

The assay was performed according to **Vizoso *et al.* (2006)**. Viable cell counts were monitored during initial exposure to gastrointestinal (GI) conditions, after 2 hours to assess the gastric transit tolerance, and after 4 h to evaluate the intestinal transit tolerance. The simulated gastric juice (SGJ) was prepared using 0.3% (w/v) pepsin (SIGMA, Milan, Italy), 0.5% (w/v) NaCl, 0.22 % (w/v) KCl, 0.12% (w/v) $NaHCO_3$ and 0.022% (w/v) $CaCl_2$; the simulated gastric juice was acidified with HCl (0.1M) to pH 2.5. Simulated intestinal juice (SIJ) was prepared with 0.1% (w/v) pancreatin and 0.128% (w/v) NaCl, 0.023% (w/v) KCl, 0.64% (w/v) $NaHCO_3$, 0.5% (w/v) bile salts (SIGMA, Mian, Italy), pH was adjusted to 7.0.
Both solutions were filtered using 0.22 µm membranes filters. Free and encapsulated *Lb. plantarum* and *Lb.casei* were exposed to simulated GI conditions and their viability was calculated for different times (0, 2 and 4) hrs.

Data analysis

Analysis was carried out in duplicate; MATLAB version 2008 was used for graphic performance. The data were presented as mean ± standard deviation (SD) values.

RESULTS AND DISCUSSION

A total of ten (10) Gram-positive, rod-shaped, catalase-negative, ADH-negative, either homofermentative or heterofermentative, able to grow at 45 °C and unable to grow at 10 °C, belonged to the *Lactobacillus* genus **(Xanthopoulos *et al.*, 2000)**. Molecular identification revealed that the two strains belonged to the species *Lb. plantarum* (Accession number: MH342626) and *Lb. casei* (Accession number: KY764324).

Tolerance of free and encapsulated bacterial cells to acidic pH stress

The effect of pH on the viability of free and encapsulated bacteria was tested at different times of incubation and at three different pH values (2, 4 and 7). Results are presented in figures 1 (a, b, c) and figure 2 (a, b, c) for *Lb. casei* and *Lb. plantarum*, respectively, they showed that the lowest viability was recorded at pH 2 and it decreased as the time of storage increased. For example, the number of *Lb. casei* free cells was reduced by 8 Log CFU/ml after 7 days and by 9 Log CFU/ml after 14 days, however, encapsulated ones were reduced only by 2Log CFU/ml after 7 days and by 2.8 Log CFU/ml after 14 days. For *Lb. plantarum*, the cell number decreased by 6.9 Log CFU/ml after 7 days and by 9 Log CFU/ml after 14 days for free cells and by 2.3 Log CFU/ml and 2.6 Log CFU/ml after 7 and 14 days, respectively for encapsulated cells. The optimal pH for storage for both isolates and in both cases (free or encapsulated) was 7. The study of **Bosnea *et al.* (2014)** reported similar results**,** since at low pH values; the viability of free cells decreased significantly, however, encapsulated ones showed a higher survival rate suggesting that sodium alginate microenvironment is offering to cells an acid resistance feature. In contrast, and after 3 h of incubation at 4 °C, no effect was observed on the viability of both free and encapsulated forms, at pH 4.0 and pH 7.0. In a report of **Krasaekoopt *et al.* (2006),** microencapsulated *Lb. acidophilus* and *Lb. casei* in sodium alginate and chitosan beads were added to yoghurt at pH 4.7 and stored at 4°C. Viability increased by one cycle Log compared to free cells in the cited conditions.
According to **Bosnea *et al.* (2014)**, the restored viability of bacterial cells at low pH environments, is attributed to the presence of membrane proton pumps or proton/cation exchange systems, their role is to maintain the cytoplasm pH near neutrality by controlling the influx of protons. In highly acidic pH conditions (H^+ concentration is very high), cells will be disrupted and will consequently lose their viability, this is actually due to the intracellular acidification resulting from the drastic decrease in pH gradient (the difference between the intracellular and the extracellular pH), caused by the dysfunction of the pH regulatory pumps. Alginate microencapsulation is providing an additional physical defense mechanism to the probiotic cells through a barrier effect.

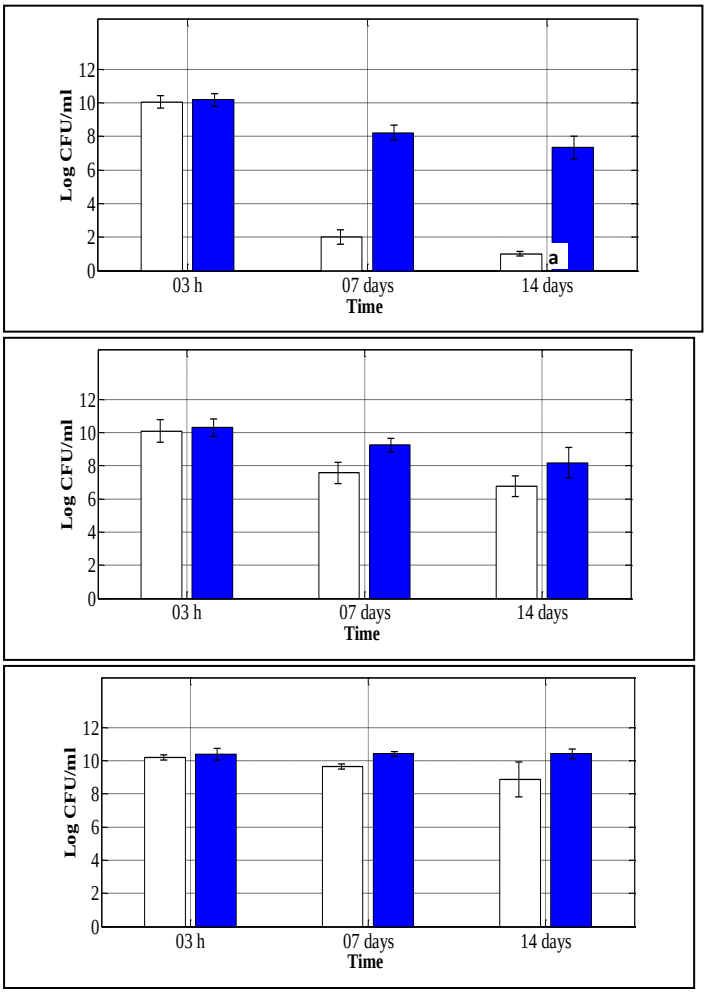


Figure 1 Viability of free (uncolored) and encapsulated (colored) *Lb. casei* at different pH values (a: pH=2, b: pH=4, c: pH=7) after their storage at 4 °C for 3 h, 07 and 14 days.

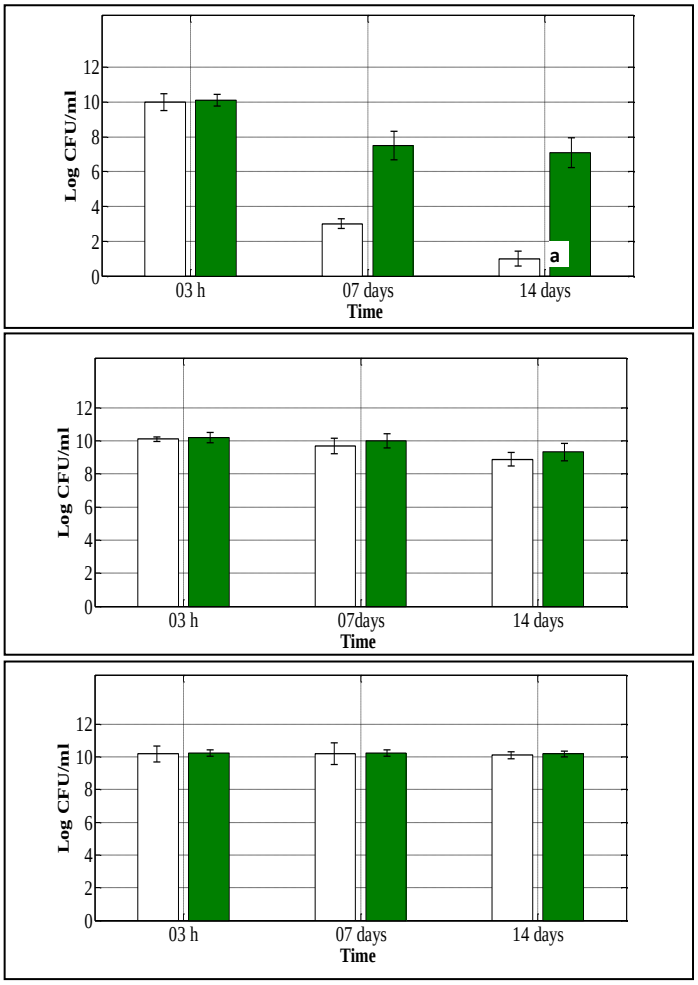


Figure 2 Viability of free (uncolored) and encapsulated (colored) *Lb. plantarum* at different pH values (a: pH=2, b: pH=4, c: pH=7) after their storage at 4 °C for, 3 h, 07 and 14 days.

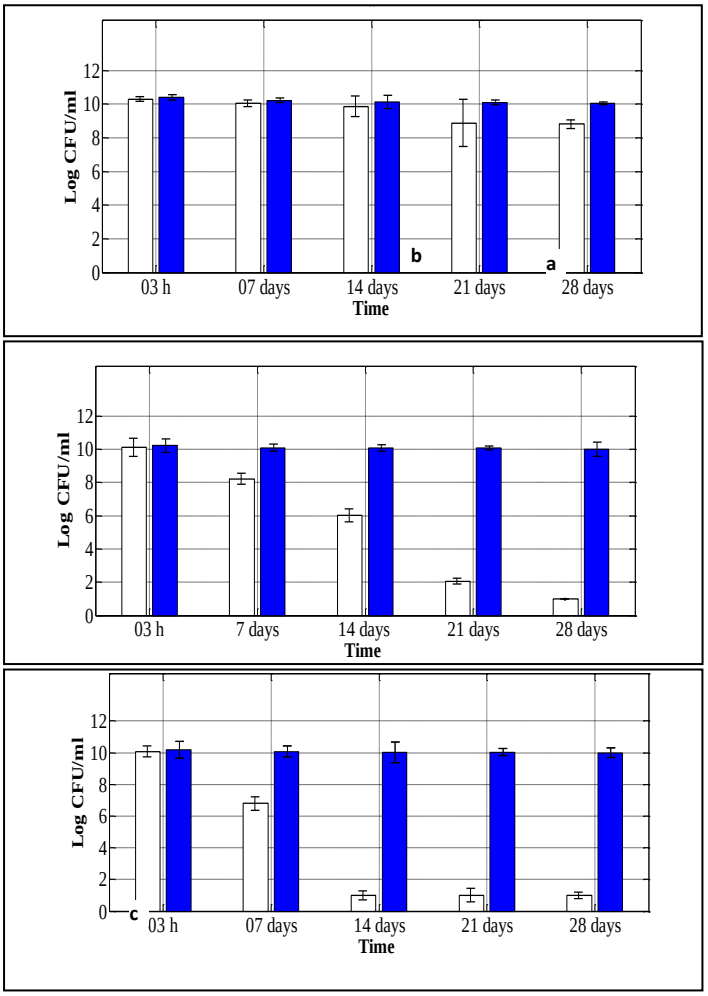


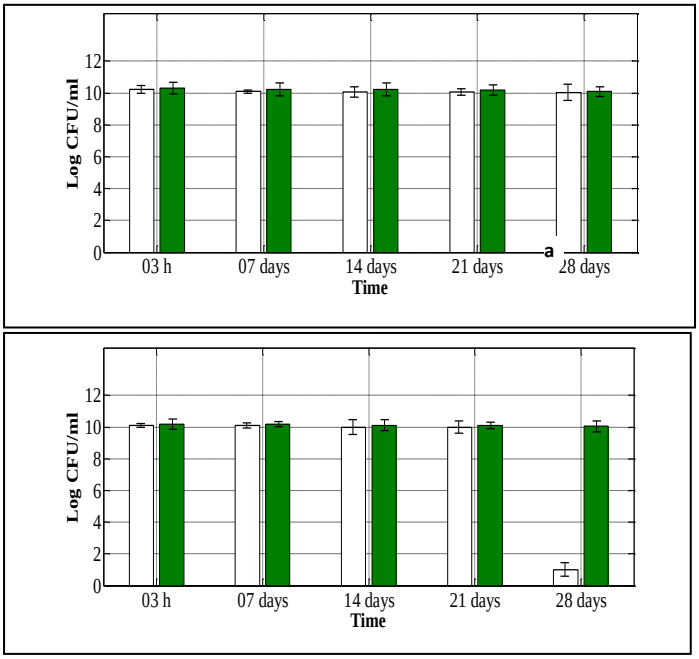
Figure 3 Viability of free (uncolored) and encapsulated (colored) *Lb. casei* at different NaCl concentrations (a: NaCl=3%, b: NaCl=6%, c: NaCl=9%) after their storage at 4 °C for, 3 h, 07, 14, 21 and 28 days.

Tolerance to NaCl

The effect of different salt (NaCl) concentrations on the survival of free and encapsulated cells are presented in figure 3 (a, b, c) for *Lb. casei* and in figure 4(a, b, c) for *Lb. plantarum*.

As clearly shown in the results, the number of encapsulated viable cells was higher than that of free ones at all salt concentrations, it remained unchangeable. Moreover, viability was better at the concentration of 3% compared to the other concentrations (6% and 9%) for *Lb. casei* free cells, where at 3% it was reduced by 1.7 Log CFU/ml after 28 days of storage and by 4 Log CFU/ml and 9 Log CFU/ml after 14 and 28 days, respectively. At 6%, it was reduced by 8.06 Log after 21 days and 9.12 cycles after 28 days and at 9%, it was reduced by 3.6 Log CFU/ml and 9 Log CFU/ml after 7 and 14 days, respectively. However, *Lb. plantarum* free cells were able to resist both 3% and 6% NaCl, but at 9%, viability was reduced by 9 Log CFU/ml after 14 days of storage at 4 °C . However, encapsulated cells for both isolates resisted all salt concentrations. These results indicated that these bacteria resist harsh conditions of osmolarity, and showed that sodium alginate gives more protection to bacterial cells to resist such conditions of stress. In addition, the concentration of 3% gave the highest resistance and viability, but it decreased by the increase of salt concentration. Our results agree with those of **Gomes *et al.* (1998)** where they reported that the number of *Lb. acidophilus* decreased by the increase of salt concentration above 3.0%. Furthermore, in a study of **Cruz *et al.* (2015)**, when free *Lb. acidophilus* cells were exposed to 6% NaCl, viability decreased and by consequence, the microorganisms become less resistant to osmotic stress. The observed decline in the survival and resistance during storage could be atributed to the reduction in water activity and to the increase in osmolarity **(Jorgensen *et al.*, 1994)**.

Salts is usually added to foods as a taste enhancer or to prevent spoilage, therefore, at higher levels, the NaCl content could negatively influence viability of the probiotic cells. However, little is known on the mechanism by which microencapsulated probiotics resist to high salt concentrations found in cheese and other salted foods **(Bosnea *et al.*, 2014)**.



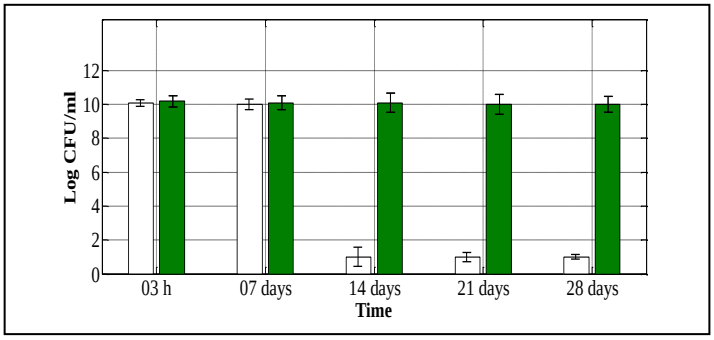


Figure 4 Viability of free (uncolored) and encapsulated (colored) *Lb. plantarum* at different NaCl concentrations (a: NaCl=3%, b: NaCl=6%, c: NaCl=9%) after their storage at 4 °C for, 3 h, 07, 14, 21 and 28 days.

Storage in strawberry juice

The survival of probiotics embedded in the food matrix could be affected during food processing and storage because they are exposed to several stress conditions namely osmotic stress, high temperature, acidic conditions…etc. For example, the presence of high sugar levels in sweetened foods affects their survival due to the high osmotic conditions **(De Prisco *et al.*, 2015)**.

In the present work, the survival of free and microencapsulated cells in strawberry juice was evaluated. A decrease in viability was observed with time for free cells only, however, the number of encapsulated cells remained unchanged, as shown in figure 5a for *Lb. casei* and figure 5b for *Lb. plantarum*, this means that encapsulation in sodium alginate enhanced the viability of bacteria in sugar stress.

The number of *Lb. casei* free cells was reduced by 1.9 Log CFU/ml, and 9 Log CFU/ml after 7 and 14 days, respectively, whereas that of encapsulated cells decreased only by 0.2 Log CFU/ml after 14 days. For *Lb. plantarum*, viability of free cells decreased after 14 days to reach only 1 Log CFU/ml after 14 days, while for encapsulated cells it was reduced by 0.2 Log CFU/ml after 14 days. The study of **De Presco *et al.* (2015)** conducted with apricot jam as a high osmotic pressure food, revealed a significant decline in the survival of free cells compared to microencapsulated ones both subjected to osmotic stress for three hours. The cell number was reduced by about 2 Log cycles for free cells and by about 0.67 Log cycle for microencapsulated ones.

Our result is in agreement with the findings of **Nualkaekul *et al.* (2013)**, the researchers compared the survival of *Lb. plantarum* and *Bifidobacterium longum* in alginate or pectin beads during storage in pomegranate and cranberry juices. They found that the survival of the cells was improved considerably after being entrapped within both matrices. However, free cells of the two strains died after one week of storage in caranberry juice. Furthermore, free cells of *Lb. plantarum* died after 4 weeks and those of *B. longum* after 1 week of storage in pomegranate juice.

During storage, the cell viability decrease for free cells indicated that probiotic cells were highly influenced by the juice composition. In a similar report, **Vinderola *et al.* (2002)** indicated that the reduced viability in fruit juices may be prinicipally caused by the presence of some inhibitory food additives such as colorings and aoma. In another study, it was shown that the acidic pH of fruit juices ranging from 2.5 and 3.7 with benzoic and lactonic acids may reduce viability of probiotics too **(Sheehan *et al.*, 2007)**.

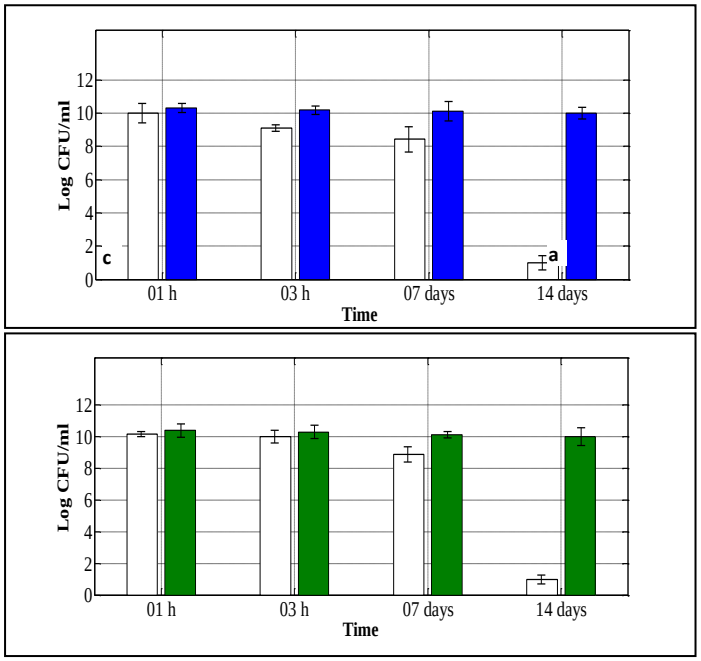


Figure 5 Viability of free (uncolored) and encapsulated (colored) *Lb. casei* (a) and *Lb. plantarum* (b) after its storage in fruit juice at 4 °C

Viability under simulated GI conditions

This test was performed to evaluate the capacity of the probiotics to overcome the stomachal barrier, for this, free and microencapsulated cells were incubated in simulated gastric juice (SGJ), their respective counts were determined. The initial number of viable free cells (approximatly 10.2 Log CFU/ml) decreased to 8. and to 8.3 Log CFU/ml for *Lb. casei* and *Lb. plantarum*, respectively following 2h exposure to SGJ (figures 6a and 6b).

These results suggested that free and encapsulated *Lb. plantarum* and *Lb. casei* cells showed a slight decrease in the number of cells in the acidic environment (pH 2.0). After 4 hours of incubation in simulated intestinal conditions, *Lb. plantarum* showed the same results for both free and encapsulated cells with a decrease of 2.1 Log CFU/ml. For *Lb. casei*, encapsulated cells were reduced in number by also 2.2 Log CFU/ml however, free cells viability was decrease by 7 Log CFU/ml.

In the study of **Dimitrellou *et al.* (2016)**, aiming to evaluate the survival of spray-dried microencapsulated *Lb. casei* ATCC 393 cells exposed to simulated gastrointestinal conditions, the researchers reported a higher survival rate of the entrapped cells compared to free ones, both were subjected to simulated gastric juice and bile salts, in this case, a continuous loss in *Lb. casei* viability was observed for free cells after exposure to simulated gasrtric conditions, the viable cell counts dropped by 4.03 Log CFU/g at pH 2.0. Similar findings were also reported by other researchers working on the same species, indeed, free *Lb. casei* ATCC 393 cells showed a decreased survival rates at low pH values **(Sidira *et al.*, 2010; Li *et al.*, 2011; Xu *et al.*, 2016)**. In addition, spray-drying microencapsulation of *Lb. plantarum* provided an efficient protective effect facing bile salts solutions when compared to freeze-drying, this was highlighted by **Rajam *et al.* (2012)**. Moreover, **Mandal *et al.* (2006)** reported also an improved viability of *Lb. casei* NCDC-298 cells after being exposed to 1% and 2% bile salts for 12 h, this improvement is resulting from alginate encapsulation, and it was proportional to the polymer concentration. In the same context, the study of **Brinques and Ayub (2011)**, using *Lb. plantarum* BL01, reported that incubation in SG medium showed no change in cell viability compared to free cells, in other words, viability of both free and immobilized cells was deeply affected by the incubation conditions, excluding the protective effect of the polymeric matrix. Other researchers failed to protect probiotic cells through immobilization too, this is the case of **Sultana *et al.* (2000)**, and **Gbassi *et al.* (2009),** who reported a lower protective effect on probiotics at pH 2.0 in the former or a total loss of viability of microencapsulated *Lb. plantarum* in the later. Moreover, **Michida *et al.* (2006)** found that *Lb. plantarum* cells tolerated pefectly the SIJ conditions even at the "free" status.

Other authors suggested that the release of bacteria from their encapsulating material may be due to factors related to bacterial cells including biomass distribution inside the bead, cell density as well as biomass distribution near the surface of the beads. Furthermore, interactions between bacterial cells and the polymers are not to be excluded, since they affect the cell release rate **(Klinkenberg *et al.*, 2001; Anal and Singh, 2007)**.

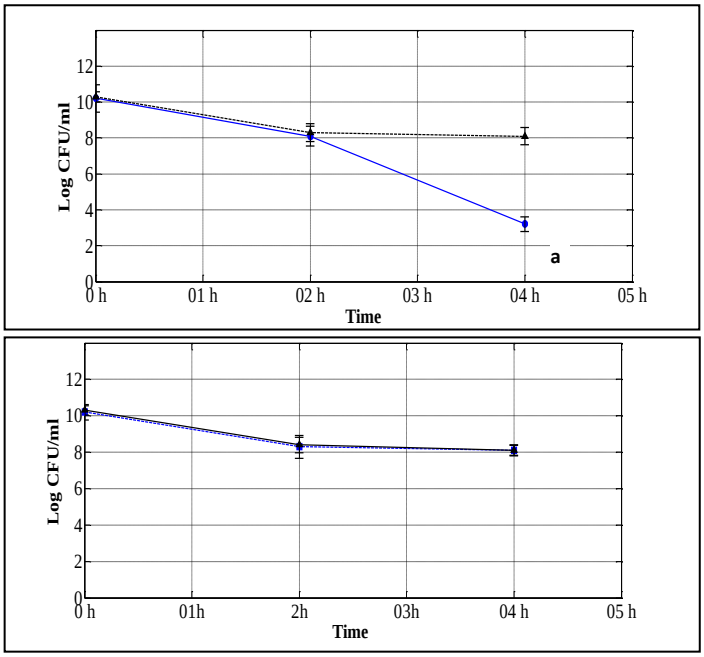


Figure 6 Viability of free (blue line) and encapsulated (black line) *Lb. casei* (a) and *Lb. plantarum* (b) under simulated gastrointestinal (SGI) conditions

CONCLUSION

This study presented the encapsulation of probiotic bacteria *Lb. casei* and *Lb. plantarum* in sodium alginate via extrusion technology. Results showed that encapsulation enhanced viability of both isolates compared to non-encapsulated ones to the tested harsh conditions; however, encapsulation was more efficient with *Lb. casei* compared to *Lb. plantarum.*

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