

Study on the use of different combined biopolymers for the microencapsulation of the probiotic bacteria *Lactobacillus curvatus* B431

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Abstract

This study has focused on the confirmation of the protective role of microencapsulation of the probiotic lactic acid bacterium *Lactobacillus curvatus* B431 isolated from traditional butter by several matrixes against the hostile gastrointestinal tract conditions. The tolerance of *Lb. curvatus* to acidic pH, bile salts and pancreatic enzymes was evaluated. In addition, the effect of cold storage and heat treatment on viability was also tested. The results showed that the strain is resistant to low pH, bile salts, digestion by pancreatic enzymes, cold storage and heat treatment, particularly when encapsulated in sodium alginate. Microencapsulation of this strain in biopolymer enhanced the viability of probiotic strain in gastrointestinal-like conditions.

Keywords: Biopolymer, alginate, *Lactobacillus curvatus*, microencapsulation, probiotic

1. Introduction

Probiotics are microorganisms that transfer a variety of health benefits to the host and have numerous applications in food and medicine. Probiotic bacteria should be safe for consumption, reach the intestine alive in large numbers and provide specific health benefits to the host. These bacteria should maintain the balance of the intestinal flora by altering favorably the gut environment in such a manner that the growth of friendly beneficial bacteria are promoted and harmful disease causing organisms are inhibited [1]. Lactic acid bacteria (LAB) are the most important probiotic microorganisms typically associated with the human gastrointestinal tract. They are mainly used as starter cultures and play an important role in food preservation. They have the capacity to inhibit spoilage and pathogenic bacteria as a result of the drop in pH and antimicrobial activity of their metabolites such as lactic, acetic acid or bacteriocin; they also play an important role in microbiological stability and production of aroma compounds in various food products [2-5]. Beneficial effects depend on the ability of the probiotic strains to maintain viability in the food during shelf-life and to survive the natural defenses of the host and multiply in the gastrointestinal tract [6]. Many factors such as acidity, oxygen content, and concentration of lactic and acetic acids affect the survival of probiotics in food and in the gastrointestinal tract of the host. Several methods have been used to enhance the viability of probiotics, including selection of resistant strains, stress adaptation, incorporation of micronutrients, and microencapsulation [7]. Encapsulation is the most developed method for cell immobilizing which involves a

large number of processes that entrap an active material in mainly spherical particles in order to immobilize it, protect it, control its release and provide new physical properties or functions. Immobilized cells have many advantages over free-cell cultures which include higher productivity as a result of high cell densities, long-term operational stability, improved control process, protection against contamination, improvement of plasmid stability and improvement of the ability to separate and reuse cells [8, 9].

The encapsulation is defined as the technology for packaging solids, liquid, or gaseous materials in capsules to protect the microencapsulated materials from the surrounding environment, or conversely to protect the environment from the active ingredient, while nutrients and products can migrate through the semi-permeable membrane of the capsule. Besides enhancing the viability of bacteria, microencapsulation facilitates handling of cells and allows a controlled dosage. The materials used for encapsulation consist of a semi-permeable, spherical, thin and strong membrane surrounding a solid or liquid core, with a diameter varying from a few microns to 1 mm. Polysaccharides like alginate, gellan, *k*-carrageenan and starch are the most commonly used materials in microencapsulation of bifidobacteria and lactobacilli [7, 9-11].

Microencapsulation of probiotic cells has been recently used as an efficient method for improving the viability of probiotic bacteria in fermented milk drinks, fermented frozen dairy desserts, ice-cream and juices, and simulated gastrointestinal tract [12]. Various researchers have studied the efficiency of encapsulation of *Lb. acidophilus* with

sodium alginate in calcium chloride (CaCl_2) to protect this organism from the harsh acidic conditions in gastric fluid [13-15].

The main purpose of this work was to investigate the effect of encapsulation with several biopolymers on the viability of a probiotic *Lactobacillus curvatus* B431 isolated from traditional butter.

2. Materials and Methods

2.1. Microencapsulation of bacterial strain in different biopolymers

The isolate used in this study was previously isolated from traditional butter and identified as *Lb. curvatus*. *Lb. curvatus* B431 was grown at 37°C in Man Rogosa Sharpe (MRS) broth (Biokar Diagnostics, France). To confirm the purity of the isolate the strain was streaked on MRS agar plates (MRS broth + 15g/L agar). *Lb. curvatus* B431 was cultivated in MRS broth at 37°C for 20h. Then the cells were harvested by centrifugation (4000g for 3 min) and washed twice with sterile normal saline and resuspended in normal saline, the final OD was 1.6 at 660 nm.

Lb. curvatus B 431 cells were microencapsulated using alginate (2 % w/v). Capsules containing the cells were prepared as follows: 80 mL of 2% sodium alginate (Louis Francois) was sterilized by autoclaving (121°C per 15min) and cooled to 40 °C to which 20 mL of a freshly prepared cell suspension was added and homogenized using a magnetic stirrer (the final solution contained approximately $8.8 \cdot 10^{11}$ CFU/mL). The resulting mixture was introduced into a sterile syringe (2.5ml) and was injected through a needle into 100 mL of CaCl_2 solution (0.05M+0.1% tween 80) previously autoclaved and cooled. The resulting beads formed were then left for 30 min to harden in the cross-linking solution, and then washed three times with sterile distilled water. Finally the beads were conserved in normal saline at 4°C for further utilization. On the other hand, 2% sodium alginate supplemented with 0.5 % modified starch (E 1442, Danone) was used for the encapsulation of *Lb. curvatus* B431 cells [16]. Furthermore, extrusion method was used for the encapsulation of *Lb. curvatus* B431 cells in 2% sodium alginate supplemented with 2 % agar-agar (Institut Pasteur- Alger) [17]. Finally, thermal gelation was employed to microencapsulate *Lb. curvatus* B431 in 2 % κ -carrageenan (Sigma) [18].

2.2. Effect of GIT-like conditions on the rate 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 660nm of the culture as described by Klinkenberg *et al.* [19]. The effect of acidic conditions on the kinetics of cell release was first tested by incubating 15 microcapsules of different materials in MRS broth (pH 2.0) for 3 hours at 37 °C; OD 660nm was recorded at 1 hour intervals over the assay period. Cell release was also studied when microcapsules were incubated in MRS supplemented with 0.3% bile salts for 7 hours.

2.3. Evaluation of probiotic skills of *Lb. curvatus* in vitro

2.3.1. Effect of acidic conditions on the survival of free and encapsulated *Lb. curvatus*

The effect of acidic conditions on the viability of free and microencapsulated cells of *Lb. curvatus* B431 was determined by incubating MRS broth (pH 2.0) inoculated with approximately 10^{10} CFU/mL (free or encapsulated cells) for 2 hours at 37 °C. A viable count on MRS agar was carried out at 1h intervals over the assay period after appropriate serial dilution in normal saline. The plates were incubated at 37 °C for 48 hours. For microencapsulated cells, the count was determined after lysis of the capsules in 2M phosphate buffer (pH 7.0). Samples were plated on MRS agar. The plates were incubated for 48 hours at 37 °C and the probiotic bacteria were enumerated as CFU/mL. Results were expressed in percent viability.

2.3.2. Effect bile salts on the survival of free and encapsulated *Lb. curvatus*

To evaluate the effect of bile salts on the viability of free and microencapsulated cells of *Lb. curvatus* B431, approximately 10^{10} CFU/mL (free or encapsulated cells) were incubated in MRS broth supplemented with 0.3 % bile salts (Institut Pasteur, Alger) for 8 hours at 37 °C. A viable count on MRS agar was carried out at 1h intervals over the assay period after appropriate serial dilution in normal saline. The plates were incubated at 37 °C for 48 hours. For microencapsulated cells, the count was determined as described before.

2.3.3. Survival of *Lb. curvatus* in simulated intestinal fluid

Simulated intestinal fluid (SIF) was prepared by dissolving bile salts 3 g/L, NaCl 9 g/L, pancreatin 10 g/L and trypsin 10 g/L in distilled water; pH was adjusted to 6.5 with NaOH [20]. An appropriate number of free and microencapsulated cells was used to inoculate the SIF, which was then incubated for 3 hours at 37 °C; a viable count on MRS agar was carried out at 1.5 h intervals over the assay period after appropriate serial dilution.

2.4. Effect of temperature and cold storage on the viability of free and encapsulated cells

The effect of temperature on the viability of *Lb. curvatus* was evaluated by incubating free and encapsulated cells at different temperatures (25 °C, 40 °C, 50 °C et 60 °C) for 20 minutes. A viable count was carried out before and after the incubation period [21]. For the effect of cold storage on the viability of free and encapsulated cells, the cells were stored in a refrigerator at 4 ± 1 °C for two weeks and a viable count was carried out each week [22].

3. Results and discussion

The probiotic *Lb. curvatus* B431 was encapsulated in different matrices; and to make in evidence the presence of bacterial cells in the beads, microscopic examination of a thin section of an alginate capsule stained with the Gram stain was performed. The observation revealed the presence of lactobacilli in the encapsulation matrix.

The number of microencapsulated cells per bead was determined according to the method of Chàvarri *et al.* [23], after lysis of beads and enumeration of viable cells on MRS agar; results were expressed as CFU/ml. The yield of encapsulation (YE), which is a combination between the encapsulation efficiency and cell survival during the process of microencapsulation was calculated as follow:

$$YE = N/N^{\circ} \times 100 \quad \text{where}$$

N: the number of viable cells liberated from capsules

N°: the number of free cells added to the matrix.

The results of the encapsulation yields of studied matrices showed that encapsulation in alginate, agar and alginate-starch was less effective (30 and 50%), however alginate presented the highest yield (91.66%).

3.1. Effect of acidic conditions on the cell release

The test of cell release was realised to determine the capacity of matrices to retain cells within the beads. results showed that the release rate of cells after 3 hours of incubation in acidic conditions vary from a matrix to another (Figure 1). The release rate of cells from alginate beads increased slowly but remained relatively stable during 3h of incubation (OD=0.015). For Alginate-agar beads, the release rate increased progressively during 3h of incubation, while for alginate-starch beads the release rate was relatively stable during the 2 first hours of incubation and increased after that. We can conclude that the rate of release was the same for this two matrices after 3h of incubation (OD=0.03). However, for carrageenan beads, released rate increased rapidly during the incubation in acidic conditions and reached an OD of 0.043. The results indicated that carrageenan beads was the weakest matrix concerning the capacity to retain cells within the beads and alginate is the strongest gel.

We observed that the size of beads changed during the 3h of incubation as well as a progressive dissolution of carrageenan beads which can explain the increase in cell release, while alginate-based beads, shrunk and became rigid. As a result, we can conclude that acidic conditions affect the permeability of beads as a function of time, and we can classify polymers based on their release rate in acidic conditions (pH 2.0) in a decreasing order as follows: alginate, alginate-starch, alginate-agar and κ -carrageenan.

Immobilized cell systems are considered to be heterogeneous systems enclosing a continuous aqueous phase (microorganisms) and a dispersed phase (the matrix), the transfer of bacterial biomass is characterized by a flow through the membrane matrix which is controlled by the size and shape of the capsule, the porosity of the matrix and the capsule thickness, and the incubation of the beads formed by different matrices in a solution at low pH increases the rate of release of the cells by modifying the flow between the interior of the capsule and the outside environment [18].

Todorov *et al.* (2012) reported that after 2.5 h in solution simulating the stomach (pH 3.0), no counts of *Lb. plantarum* ST16Pa were obtained when they were encapsulated in alginate, indicating that the cells remained inside the capsules

and were not affected by the low pH. In counterpart, the counts of free cells after the same treatment were 2 log lower than the initial counts [6].

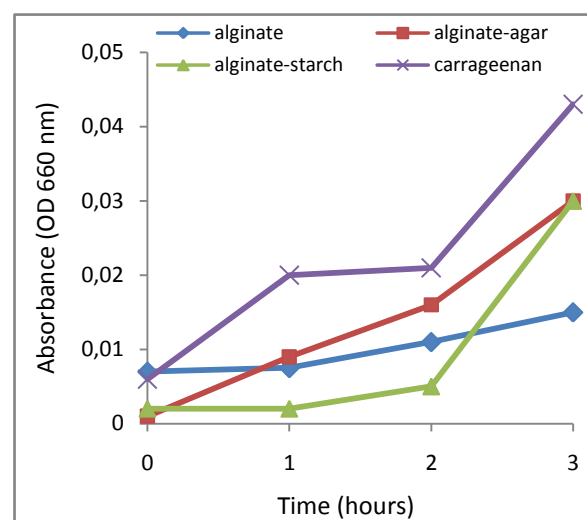


Figure 1. Effect of acidic conditions (pH 2.0) on the release of *Lb. curvatus* encapsulated in different matrices.

3.2. Release of cells from matrices in the presence of 0.3 % of bile salts

Results illustrated in Figure 2 represent the release rate of different matrices used for the encapsulation of *Lb. curvatus* in presence of bile salts. Cell release from alginate beads increased gradually during the incubation period and it reached an OD of 0.2 at the end of incubation. No cell release was observed during the first 3h of incubation from alginate-agar and carrageenan beads, where it increased gradually to reach approximately 0.34 and 0.32 after 7 hours of incubation, respectively. On the other hand, alginate-Starch beads lost easily their content in the first three hours of incubation and after that they lost all of them rapidly after 5h of incubation to reach an absorbance of 0.37 at the end of the experiment.

In general, we observed a change in the size of the beads for the different matrices. Thus, for beads containing alginate (alone, alginate-agar and alginate-starch) there was a swelling with a softening over the experience time; while for κ -carrageenan, the gel undergoes significant dissolution in function of the time of exposure to bile salts.

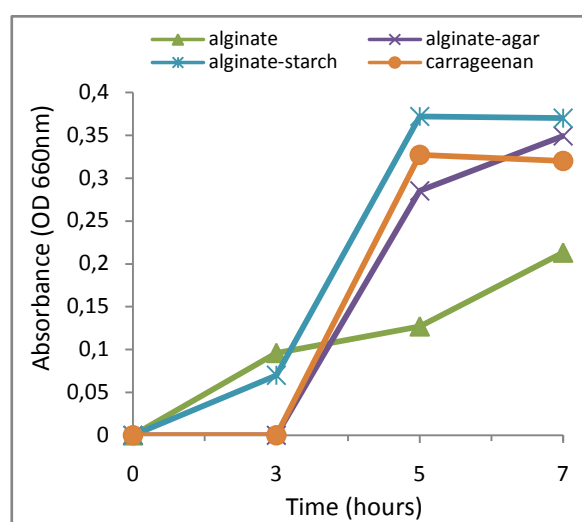


Figure 2. Effect of bile salts on the kinetic of cell release of *Lb. curvatus* B431 encapsulated in different matrices.

Similar results were reported by Todorov *et al.* [6] for alginate-based microencapsulated cells of *Lb. plantarum* ST16Pa when transferred from the low pH to the new stomach solution added of pancreatic components (digestive enzymes and bile salts), the protection given by the encapsulation weakened along exposure to this environment as the number of released cells increased gradually with time. However, the number of viable cells obtained for non-encapsulated *Lb. plantarum* ST16Pa was always lower than that recorded for their encapsulated counterparts.

Study of the effect of acidic conditions and bile salts on the release of cells from different matrices showed that 2% sodium alginate present an acceptable rate of release and it was selected for the study of the effect of the encapsulation on probiotic and technological properties of *Lb. curvatus* B431.

3.3. Evaluation of probiotic skills of *Lb. curvatus* B431 *in vitro*

3.3.1. Tolerance of free and encapsulated *Lb. curvatus* to acid conditions

Bacteria used as probiotics supplements are usually delivered in a food system and will therefore begin their travel to the lower intestinal tract through the mouth, the time between the entry and release of the stomach is about 90 min. Although the cellular stress begins in the stomach, which has a pH as low as 1.5, most *in vitro* assays are carried out at pH 3.0. thus, the screening of all cultures studied for their tolerance to low pH of 2.0 and 3.0 is required. In addition, several studies indicated that bacteria do not survive in sufficient numbers during their passage through the gastrointestinal tract [24].

Free and alginate microencapsulated *Lb. curvatus* cells were subjected to acidic conditions in order to simulate the gastric juice effect. The obtained results are presented in Table 1. The initial cell number was approximately 7×10^{10} CFU/ml for free cells and about 12×10^{10} CFU/ml for

encapsulated cells. After 1 hour of incubation in acid conditions (pH 2.0), the number of viable cells observed for the free form was 17×10^4 CFU with a survival rate of 48% and after 2h of exposition to acid conditions, the number of viable cells decreased to reach 15×10^3 CFU/ml which represent 38.4%. For the microencapsulated *Lb. curvatus* cells, a decrease in the number of viable cells was observed after 1 hours of incubation (10^7 CFU/ml) with a survival rate of about 64%. The decrease in viable cells was continued to reach 58×10^4 CFU/ml with a survival rate of 52% after 2h of incubation. The results suggest that microencapsulation of *Lb. curvatus* in 2% sodium alginate biopolymer protected efficiently this bacteria against acid conditions (pH 2.0) similar to those of stomach by increasing the survival of *Lb. curvatus* by about 14% after 2h of incubation.

Time (h)	0	1	2
Survival rate (%)			
Survival of encapsulated cells	100	64	52
Survival of free cells	100	48	38

Table 1. Effect of acidic conditions (pH 2) on the survival of free and encapsulated *Lb. curvatus* B431.

Similarly, the counts of encapsulated *Lb. plantarum* ST16Pa after exposure to simulated gastric conditions (pH 1.6) for 3 h was 1.2 log lower than the initial count, while for non-encapsulated cells, a five-log reduction was observed [6]. Mandal *et al.* showed that the survival rate of *Lb. casei* NCDC-298 encapsulated in 2% sodium alginate was about 57% after 3 hours of incubation under acidic conditions [21].

3.3.2. Tolerance of free and encapsulated *Lb. curvatus* to bile salts

Results in Table 2 showed that the initial cell number of both the free (4×10^{10} CFU/ml) and microencapsulated forms (7×10^{10} CFU/ml) decreased to reach 21×10^6 CFU/ml (67% of survival) and 26×10^7 CFU/ml (survival rate 78%) after 4 hours of exposition to 0.3% of bile salts, respectively. After 8 hours of incubation the cell number reached 24×10^3 CFU/ml (survival rate 60%) for free cells and 9×10^6 UFC/ml (survival rate 65%) for microencapsulated form. Based on the obtained results, microencapsulation in alginate biopolymer improved cell viability of *Lb. curvatus* by 5%.

It was reported that exposure to 1 and 2% oxbile for 12 h caused less than two log reduction in the counts of *Lb. plantarum* ST16Pa when encapsulated in 2, 3 or 4% alginate, while the number of non-encapsulated cells decreased five logs [6]. The survival of *Lb. plantarum* and *Lb. rhamnosus* was 68.6 and 65.4%, respectively, after exposure to 3 % bile salts. Tolerance of probiotic to bile is very important because it displays a strong antimicrobial activity due to its action that

emulsifies and breaks down lipids of bacterial membranes [25].

Time (h) \ Survival rate (%)	0	4	8
Survival of encapsulated cells	100	78	65
Survival of free cells	100	67	60

Table 2. Effect of bile salts (0.3%) on the survival of free and encapsulated *Lb. curvatus* B431.

3.3.3. Tolerance of free and encapsulated *Lb. curvatus* to simulated intestinal fluid

Because the pancreatic juice modifies the intestinal fluid, an experiment was carried out to study the viability of free and microencapsulated probiotic bacteria *Lb. curvatus* in these particular conditions by simulating the intestinal conditions through incubating both free and microencapsulated cells in a fluid containing pancreatic enzymes, bile salts, trypsin and NaCl for 3 hours. Results illustrated in Table 3 indicated that the survival of encapsulated cells decreased after 1.5 hours of incubation in the simulated intestinal fluid (survival rate 89.4%) and reached 51.4% after 3 hours of incubation. Despite of the slight protection of bacterial cells by microencapsulation, it was not very efficient since the improvement in cell viability was not important (6.7%) comparing with free cells which present a survival rate of 44.7% after 3 hours of incubation. 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.

Comparing the effect of bile salts alone with the effect of simulated intestinal juice on the viability of encapsulated cells it appears that after exposure to simulated intestinal juice for 3 hours only, the survival rate is lower than that obtained after incubation with bile salts alone for 8 hours; hence, the relevance of this test proves that the pancreatic juice has a negative effect on the survival of this probiotic bacteria in the intestinal tract.

Time (h) \ Survival rate (%)	0	1.5	3
Survival of encapsulated cells	100	89,4	51,4
Survival of free cells	100	86	44,7

Table 3. Effect of pancreatin on the survival of free and encapsulated *Lb. curvatus* B431.

The obtained results are similar to those obtained with *Lb. plantarum* CMU-FP002 encapsulated with 2% alginate and incubated for 3 h in the same solution of simulated intestinal fluid, where survival rate was of approximately 60% [20].

3.4. Evaluation of technological skills of *Lb. curvatus* in vitro

3.4.1. Effect of heat treatment on free and microencapsulated cells

As the main aim of the heat treatment is to inactivate harmful microorganisms and pathogens responsible for deterioration of food in order to extend its shelf life and food safety, it is inevitable to kill beneficial organisms that offer advantages over health. Therefore, it may be useful to use the microencapsulation to protect cells against heat treatments such as pasteurization, so we tested the resistance of free and encapsulated *Lb. curvatus* to heat treatment.

The results of the heat resistance test of free and encapsulated *Lb. curvatus* in different temperatures are illustrated in Figure 3. Cell viability of *Lb. curvatus* in the free form, showed a decrease in function of temperature increase, 83.72%, 65.48% and 46% of the cells survived after exposure to 40, 50 and 60 °C, respectively. In addition, encapsulated cells are more resistant and present the following survival rate: 90.9%, 78.65% and 52% for temperatures 40, 50 and 60 °C, respectively. Relatively low protection of *Lb. curvatus* cells was found by comparing the different values obtained in free or encapsulated, thereby protection was improved as follows: 7.13, 17 and 6% at 40, 50 and 60 °C, respectively.

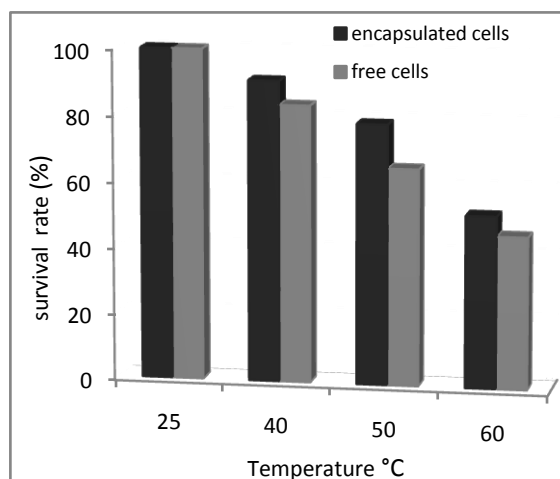


Figure 3. Effect of temperature on the survival of free and encapsulated *Lb. curvatus* B431.

In a similar report, *Lb. acidophilus* ATCC 43121 showed a survival rate of approximately 90% when encapsulated in 2% alginate and exposed to 65°C for 30 min [26]. The effect of temperature on the strain *Lb. casei*

NCDC-296 encapsulated in alginate at various concentrations: 2, 3 and 4% was tested and results showed that the survival rate was of about 70, 75 and 82%, respectively [21], these finding are in agreement on the fact that 2% alginate significantly improves the viability of encapsulated cells of different species of *Lactobacillus* compared to free one.

3.4.2. Resistance to cold storage

As there is a significant loss of bacterial cells during storage, an experiment was conducted to evaluate the efficiency of encapsulation in 2% sodium alginate to reduce the loss of viability of probiotic caused by refrigeration; Table 4 illustrated the results obtained for *Lb. curvatus* after 15 days of storage at 4 °C. The results showed that free cells of *Lb. curvatus* are less resistant to storage at 4°C (66% were viable after two weeks of incubation) comparing with encapsulated form where the survival after two weeks remains relatively high (86% survived after two weeks). The microencapsulation was able to protect cells of *Lb. curvatus* against cold storage and the efficiency of the protection was equal to 20%.

Time (week)	0	1	2
Survival rate (%)			
Survival of encapsulated cells	100	98	86,6
Survival of free cells	100	86	66

Table 4. Effect of cold storage at 4°C on the survival of free and encapsulated *Lb. curvatus* B431.

In a recent study, 75 % of the cells survived after 8 weeks of cold storage of *Lb. plantarum* CMUF-002 encapsulated in 2% alginate [20]. On the other hand, viability of immobilized cells of *Lb. plantarum* BL011 was greatly enhanced compared to the free microorganisms under refrigerated storage, and the treatments showing the lowest loss of viability were those of 4% (w/v) pectin, 3% (w/v) sodium alginate coated with chitosan and a mixture of 2% (w/v) sodium alginate and 2% (w/v) pectin, respectively [22].

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