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Original article

## Calcium alginate-resistant starch mixed gel improved the survival of *Bifidobacterium animalis* subsp. *lactis* Bb12 and *Lactobacillus rhamnosus* LBRE-LSAS in yogurt and simulated gastrointestinal conditions

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**Summary** The aim of this study was to investigate the protective effect of microencapsulation in calcium alginate-resistant starch mixed gel of a new human isolated strain of *Lactobacillus rhamnosus* LBRE-LSAS compared with the probiotic strain of *Bifidobacterium animalis* subsp. *lactis* Bb12. Influence of microencapsulation was tested under deleterious digestive environment, when challenged to salivary  $\alpha$ -amylase, to simulated gastric fluid and to simulated intestinal fluid. Bacterial survival, post-acidifying activity and exopolysaccharides (EPS) content in stored mix yogurt were assessed. Integrity of the beads was acceptable under  $\alpha$ -amylase levels largely higher than those found in human saliva. Under simulated gastrointestinal model, viable cell counts of encapsulated cells were significantly higher than those observed with free cells and remained at the recommended levels. Additionally, microencapsulation allowed an improved viability of bacteria and generated higher EPS amounts in mix yogurt stored at 4 °C. Our results indicate that calcium alginate-resistant starch beads extend survival under digestive conditions and in yogurt and could be used as an efficient delivery system for probiotics.

**Keywords** *Bifidobacterium animalis* subsp. *lactis* Bb12, calcium alginate-resistant starch beads, *Lactobacillus rhamnosus* LBRE-LSAS, simulated digestive environment, viability, yogurt.

### Introduction

Probiotic foods are supplements, which contain sufficient amounts of viable and active micro-organisms (single or associated culture) that have beneficial effects on the consumer, such as improving lactose tolerance, control of gastrointestinal infections, reduction in serum cholesterol, increasing the digestibility of foods, immune stimulation and suppression of cancer (Sanders & Klaenhammer, 2001). Members of the genera *Lactobacillus* and *Bifidobacterium* are mainly used, but not exclusively, as probiotic micro-organisms. However, reproducing all these benefits depends on high survival rates of potential probiotic bacteria during manufacture, storage and gastrointestinal passage. Therefore, International Dairy Federation (IDF) has suggested that a minimum of  $10^7$  probiotic bacterial cells should be alive at the time of consumption per gram of the product

(Kailasapathy & Chin, 2000). The main challenges to probiotics whilst passing through the digestive tract are acidic pH of the stomach and bile salts released in duodenum (Lo *et al.*, 2004).

The physical protection *via* microencapsulation is a new approach to increase the survival of probiotic bacteria in food products and the gastrointestinal tract (GIT) (Fávaro-Trindade & Grosso, 2002). In addition, encapsulation may enhance microbial survival during fermentation (Champagne *et al.*, 1994). Encapsulation involves the envelopment of tissues or biologically active substances in a semi-permeable membrane to protect the enclosed biological structures from potential hazardous processes in the direct environment. Alginate, a non-toxic polymer extracted from seaweed, is an ideal encapsulation agent because it is entirely biocompatible, inexpensive and widely used in many dairy preparations. The ease of solubilising alginate gel (by  $\text{Ca}^{2+}$  sequestration) and its release of entrapped cells within the human intestine are additional benefits (Nazzaro *et al.*, 2009). Furthermore, incorporating both prebiotics (i.e.

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resistant starch) and calcium alginate in coating materials may improve protection of active bacteria in food systems and in the upper gastrointestinal tract (Chen *et al.*, 2005). By adopting microencapsulation methods to enhance the survival and stability of beneficial bacteria including their protection in biopolymeric structures, an increased delivery of viable cells in human intestine can be achieved. Nevertheless, although microencapsulation is increasingly used, only a few parameters were taken into account in previous studies describing the beneficial effect of this technique. As an example, *in vitro* methods studying the survival of encapsulated cells in starch-based beads often neglected the starch digestion by  $\alpha$ -amylase in the mouth. Finally, the encapsulation procedure using starch reinforced alginate capsule, and the protective properties of the resulted beads against adverse gastrointestinal conditions and in products' shelf life are still not clearly defined.

In this study, our objectives were to extensively characterise the effect of microencapsulation on probiotic survival and activities. Resistant starch was mixed with sodium alginate (Ca-alginate-RS mixed gel) to encapsulate separately a new human isolated strain of *Lactobacillus rhamnosus* LBRE-LSAS and the well-known probiotic strain *Bifidobacterium lactis* Bb12. The ability of these starch-containing beads to withstand high concentrations of salivary  $\alpha$ -amylase and gastrointestinal conditions in simulated food matrix to imitate the food intake was investigated. Their survival rates, post-acidifying activities and exopolysaccharides (EPS) production capacities in stored yogurt were also assessed.

## Materials and methods

### Bacterial strains and materials

*Lactobacillus rhamnosus* LBRE-LSAS (*Lactobacillus Rhamnosus* Expérimentale-Laboratoire de Sécurité Alimentaire et Santé) was obtained from our laboratory's collection (faecal strains isolated from healthy Algerian neonates). It was genetically identified by 16S RNA sequencing method and studied herein as an experimental strain. This strain was a good milk fermenter and produced a very low quantity of exopolysaccharides. LBRE-LSAS was also evaluated for a number of probiotic characteristics *in vitro* and *in vivo*. *In vitro*, this strain displayed adherence to hexadecane support, bile salt resistance, salt tolerance, low pH survival, cholesterol assimilation and antimicrobial activity. It also adhered to mouse intestinal mucosa and reduced the incidence of *Shigella dysenteriae* symptoms (data not shown).

The well-known probiotic strain of *Bifidobacterium animalis* subsp. *lactis* Bb12 (Chr. Hansen, ATCC 27536)

was obtained in frozen form from Chris Hansen (Hørsholm, Denmark).

Sodium alginate E401 (GRINDSTED R. Alginate FD, Material no 120059912) and resistant starch (Hi-maize™ 260, RS<sub>2</sub>) were provided free of cost by Danisco (Algiers, Algeria).

### Encapsulation procedure

Individual bacterial cell suspensions were prepared by centrifuging 80 mL of a 24 h culture at 5000 g. Following centrifugation, the cells were washed twice with saline solution (20 mL). The washed bacterial cells were mixed individually with 10 mL of 18 g L<sup>-1</sup> of sodium alginate and 20 g L<sup>-1</sup> resistant starch (RS). Concentrations of sodium alginate and RS were selected after preliminary studies. The RS was incorporated in the alginate to improve beads rigidity. One millilitre of the microbial suspension was introduced into 5 mL sterile syringe fitted on a 27.5 G needle (Terumo, Leuven, Belgium). The suspension was dropped aseptically into 100 mL of vegetable oil (canola and sunflower oil) containing 1 mL polysorbate 80 (E 433; A and Z Food Additives Co. Ltd, China). After 20 min of agitation at 200–400 rpm, 100–200 mL of 0.1 M calcium chloride was added quickly along the side of the beaker, when the phase separation of oil/water emulsion occurred. Beads of Bb12 or LBRE-LSAS were separated by decantation and rinsed with a solution containing 0.1 M calcium chloride and 5% glycerol.

The distance between the syringe and the CaCl<sub>2</sub> solution was kept to 20 cm, to yield capsules of 0.2–0.3 mm in diameter. The beads were then aliquoted into separate vials and used within 2 days.

### Effect of salivary $\alpha$ -amylase on the integrity of beads containing microencapsulated cells

Bead integrity was evaluated according to the method of Haider & Husain (2008) by calculating the percentages of released cells upon  $\alpha$ -amylase exposure. Briefly, Beads containing *B. lactis* Bb12 or *L. rhamnosus* LBRE-LSAS were incubated with increasing concentrations of salivary  $\alpha$ -amylase (Alpha-amylase from human saliva; Sigma, Schnellendorf, Germany) (0, 50, 75, 150 and 250 U) in 0.1 M sodium acetate buffer, pH 5.6 for 4 h at 37 °C. The activity of the enzyme without salivary amylase was considered as control (100%) for the calculation of the remaining activity.

### Viability of free and microencapsulated cells in simulated gastrointestinal model

The beads were first exposed to the stomach compartment (simulated gastric fluid: SGF) consisting of a food suspension adjusted to pH 2 at 37 °C for 2 h under

100 rpm and on anaerobiosis (Anaerocult; Merck, Darmstadt, Germany) as described by Martoni *et al.* (2007).

The food suspension consisted of starch (3 g L<sup>-1</sup>), pectin (2 g L<sup>-1</sup>), mucin (4 g L<sup>-1</sup>), glucose (0.4 g L<sup>-1</sup>), yeast extract (3 g L<sup>-1</sup>), peptone (1 g L<sup>-1</sup>) and cysteine (0.5 g L<sup>-1</sup>). The control isotonic medium regarding SGF was prepared by culturing beads containing bacteria (Bb12 or LBRE-LSAS) in NaCl solution (0.86%, w/v, pH 6.5) during 2 h at 37 °C as described by Xiao *et al.* (2003).

After 2 h exposure, beads from the food suspension in the SGF were washed with phosphate buffer (PBS) by centrifugation at 3200 g for 10 min and the pellet was transferred to the small intestine compartment (simulated intestinal fluid: SIF) consisting of the same food suspension supplemented with 0.5% oxgall, and 2.5 g L<sup>-1</sup> sodium bicarbonate and readjusted to pH 6.5. This preparation was incubated in the simulated small intestine conditions for 16 h at 37 °C on anaerobiosis. The non-incubated food suspension was considered as a second control medium with respect to SIF.

Control cultures with free cells were subjected to treatment similar to encapsulated cells. Viability of free or encapsulated cells of Bb12 and LBRE-LSAS was assessed at the end of the exposure periods to SGF and SIF.

#### Preparation of yogurt containing encapsulated cells

The micro-organisms were first activated by three successive transfers for the preparation of mother cultures for yogurt making. Four batches of yogurt were prepared by aerobic fermentation at 42 °C in tyndallized (steamed repeatedly for 30 min on three successive days) reconstituted skimmed milk (RSM 18%, p/v). Each batch contained mixed culture of free or encapsulated cells of Bb 12 or LBRE-LSAS and yogurt starters, each inoculated at the concentration of 1%. The yogurt starters (Danisco, France) *Lactobacillus delbrueckii* subsp. *bulgaricus* LB 340, which did not produce EPS and *Streptococcus thermophilus* TA040, a fair EPS-producing strain (Foligne *et al.*, 2007), will hereafter be termed Lb and St, respectively, in the text. All cultures were added at the beginning of fermentation at final counts of  $\sim 1 \times 10^7$  CFU mL<sup>-1</sup> in milk. Afterwards, the fermentation was stopped as soon as pH of yogurt reached the value of 4.7, followed by cooling to 4 °C and storing for 4 weeks.

#### Viability and post-acidification assessment

Viable encapsulated cell counts were monitored by sampling dilutions from pellets (SGF or SIF) on MRS-Cysteine agar after being released from Ca-algi-

nate-RS beads by sequestering calcium ions with sodium PBS.

Samples of different yogurts (containing free or encapsulated cells) were assessed for viability during refrigerated storage. One gram of each sample was diluted with 99 mL of sterile PBS buffer (0.2 M, pH 7, 20 min vigorous disruption at 4 °C) before making serial dilutions in 0.1% (w/v) sterile peptone buffered water (Difco laboratories, Detroit, MI, USA). Control cultures (with free cells) were subjected to similar treatment.

An aliquot of 100 µL of each sample was taken to enumerate the viable cell counts. Viability tests were performed in triplicate, and cell counts were calculated from the colonies on appropriate medium plates after suitable incubation. *B. lactis* and *L. rhamnosus* were enumerated in Lactobacilli MRS agar (Difco Laboratories) containing 0.1% (p/v) lithium chloride and 0.3% sodium propionate (p/v) (GasPak anaerobic system 37 °C for 72 h). Lactobacilli MRS agar, pH 5.2 (anaerobic environment 42 °C for 72 h) and *Streptococcus thermophilus* agar (ST agar, aerobic environment 37 °C for 72 h), respectively, were used to enumerate *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* (Tharmaraj & Shah, 2003).

Viable cells were expressed as colony forming units per gram (Log CFU g<sup>-1</sup>).

Post-acidifying activities were measured on samples taken from the different preparations of yogurt during storage at 4 °C using a digital pH-meter (microprocessor pH-meter, model pH 537; WTW, Weilheim, Germany).

#### EPS production in stored yogurt

Proteins in 50 mL of diluted yogurt sample (1:1 yogurt: Milli-Q water) were precipitated by adding 4 mL of 20% (w/v) TCA (Sigma Chemical Co.). Precipitated proteins were separated by centrifugation at 3313 g for 30 min at 4 °C. The pH of supernatant was adjusted to 6.8 with 40% (w/v) NaOH followed by boiling at 100 °C for 30 min to denature whey proteins. Denatured whey proteins were separated by centrifugation at 3313 g for 30 min at 4 °C. Equal volume of cold absolute ethanol was mixed with the supernatant to precipitate the carbohydrate from the supernatant. The precipitation was carried out overnight at 4 °C, and the precipitate was separated by centrifugation at 3313 g for 30 min at 4 °C. The resultant carbohydrate pellet was completely dissolved by adding 10 mL of Milli-Q water, and the resultant suspension was subjected to sonication for 1 h at room temperature. Then, the solution was dialysed at 4 °C in a dialysis membrane tube with a molecular weight cut-off of 13 000 Da against tap water over a 2-week period. Water was changed twice a day. The EPS concentration was quantified using the

phenol-sulphuric method of Dubois *et al.* (1956) and was expressed as glucose equivalent.

All food composition materials were purchased from Sigma (Sigma-Aldrich, Chimie) and chemicals from Difco (Difco Laboratories), unless otherwise specified.

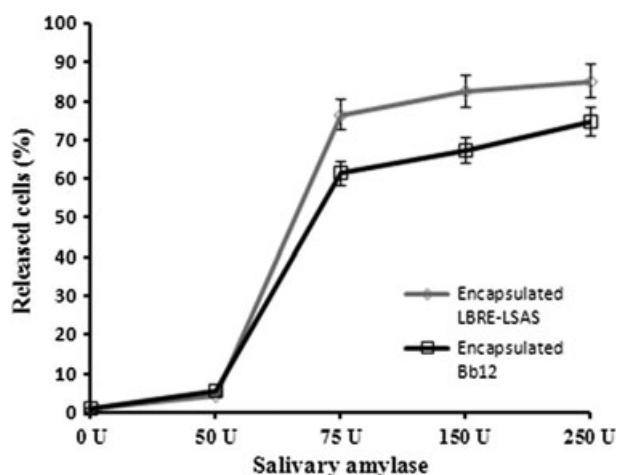
### Statistical analysis

Data analysis was performed using STATISTICA data analysis software system, version 8.0 (StatSoft Inc., Tulsa, OK, USA, 2008). One way ANOVA analysis was used to evaluate the significant differences between sample means, with significance level set at  $P < 0.05$ . Mean comparisons were assessed by Tukey's test. All data presented are mean values of triplicates obtained from three separate runs ( $n = 9$ ).

## Results

### Effect of salivary $\alpha$ -amylase on Ca-alginate-RS beads

The effect of salivary  $\alpha$ -amylase on integrity of Ca-alginate-RS beads is shown in Fig. 1. In absence of amylase, there was a slight cell release ( $< \text{Log } 5 \text{ CFU mL}^{-1}$ ) for both assayed strains (data not shown). The percentages of liberated cells from Ca-alginate-RS beads were not statistically ( $P > 0.05$ ) significant when treated with 0–50 U of  $\alpha$ -amylase for 4 h at 37 °C. When treated with 75 U of  $\alpha$ -amylase, 38.7 and 23.5% of enclosed Bb12 and LBRE-LSAS were released,



**Figure 1** Effect of salivary amylase on Ca-alginate-RS beads. Beads were inoculated with increasing concentrations of salivary amylase (50–250 U) in 0.1 M sodium acetate buffer, pH 5.6 for 4 h at 37°C. Control for the calculation of released cells per cent after  $\alpha$ -amylase exposure was count without salivary amylase treatment.

respectively. Following exposure to higher concentration of  $\alpha$ -amylase (250 U), the beads became weak and approximately 74.6 of encapsulated Bb12 and 84.1% of LBRE-SAS were released ( $P < 0.05$ ).

### Survival of free and encapsulated Bb12 and LBRE-LSAS in SGF and SIF

The viability of free and encapsulated bacteria under simulated gastrointestinal conditions is represented in Fig. 2.

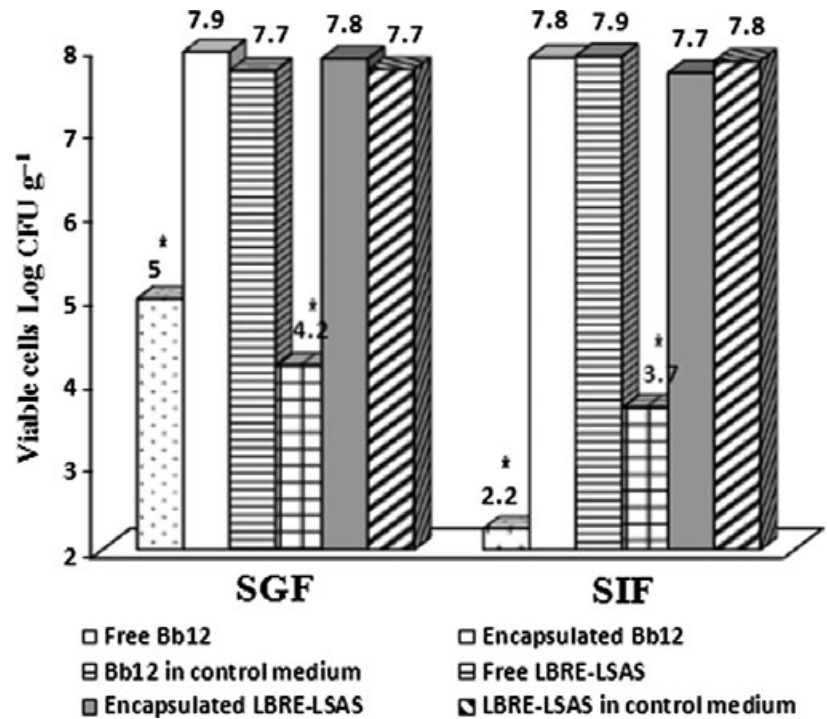
Food suspension containing encapsulated cells showed no significant ( $P > 0.05$ ) decline in population from initial inoculated counts for both strains after exposure to SGF as compared to the isotonic control medium. High viable loads of 7.9 and 7.8 of  $\sim 8 \text{ Log CFU g}^{-1}$  of Bb12 and LBRE-LSAS (Fig. 2) were registered, respectively. Conversely, the non-encapsulated bacterial levels strongly ( $P < 0.05$ ) decreased during exposure to SGF, displaying a reduced viability of 3–3.8 logs.

After 2 h in SGF, cell pellets of Bb12 and LBRE-LSAS were transferred separately to SIF. The load of bacteria was not influenced ( $P > 0.05$ ) by oxgall present at the level of 0.5% after 16 h of incubation in SIF. After 16 h of that residence, cells were released automatically and 7.8 and 7.7 viable Log CFU  $\text{g}^{-1}$  of Bb12 and LBRE-LSAS (Fig. 2) were detected, respectively, demonstrating a viability very similar to the one obtained in control medium.

In comparison, viability of free cells was markedly ( $P < 0.05$ ) impaired after SIF residence resulting in 2.2 and 3.7 viable Log CFU  $\text{g}^{-1}$  of Bb12 and LBRE-LSAS, respectively.

### Viability of free and encapsulated *B. lactis* Bb12 and *L. rhamnosus* LBRE-LSAS in stored yogurt

Viability of bacteria in yogurt is reported in Table 1. The free or encapsulated *B. lactis* Bb12 and *L. rhamnosus* LBRE-LSAS counts were converted to decimal logarithm scale before the data were analysed statistically. No differences in bacterial counts were observed between encapsulated and free cells at the beginning of the storage period. Conversely, the numbers of viable bacteria were significantly different ( $P < 0.05$ ) between the encapsulated and free cells from first week onwards. In control yogurts containing free cells, viable counts decreased significantly by 1.6 (LBRE-LSAS) and 1.88 (Bb12) log cycles from week one to week 4 of the refrigerated storage period. On the other hand, losses in bacterial viability were  $< 1$  log cycle (from week one to week 4) with the use of encapsulated cells. Interestingly, viable counts of encapsulated cells of both organisms remained higher than 8.5 Log CFU  $\text{g}^{-1}$  at the end of 4 weeks storage, whereas 7.2



**Figure 2** Viable free or encapsulated cells of *Bifidobacterium animalis* subsp. *lactis* Bb12 and *Lactobacillus rhamnosus* LBRE-LSAS at the end of the exposure period to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). Controls are NaCl solution (0.86%, pH 6.5) at 37°C/2 h (SGF) and non-incubated food suspension SIF. Inoculated counts were  $8 \pm 0.5$  Log CFU g<sup>-1</sup>. \*Denotes statistically significant ( $P < 0.05$ ).

**Table 1** Number of viable cells (Log CFU g<sup>-1</sup>) of *Bifidobacterium animalis* subsp. *lactis* Bb12 and *Lactobacillus rhamnosus* LBRE-LSAS under free and encapsulated states at 4 °C in yogurt\*

Storage period (weeks)	Bb 12		LBRE-LSAS	
	Free	Encapsulated	Free	Encapsulated
0	10.2 ± 0.1dA	10.2 ± 0.1dA	10.4 ± 0.3dA	10.4 ± 0.3dA
1	6.9 ± 0.1cA	9.7 ± 0.3dD	8.8 ± 0.2cB	9.3 ± 0.9cC
2	5.6 ± 0.3bA	9.1 ± 0.5bC	8.7 ± 0.2cB	9.2 ± 1.2bcC
3	5.3 ± 0.3bA	9.3 ± 0.8cD	8.4 ± 0.1bB	9.1 ± 0.9bC
4	5.1 ± 0.1aA	8.9 ± 0.6bC	7.2 ± 0.2aB	8.6 ± 0.7aC

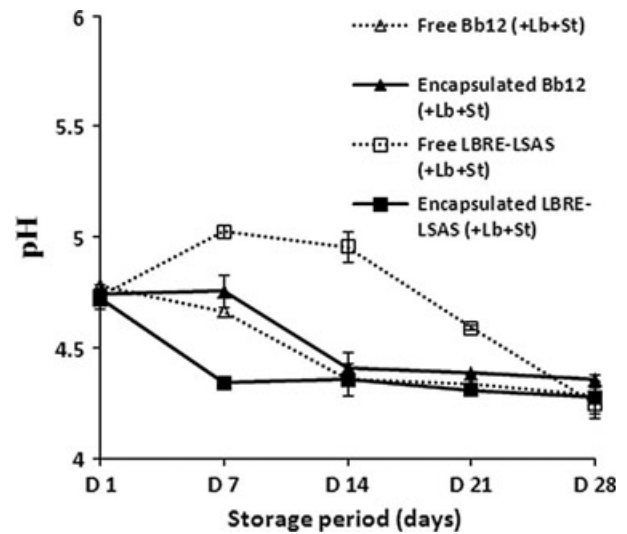
Survival represents the mean ± SD ( $n = 9$ ). For comparative purposes, all counts were adjusted to 'the day 0' values of free treatments of Bb12 or LBRE-LSAS.

\*Yogurt was made by associated culture between one strain of Bb12 or LBRE-LSAS and yogurt starters.

<sup>abcd</sup>Means in the same column with different small letter superscripts are significantly different ( $P < 0.05$ ).

<sup>ABCD</sup> Means in the same row for a particular strain with different capital letter superscripts are significantly different ( $P < 0.05$ ).

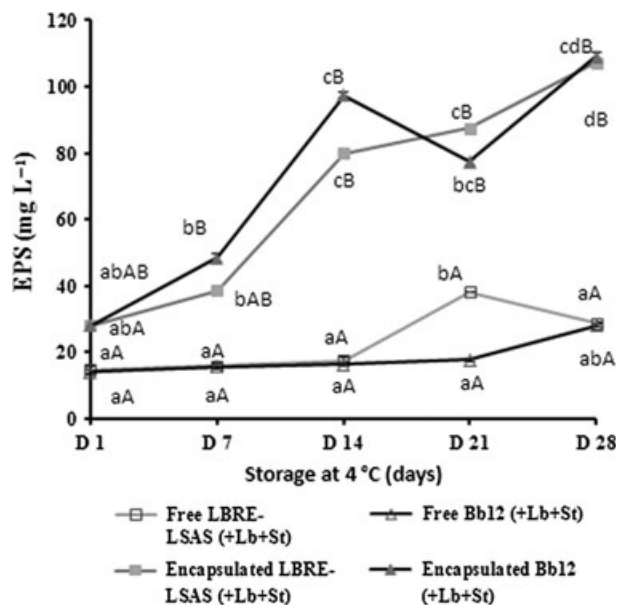
and 5.1 Log CFU g<sup>-1</sup> were detected for free LBRE-LSAS and free probiotic strain of Bb12, respectively (Table 1). Consequently, the gain in viable biomass as a result of microencapsulation in Ca-alginate-RS gel was notably higher for the probiotic strain of Bb12, compared with LBRE-LSAS.



**Figure 3** Effect of microencapsulation on post-acidifying activity of *Bifidobacterium animalis* subsp. *lactis* Bb12 and *Lactobacillus rhamnosus* LBRE-LSAS in stored yogurt, expressed as pH.

**Changes in pH**

Figure 3 shows the changes in pH of the different preparations of yogurt during storage at 4 °C for 28 days. Microencapsulation of Bb12 has no effect



**Figure 4** Exopolysaccharides amounts ( $\text{mg L}^{-1}$ ) produced in stored yogurt containing free and encapsulated *Bifidobacterium animalis* subsp. *lactis* Bb12 or *Lactobacillus rhamnosus* LBRE-LSAS and in the presence of yogurt starters. (abcd) Means with different small letter super-scripts are significantly different within each type of yogurt; ABCD Means with different capital letter super-scripts are significantly different between each type of yogurt for a particular day of storage.

( $P > 0.05$ ) on pH values measured from day 1 to 28 (D1 to D28) (Fig. 3). Conversely, in yogurt with LBRE-LSAS, a significant ( $P < 0.05$ ) decrease in pH is only obtained from D7 with encapsulated cells (Fig. 3). pH values were then significantly ( $P < 0.05$ ) lower in yogurt with encapsulated LBRE-LSAS compared with yogurt with free LBRE-LSAS from D7 to D14. After 28 days, final pH values were almost similar ( $P > 0.05$ ) in all batches, ranging from 4.2 to 4.3.

### EPS production

Results relating to the EPS production of microencapsulated cells during yogurt storage are shown in Fig. 4. After 1 day at 4 °C, the quantities of EPS were slightly higher in yogurt made with encapsulated cells (Fig. 4). EPS production increased significantly during storage of yogurts containing encapsulated cells. Indeed, amounts of EPS were nearly quadrupled ( $P < 0.05$ ) in yogurts containing encapsulated Bb12 or LBRE-LSAS during the storage, reaching 109 and 107  $\text{mg L}^{-1}$ , respectively. On the contrary, no (Bb12) or a weak (LBRE-LSAS) increase in EPS amounts was observed in yogurt containing free cells during the 4 weeks of storage.

### Discussion

Probiotic bacteria must survive transit through the digestive tract and reach the colon in large quantities (Mainville *et al.*, 2005). An attempt has been made herein to examine the effectiveness of Ca-alginate-RS beads in adverse conditions when used to protect beneficial bacteria taken *via* an oral route.

In our study, resistant starch was incorporated at the concentration of 2% (w/v). This concentration is higher than that used in previous studies on encapsulating probiotic bacteria in starch-based matrix (Sultana *et al.*, 2000; Iyer & Kailasapathy, 2005; Zou *et al.*, 2011) and may ensure a better protection against salivary  $\alpha$ -amylase. Therefore, our results indicate that our Ca-alginate-RS beads were stable enough under higher salivary  $\alpha$ -amylase concentrations and longer residence time than that occurring in adult human's buccal cavity. This suggests that the modified starch employed herein was advantageously incorporated in the alginate matrix, allowing more rigidity to beads, and confirms the study by Haider & Husain (2008), where the concanavalin A layered calcium alginate-starch beads immobilised  $\beta$ -galactosidase were significantly stable after 4 h (37 °C, pH 4.6) exposure to 200 U of salivary amylase.

Viability of probiotic cultures in the GIT is affected mainly by gastric acid present in the stomach and bile in the duodenum (Lo *et al.*, 2004). The effect of encapsulation on the survival of bacteria under adverse conditions has been reported with contrasting results. Indeed, the advantage of encapsulating lactic acid bacteria over that of free cells under *in vitro* gastric conditions has been reported (Chandramouli *et al.*, 2004; Iyer & Kailasapathy, 2005; Muthukumarasamy *et al.*, 2006) whereas other studies showed that encapsulation does not effectively protect these bacteria from strong acidic conditions (Gbassi *et al.*, 2009; Zou *et al.*, 2011). Similarly, Sultana *et al.* (2000) reported that survival of probiotics in alginate-starch microspheres did not improve after exposure to acidic and bile salt solutions.

The addition of starch to alginate matrix was recently found inefficient to improve the survival of encapsulated cells (Zou *et al.*, 2011), whereas the microencapsulation of probiotics using alginate with starch by extrusion provided greater protection against gastric juice than using pure alginate beads (Iyer & Kailasapathy, 2005). Here, we assessed the survival of a new human isolated lactobacilli LBRE-LSAS compared with the probiotic strain of *Bifidobacterium* Bb12 under simulated conditions of GIT and highlighted the role of the protecting capacity of Ca-alginate-RS matrix in improving survival of cells in presence of food matrix. Interestingly, our microencapsulation method was able to protect both probiotic strains with the same efficiency despite probable different acid and bile salt tolerances of these two strains. This suggests that our Ca-alginate-RS

matrix could be used to encapsulate and protect a large panel of probiotic strains. Therefore, we believe that our microencapsulation procedure consisting of mixing the sodium alginate gel with resistant starch confers stability to beads and leads to a better protection against harmful environmental conditions. This could be explained by the fact that resistant starch and sodium alginate tend to be synergistic in gelling and as a result may help in providing additional protection to the entrapped bacterial cells. In addition, we postulate that the diffusion of bile salts into the beads may be limited by the sodium alginate gel matrix reinforced herein by incorporation of resistant starch.

In addition, resistant starches added to foods are classified as functional fibre under the American Association of Cereal Chemists (AACC) and the National Academy of Sciences (NAS) definition (Sajilata *et al.*, 2006). Resistant starch is largely undegraded in the small intestine and constitutes a major part of the carbohydrate available in the human colon for bacterial fermentation leading to the production of short chain fatty acids and gases (Brown, 1996). In this study, we used Type II resistant starch, representing starch with a granular structure characterised by B-type crystallinity. These granules, with water in the centre of the array, display strong resistance to enzymatic digestion (Brown, 1994) implying they can enter the colon and be used as substrates for fermentation by the colonic microbial community. Thus, besides increasing the protection of probiotic bacteria from adverse conditions, starch included in the microcapsule may also confers health benefits through fermentation products. In this work, the beads of Ca-alginate-RS containing entrapped cells fulfilled the established criterion of a minimum of  $6 \text{ Log CFU g}^{-1}$  viable beneficial cells, frequently cited to gain the therapeutic benefit in the intestine (Gardiner *et al.*, 2000). Indeed, the slow release of encapsulated cells from Ca-alginate-RS beads should protect the probiotic bacteria during the passage in the small intestine whose duration is about 3–4 h in humans (Chan & Zhang, 2005). Moreover, as the transit time in the colon can range from 0.8 to more than 20 h (Vandamme *et al.*, 2002), our Ca-alginate-RS beads should assure an efficient delivery in this distal part of the digestive tract.

The enhancement in bacterial survival in yogurt is an attractive aspect for any commercial exploitation because bacteria will remain intact in the food until consumption. The effects of microencapsulation on bacterial survival in yogurt largely depend on the method of encapsulation. Indeed, alginate microspheres blended with pectin did not improve survival of encapsulated cells after 1 month of storage at 4 °C, whereas the alginate microspheres blended with starch or coated with chitosan maintained the initial level of survival (Zou *et al.*, 2011). In this study, microencapsulation in

Ca-alginate-RS was found to improve the viability and to maintain a suitable post-acidifying activity of beneficial organisms in yogurt after 1 month of storage at 4 °C reinforcing the valuable role of adding starch to microcapsule for an efficient protection of probiotics. Moreover, this is the first study to our knowledge where alginate-resistant starch-encapsulated probiotic strain (Bb12) and/or intestinal human isolated strain (*L. rhamnosus*) were incorporated in yogurt mix in the presence of *S. thermophilus* and *L. bulgaricus*, the traditional starters of yogurt. From a biotechnological point of view, the appropriate production process depends on fermentability and acid tolerance of the added probiotic micro-organisms. Only a few can be used as sole starter cultures. In most of the cases, fermentation occurs exclusively or predominantly through an associated culture between probiotic and conventional starter cultures (*Streptococcus thermophilus* and others). Here, the survival of oxygen-sensitive bacteria (e.g. bifidobacteria) in the product is favoured by oxygen-consuming conventional starter cultures (*S. thermophilus*), and by the lowering of the redox potential (de Vrese & Schrezenmeir, 2008). Furthermore, in our study, microencapsulation resulted in dramatic increase in EPS amounts in yogurt. Literature reporting EPS production by mixed culture containing encapsulated probiotic cells in presence of starter of yogurt is, to our knowledge, very scarce. EPS are produced by a great variety of bacteria, including lactic acid bacteria (LAB). The greatest potential of bacterial EPS is related to their use in high value market niches, such as cosmetics, pharmaceuticals and biomedicine. Therefore, EPS-producing strains are of commercial value for both their technological and presumed probiotic properties (Zhang *et al.*, 2011). By incorporating resistant starch to encapsulate bacteria, it may be possible not only to increase survival but also to improve thickening and gelling properties of yogurt. Our hypothesis that the encapsulation of Bb12 or LBRE-LSAS may enhance EPS production by streptococci culture, which is recognised as an EPS producer strain, was proven during mix yogurt storage. The population of St in the mix was stimulated in presence of encapsulated cells and maintained higher survival rates during refrigerated storage compared with plain yogurt (data not shown). Finally, the increase in EPS amounts in yogurt may also have beneficial effects on human physiology. Apart from their prebiotic potentialities, EPS have been identified as blood cholesterolemia reducer, immunomodulator, antitumoral and antiulcer agents (Badel *et al.*, 2011). EPS may also allow the bacteria to better withstand stomach acid and bile salts thanks to their protective coating effect. Indeed, a likely relationship between EPS production and resistance to bile salts or tolerance to low pH of strains has been recently underlined (Alp & Aslim, 2010).



## Conclusion

In this study, we have demonstrated that the Ca-alginate-resistant starch beads displayed strong resistance when treated with high salivary amylase. In addition, both encapsulated assayed strains showed increased survival to simulated gastric fluid and simulated intestinal fluid compared with free cells. Such data may allow us to gain further knowledge on the controlled-release application of the tested bacteria when administered *via* the oral route. Indeed, the beads developed would provide a suitable form to adapt for sensitive beneficial bacteria that cannot survive the highly challenging conditions of the digestive tract. Furthermore, we hypothesise that food components appear to have a protective effect as an isotonic medium regarding bacterial cell viability. The microencapsulation in Ca-alginate-RS was also effective in maintaining higher viable probiotic bacteria during refrigerated storage. Moreover, appreciable amounts of EPS produced in yogurt made with milk containing encapsulated cells could constitute an interesting benefit for industries. We believe that this study leads to a better understanding of the real behaviour of microencapsulated bacteria in the gut and in a favourable food carrier such as yogurt and emphasises the beneficial effects of microencapsulation of probiotic strains.

Finally, the *in vitro* bacterial survival observations need to be confirmed by testing encapsulated acid sensitive beneficial strains *in vivo*.

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