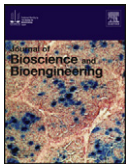

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Stability of lactobacilli encapsulated in various microbial polymers

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Various microbial polymers, namely xanthan gum, gellan gum, pullulan gum and jamilan, were tested as a suitable encapsulating material for *Lactobacillus plantarum* CRL 1815 and *Lactobacillus rhamnosus* ATCC 53103. Resulting capsules were also studied for their pH and simulated gastrointestinal conditions tolerance. The morphology of the microcapsules was studied using scanning electron microscopy. pH tolerance was tested at pH 2.0, 3.5, 5.0 and 6.5 over a 6 h incubation period. Simulated gastrointestinal conditions were assayed with simulated gastric and pancreatic juices and simulated bile over a 24 h incubation period. Suspensions of probiotic organisms were used as a control. The results from encapsulation with microbial polymers indicate that mixtures of 1% xanthan gum with 0.75% gellan gum and 1% jamilan with 1% gellan gum were the most suitable for microencapsulation. Results for the pH tolerance tests showed no improvement in the viability of cells in relation to the control, except for pH 2.0 where lactobacilli encapsulated in xanthan:gellan gum (1%:0.75%) prolonged their viability by 6 h exposure. Xanthan:gellan gum (1%:0.75%) was the most effective of the encapsulating materials tested in protecting *L. plantarum* and *L. rhamnosus* against simulated bile, improving its viability in 1–2 log CFU when compared with control. The results of this study suggest that microbial polymers are an interesting source of encapsulating material that should be taken into account for prospective studies of probiotic encapsulation for oral delivery applications.

[**Key words:** *Lactobacillus*; Microencapsulation; Exopolysaccharide; Xanthan gum; Gellan gum; Pullulan gum; Jamilan; Simulated gastrointestinal conditions]

The *Lactobacillus* genera have been widely employed for their beneficial properties on the human host with a long history of safe use in the manufacture and human consumption of dairy products (1). In fact, it is one of the most commonly genera belonging to the lactic acid bacteria, together with *Bifidobacterium*, used as probiotic. Probiotics are "live microorganisms, which when administered in adequate numbers, confer a health benefit on the host" (2) and their use has been associated with prevention of diarrheal diseases, control of intestinal infections, prevention of colon cancer, modifications of serum cholesterol levels, improvement in lactose utilization, prevention of upper gastrointestinal diseases, and enhancement of immune function (3–5). Food and pharmaceutical industries want to profit from these benefits and different formulations and dairy products have incorporated them (6–8). Cell viability in these products is often low and the ability to survive and multiply in the digestive tract after an oral delivery strongly influences the benefits that probiotics can produce in the host (9).

Over the last years, research has focused on microencapsulation for the improvement of probiotic survival, because it is a technological process that aims to protect probiotic bacteria, offering a great potentiality in the delivery of viable cells (10). Numerous studies have been conducted in this direction, mainly aimed to an improvement of the survival of probiotic bacteria during the oral delivery or incorporated into the matrix of the food product (11–14). A wide range of encapsulating materials has been employed for these purposes. Polysaccharides have been frequently used as they constitute a matrix which can be degraded by microorganisms of the intestinal microbiota and, thus, allow a targeted delivery of probiotics to the human intestine (15). Although a variety of polysaccharides have been described for lactic acid bacteria encapsulation, by far, the most commonly used polysaccharide is alginate as it provides non-toxic matrices with calcium chloride, suitable for sensitive bacteria (16). However, the gel is susceptible to disintegration in the presence of excess monovalent ions, Ca^{2+}-chelating agents and harsh chemical environments (17). Also, although high survival rates were reported with lactobacilli immobilized in alginate (18,19) these results are in conflict with other studies, where alginate has been reported ineffective in protecting probiotics in highly acidic

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Abbreviations: EPS, Exopolysaccharides; SEM, Scanning electron microscopy; X:G (1%:0.75%), Xanthan:gellan gum (1%:0.75%); J:G (1%:1%), Jamilan:gellan gum (1%:1%); LGG, *Lactobacillus rhamnosus* GG or ATCC 53103.

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environments (20,21). Few studies have been performed, to date, exploring various encapsulating materials; hence, there is an open field of research in this area.

Exopolysaccharides (EPS) obtained from different bacteria constitute an invaluable source of polysaccharidic material. These EPS have been employed widely in the food industry and have been proven safe for human consumption. In fact, exopolysaccharides as xanthan and gellan gum have been employed for many years in various food products worldwide. In this sense, previous studies have described that mixtures of microbial EPS, xanthan gum and gellan gum, provide an effective protection against simulated gastric juice (22). Xanthan gum by itself also improves probiotic bacteria survival when their tolerance to acid and bile exposure was tested (23). Thus, EPS seem to constitute a useful resource of encapsulating material suitable for probiotic encapsulation. To this effect, pullulan gum obtained from *Aureobasidium pullulans*, may constitute a good candidate. It is employed in the food industry forming transparent films suitable for food preservation and constitutes an effective barrier to prevent unwanted mass transfers (24). Also in this sense, an EPS, named "Jamilan", obtained from *Paenibacillus jamilae*, was described by our research group (25). This EPS can be applied for heavy metal biosorption (26) and, more interestingly, is able to form films (Garcia-Ribera, R., Ph.D. thesis, University of Granada, Granada, 2003) which led us to think that it could be also a good candidate for microencapsulation.

The aims of the present work were to ascertain whether various microbial EPS were a suitable encapsulating material for probiotic bacteria and if these encapsulation materials may affect lactobacilli survival under a pH range and simulated gastrointestinal conditions.

MATERIALS AND METHODS

Bacterial strains and culture conditions For this study the lactobacilli strains used were: *Lactobacillus plantarum* CRL 1815 (CERELA Culture Collection) and *Lactobacillus rhamnosus* ATCC 53103 (American Type Culture Collection). All the microorganisms were stored individually in milk-yeast extract at −20°C and subcultured twice in appropriate medium prior to final inoculation in 400 mL MRS broth (Merck KGaA, Darmstadt, Germany) at 37°C during 18–20 h.

Microbial polymer solution preparations Four different microbial polymers were assayed: gellan gum (Sigma, St. Louis, MO, USA), xanthan gum (Sigma), pullulan gum (Fluka, St. Louis, MO, USA) and jamilan, being this obtained in our laboratory from *P. jamilae* CECT 5266 (Spanish Type Culture Collection). Culture conditions of *P. jamilae* CECT 5266 and EPS separation were performed as described by Morillo Perez et al. (26), but 50 g of D(+)-saccharose was employed for the culture media. Briefly, after 72 h growth in a 2 L jar-fermentor BIOSTAT M (Braun-Biotech) containing 1 L of culture medium (30°C, pH 7, 150 rpm, aeration rate of 2 mL/min), the EPS was recovered from cell-free supernatant by precipitation with two volumes of −20°C ethanol with a prior addition of 1% (w/v) NaCl. The solution was kept at −20°C overnight and the precipitated polymer was recovered and subsequently dissolved in distilled water for an extensive dialysis against distilled water for 48 h at 4°C. Dry weight of polymer was obtained by freeze-drying.

Various polymer concentrations were assayed individually (2%–0.5% w/v), as well as mixtures of gellan gum (2%–0.5% w/v) with the remaining polymers (0.2%–1.5% w/v). In this stage, adequate amounts of polymer powders and freeze-dried jamilan were dispersed in 15 mL of deionized preheated water (80°C) by magnetic stirring and kept at 80°C until complete polymer hydration was achieved. For mixtures preparation, each individual concentration was calculated and then included in the powder form prior to hydration. Solutions were autoclaved for 15 min at 121°C and stored at 4°C, maximum 4 days.

Microencapsulation procedure Bacteria grown in 400 mL of culture medium were harvested by centrifugation (4000×*g*, 15 min), washed twice with sterile saline solution 0.9% and resuspended in 5 mL of saline solution. One mL of bacterial suspension was added to 15 mL of polymer solution and stirred magnetically for 30 s.

An electrostatic droplet generator was employed for encapsulation. The mixture was extruded with the aid of a syringe pump (kdScientific, MA, USA), at a flow rate of 120 mL/h, through a 20 mL syringe (Terumo®, Leuven, Belgium) connected to a needle (TIP 25 GA. 0.010 in. ESD SAFE, EFD®, RI, USA) through a metallic connector, which helps to conduce the electrostatic potential. Beads were dropped on a recovery solution of calcium chloride (Panreac, Barcelona, Spain) 0.1 M, at 4°C, gently stirred magnetically. An electrostatic potential of 6.5 kV was applied between the needle and the collecting solution.

Beads formed were hardened in the $CaCl_2$ solution during 30 min and separated from the solution with a sterile stainless steel filter. Beads were kept in sterile Petri dishes at 4°C until use, for 1 day maximum.

Microcapsule morphology The external morphology of the capsules with bacteria entrapped was analyzed by scanning electron microscopy (SEM). Beads were fixed with glutaraldehyde 2.5%, dehydrated by increasing concentrations of ethanol solutions (50%, 70%, 80%, 90% and 100% v/v ethanol) and dried up by using a critical point dryer Polaron CPD7501. Samples were carbon-coated and observed with the scanning electron microscope (DSM 950 Zeiss LEO 1530). The determination of bead size was performed with a Visilog 6.2 (Noesis, Les Ulis, Courtaboeuf, France) image analyses software. Pictures were taken by amplification of the samples with a stereo microscope SZT 300 (VWR International Eurolab, Barcelona, Spain) and by using a Moticam 1000 camera (Motic Instruments Inc., Canada).

pH tolerance Non-encapsulated (assayed at a level of 8.79 ± 0.42 log CFU/mL) and encapsulated bacteria (1 g of beads corresponding to 9.22 ± 0.50 log CFU/g beads) were exposed at 37°C to 9 mL of MRS broth adjusted to pH 2.0, 3.5, 5.0, and 6.5 (control) with 12 M HCl and 1 M NaOH. Samples were taken from microbial suspensions and encapsulated cells after 2, 4 and 6 h exposure. Subsequent serial dilutions were performed in phosphate buffered saline (PBS) (Sigma) for the enumeration of viable microorganisms before spreading 0.1 mL of appropriated dilutions, in triplicate, onto MRS agar plates. For the enumeration of encapsulated bacteria, prior to serial dilutions, microorganisms were released by dissolving the capsules in 0.05 M sodium phosphate buffer pH 7.0 using a stomacher for 6 min. Spread *Lactobacillus* spp. plates were incubated for 30 h at 37°C with 5% CO_2 by using CO_2 Gen® envelopes (Oxoid, Cambridge, UK) in an anaerobic jar. All tolerance tests were carried out by duplicate.

Simulated gastrointestinal juices tolerance Tolerance of probiotic bacteria to simulated gastrointestinal juices was determined by exposing free (at a level of 8.79 ± 0.42 log CFU/mL) and encapsulated bacteria (1 g of beads corresponding to 9.22 ± 0.50 log CFU/g beads) at 37°C to 9 mL of simulated gastric juice (2 h), simulated pancreatic juice (2 h) and simulated bile (up to 24 h) successively, and monitoring changes in total viable counts. MRS broth containing 0.03% (w/v) pepsin (Sigma) was adjusted to pH 3.5 with 12 M HCl for simulated gastric juice preparation. Simulated pancreatic juice was prepared with 0.2% (w/v) pancreatin (Sigma) dissolved in MRS broth adjusted to pH 6.5. MRS broth pH 6.5 containing 3% bile (Sigma) was employed as simulated bile. All the simulated gastrointestinal conditions were previously established by spectrophotometric assays (data not shown).

After each incubation time and before the following step, supernatants of each tube were discarded and the pellets were washed with 0.5% peptone water pH 6.5 prior to the addition of the next medium. Samples were taken at time 0 and after each incubation time, except for bile exposure, where sampling was also performed after 2 h incubation. Microbial cell numbers were determined as previously described for pH tolerance. Assays were carried out by duplicate.

Statistical analysis The mean values and the standard deviation were calculated from the experimental data obtained from duplicate trials for the tolerance assays. Factorial designs were applied for each experiment and statistical analysis was conducted by using ANOVA and, if statistical differences were detected ($P<0.05$), multiple comparison tests were performed and differences were quantified by mean difference estimation (standard error). Full factorial designs were applied by using two-way ANOVA, for tolerance assays. Two-way interaction tests were computed, as previously, for a complete factorial design. If any incomplete design was obtained (missing data), no interaction between factors was assumed.

RESULTS AND DISCUSSION

Microcapsule formation Individual solutions of xanthan gum, pullulan gum and jamilan, independently of their concentration, were unable to form capsules with the methodology employed in this work. Previous studies showed the ability of xanthan gum to form beads individually, but the concentrations used were higher and the encapsulation methodology was different from the dripping method employed at the present work (23). Gellan gum was able to form spherical beads but concentrations higher than 1% were needed to obtain the beads (data not shown). Therefore, mixtures of gellan gum with the other polymers were produced, facilitating the polymer manipulation during encapsulation.

Solutions containing different percentages of gellan gum and pullulan gum were unable to produce complete microcapsules, but formed semi-spheres when the drop got into the calcium chloride solution. Hence, they were not subsequently considered for microbial encapsulation. Xanthan–gellan gum and jamilan–gellan gum solutions did form microcapsules, but no perfect spheres were obtained with this methodology. Nevertheless, capsules obtained from the

combination of 1% gellan gum with 1% jamilan (J:G) and 0.75% gellan gum with 1% xanthan gum (X:G) showed the most spherical shape and lowest diameter and, therefore, were selected for the following tests.

The average diameter was of 1.71 ± 0.48 mm for X:G (1%:0.75%) capsules and 2.47 ± 0.54 mm for J:G (1%:1%) capsules. Scanning electron microscopy images showed a homogeneous surface, being the X:G (1%:0.75%) beads surface smother and less porous than J:G (1%:1%) ones (Fig. 1). No surface cavities or fractures were detected and bacterial cells appeared included and homogeneously distributed within the polymeric matrix, layering from the surface to the core of the capsule (Fig. 2). Thus, the capsules provided an intact physical barrier to the encapsulated cells.

Polysaccharides are already highly viscous in the liquid form at low concentration, which was a limiting factor for obtaining smaller capsules (27). The size and shape of the microcapsules formed in the present work are similar to those prepared with microbial polymers in other studies employing the same methodology (22,28). The size range obtained, although could not be optimal for certain dairy product applications, may represent an advantage for their implementation for pharmaceutical/nutraceutical purposes, i.e., oral delivery.

pH tolerance The effect of various pHs on the viability of free probiotic microorganisms is shown in Table 1. All the probiotic species assayed showed a loss in viability after the high acidic exposure (pH 2.0) with an average reduction of 4.5 log CFU after 2 h and a complete loss of detectable viability after 6 h exposure. An acidic pH of 3.5 did not reduce the viability of any of the bacteria tested and higher pH (5.0 and 6.5) allowed them to increase their numbers. The effect of pH on the viability of lactobacilli encapsulated in X:G (1%:0.75%) and J:G (1%:1%) is shown in Tables 2 and 3, respectively. The encapsulation conditions provided a stable number of viable microorganisms during the assay, with no significant differences between the encapsulating materials, except for the high acidic conditions exposure. At pH 2.0, losses in the viability of lactobacilli were at the level of 5 log CFU. However, the *Lactobacillus* species encapsulated in X:G (1%:0.75%) prolonged their viability by 6 h.

These results agree with Muthukumarasamy et al. (28) study, who found a low survival after simulated gastric juice exposure of *Lactobacillus reuteri* when encapsulation was performed with xanthan gum and an extrusion method. Gbassi et al. (20) reported that, after 90 min of incubation, three different strains of *L. plantarum* encapsulated in calcium alginate showed a total loss of viability. Similarly, Brinques and Ayub (29) observed a drastic decrease in the total number of survivors after simulated gastric juice exposure, so encapsulation in several alginate formulations was not effective in protecting *L. plantarum* with the extrusion method. However, other encapsulation methodologies, i.e., emulsion, were found effective on lactobacilli protection at pH 2.0 employing both alginate and xanthan gum (23). These reports reaffirm the difficulty published by different authors when comparing the results of probiotic survivability experiments, since the strains, encapsulating material, methodology and simulated media are completely different in all the assays (20,30).

All encapsulating materials employed in this study were exopolysaccharides but have different structural characteristics. Actually, X:G (1%:0.75%) seems to be more effective on bacterial protection at low

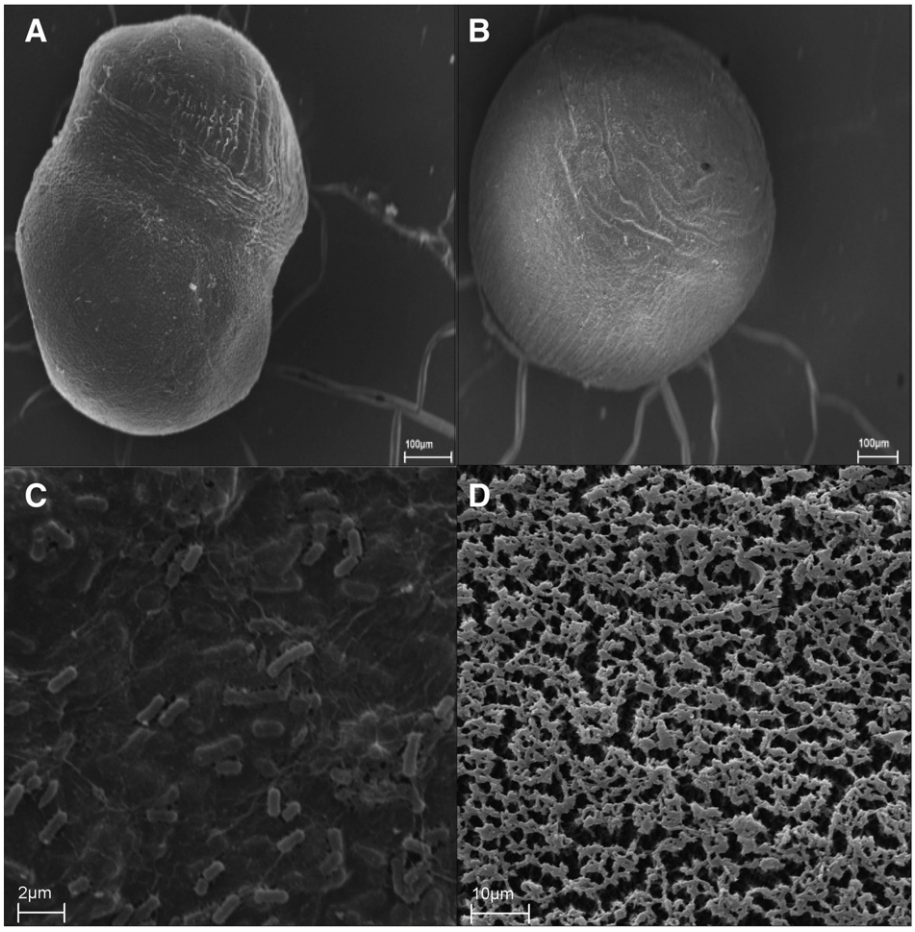

FIG. 1. SEM image of electrostatic bead generator produced capsules: (A, B) Capsules prepared with xanthan:gellan gum (1%:0.75%); (C, D) capsules prepared with jamilan:gellan gum (1%:1%).

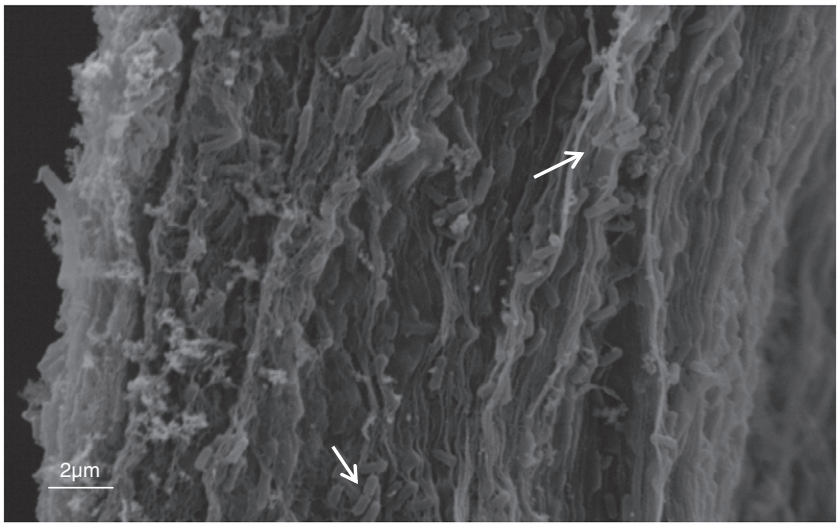

FIG. 2. Visible bacterial cells included in the polymeric matrix of xanthan:gellan gum (1%:0.75%) capsules. SEM images of capsule's section. Arrows stand out lactobacilli cells within the polymeric matrix.

pH (2.0). Differences in protection provided by the encapsulating materials used may be related to structural differences between xanthan gum and jamilan, since gellan gum was added to all the capsules obtained successfully. These structural and physical properties may affect the exopolysaccharides protective properties (27,31). Both xanthan and gellan gum are anionic polymers and therefore they could have the ability of capturing some of the medium's protons. In fact, at pH 1, a suppression of the polyelectrolytic behavior of gellan chains occurs, because the carboxylate groups are in their acidic form (32). Although the chemical structure of jamilan has not been determined yet, its chemical composition has been described. Jamilan has demonstrated the ability of precipitate heavy metal ions (26), and therefore should have an anionic character. Then, we infer that the ability to bind H^+ of the acidic environment, hence reducing their effect on bacterial metabolism, may be conditioned by the EPS structure, determined by the number of radicals available for the bonding.

Simulated gastrointestinal juices tolerance The effect of simulated gastrointestinal conditions on free and encapsulated *L. plantarum* and *L. rhamnosus* viability is shown in Figs. 3A and B, respectively. Both strains were especially susceptible to simulated bile exposure as planktonic bacteria. In this condition, *L. rhamnosus* showed a reduction of >3 log CFU after a 2 h exposure, but *L. plantarum* only >2 log CFU after 20 h simulated bile exposure. Both of the encapsulating materials used in this work improved the lactobacilli survival at these conditions, and the results agree with other studies that showed microencapsulation as an effective way of protecting probiotic bacteria against the same concentration of bile salts (3%) (18,23).

X:G (1%:0.75%) was the most effective of the encapsulating materials assayed in protecting *L. rhamnosus* and *L. plantarum*, because a decrease on <1 log CFU was registered after the bile exposure ($P<0.05$). In contrast, J:G (1%:1%) was only effective in protecting *L. rhamnosus* after a 2 h exposure, with 1 log CFU reduction ($P<0.05$). The lack of protection of *L. plantarum* by the J:G (1%:1%) capsules may be due to a biological incompatibility between this strain and J:G (1%:1%) as encapsulating material, according to previously described by Rodrigues et al. (10). This paper demonstrated that the biocompatibility of a certain polymer with a certain bacteria for encapsulation purposes may differ even between strains of the same probiotic specie, although the molecular basis of these interactions still remains unknown.

It has been demonstrated for several lactic acid bacteria that the exposure to bile induces the expression of proteins as well as up- and down-regulate the expression of genes related with structural integrity and protection against oxidative process (33). Previous studies with *L. plantarum* demonstrated a loss of cell wall integrity, possibly leading to leakage of intracellular material from the cells and a disturbed energy balance, even though the bile concentration employed was much lower (0.1%) (34). In our work, *L. plantarum* free cells reduced their viability after bile exposure, more likely due to the

TABLE 1. Effect of pH on viability of free lactobacilli.

pH	Strain	Viable cell count (log CFU/mL)			
		0 h	2 h	4 h	6 h
2	*L. plantarum*	$9.25 \pm 0.11^{b,c,d}$	4.92 ± 0.93^{a}	4.06 ± 0.93^{a}	0
	L. rhamnosus	$8.53 \pm 0.21^{b,c,d}$	4.18 ± 0.59^{a}	3.23 ± 0.56^{a}	0
3.5	*L. plantarum*	$9.25 \pm 0.11^{b,c,d}$	9.91 ± 0.10^{a}	9.89 ± 0.10^{a}	9.90 ± 0.13^{a}
	L. rhamnosus	$8.53 \pm 0.21^{b,c,d}$	9.60 ± 0.11^{a}	9.37 ± 0.43^{a}	9.48 ± 0.37^{a}
5	*L. plantarum*	$9.25 \pm 0.11^{b,c,d}$	$10.08 \pm 0.10^{a,c,d}$	$10.40 \pm 0.10^{a,b}$	$10.46 \pm 0.15^{a,b}$
	L. rhamnosus	$8.53 \pm 0.21^{b,c,d}$	9.62 ± 0.09^{a}	9.71 ± 0.15^{a}	9.88 ± 0.10^{a}
6.5	*L. plantarum*	$9.25 \pm 0.11^{b,c,d}$	10.13 ± 0.20^{a}	10.43 ± 0.20^{a}	10.34 ± 0.45^{a}
	L. rhamnosus	$8.53 \pm 0.21^{b,c,d}$	$9.65 \pm 0.11^{a,c,d}$	$9.92 \pm 0.11^{a,b}$	$10.04 \pm 0.06^{a,b}$

[a] Significant difference from time point 0 ($P<0.05$).
[b] Significant difference from time point 2 ($P<0.05$).
[c] Significant difference from time point 4 ($P<0.05$).
[d] Significant difference from time point 6 ($P<0.05$).
Results are expressed as mean ± SD of $\log_{10}$ colony-forming units (CFU)/mL.
Lowest limit of detection was ≤10 CFU/mL.

TABLE 2. Effect of pH on viability of lactobacilli encapsulated with xanthan:gellan gum (1%:0.75%).

pH	Strain	Viable cell count (log CFU/g)			
		0 h	2 h	4 h	6 h
2	*L. plantarum*	$9.53 \pm 0.07^{b,c,d}$	3.79 ± 0.66^{a}	3.42 ± 0.60^{a}	3.73 ± 0.25^{a}
	L. rhamnosus	$8.75 \pm 0.23^{b,c,d}$	2.42 ± 0.60^{a}	3.06 ± 0.97^{a}	4.20 ± 0.01^{a}
3.5	*L. plantarum*	9.53 ± 0.07^{b}	9.64 ± 0.11^{a}	9.44 ± 0.31	9.47 ± 0.43
	L. rhamnosus	8.75 ± 0.23	9.05 ± 0.13^{c}	9.14 ± 0.05^{b}	9.20 ± 0.31
5	*L. plantarum*	9.53 ± 0.07	9.85 ± 0.14	9.85 ± 0.15	10.00 ± 0.10
	L. rhamnosus	8.75 ± 0.23	9.35 ± 0.15	9.58 ± 0.07^{d}	10.19 ± 0.05^{c}
6.5	*L. plantarum*	9.53 ± 0.07^{d}	9.72 ± 0.16^{c}	10.01 ± 0.14^{b}	10.13 ± 0.07^{a}
	L. rhamnosus	8.75 ± 0.23	9.53 ± 0.05^{d}	9.78 ± 0.05	9.87 ± 0.10^{b}

[a] Significant difference from time point 0 ($P<0.05$).
[b] Significant difference from time point 2 ($P<0.05$).
[c] Significant difference from time point 4 ($P<0.05$).
[d] Significant difference from time point 6 ($P<0.05$).
Results are expressed as mean ± SD of $\log_{10}$ colony-forming units (CFU)/mL.
Lowest limit of detection was ≤100 CFU/mL.

TABLE 3. Effect of pH on viability of lactobacilli encapsulated with jamilan:gellan gum (1%:1%).

pH	Strain	Viable cell count (log CFU/g)			
		0 h	2 h	4 h	6 h
2	*L. plantarum*	$9.40 \pm 0.19^{b,c}$	3.68 ± 0.53^{a}	3.89 ± 0.16^{a}	0
	L. rhamnosus	$9.61 \pm 0.40^{b,c}$	4.30 ± 0.46^{a}	4.16 ± 0.30^{a}	0
3.5	*L. plantarum*	9.40 ± 0.19	9.48 ± 0.28	9.49 ± 0.37	9.31 ± 0.41
	L. rhamnosus	9.61 ± 0.40	9.13 ± 0.33	9.02 ± 0.19	9.30 ± 0.37
5	*L. plantarum*	9.40 ± 0.19	9.72 ± 0.04	9.78 ± 0.07	9.82 ± 0.10
	L. rhamnosus	9.61 ± 0.40	9.40 ± 0.14	9.56 ± 0.10	9.99 ± 0.08
6.5	*L. plantarum*	9.40 ± 0.19	9.93 ± 0.26	9.99 ± 0.07	10.0 ± 0.05
	L. rhamnosus	9.61 ± 0.40	9.49 ± 0.10	9.65 ± 0.26	9.90 ± 0.05

[a] Significant difference from time point 0 ($P<0.05$).
[b] Significant difference from time point 2 ($P<0.05$).
[c] Significant difference from time point 4 ($P<0.05$).
[d] Significant difference from time point 6 ($P<0.05$).
Results are expressed as mean ± SD of $\log_{10}$ colony-forming units (CFU)/mL.
Lowest limit of detection was ≤100 CFU/mL.

previously mentioned loss of cell wall integrity as a result of the detergent action of the bile salts. However, this strain encapsulated in X:G (1%:0.75%) did not reduce its viability. We infer that a thicker and less porous structure may be the cause of this protection, preventing from bile entrance and accumulation within the capsules and therefore reducing the bacterial stress. Xanthan gum in combination with gellan gum, after hydration and capsule formation, formed a homogeneous surface, barely porous, as seen in Fig. 1, but J:G (1%:1%) capsules were more porous and therefore unable to avoid the harmful action of the bile salts on *L. plantarum* cell wall.

On the other hand, *L. rhamnosus* viable cell numbers were higher after 24 h bile exposure than after 2 h. Lebeer et al. (35) demonstrated

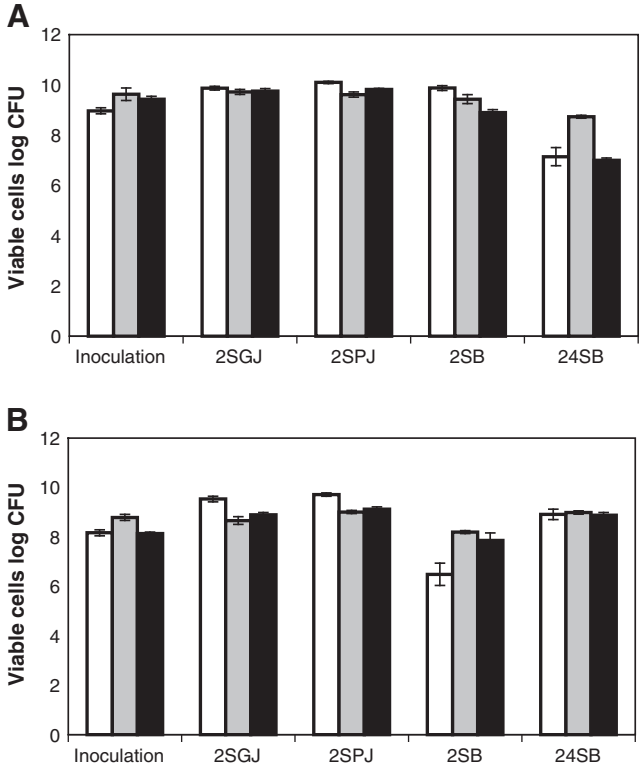

FIG. 3. Changes in viable cell counts of free and encapsulated bacteria in xanthan:gellan gum (1%:0.75%) and jamilan:gellan gum (1%:1%) during simulated gastrointestinal conditions exposure of (A) *L. plantarum* and (B) *L. rhamnosus*. Error bars represent standard deviation. White bars: free bacteria; Gray bars: X:G (1%:0.75%) encapsulated bacteria; Black bars: J:G (1%:1%) encapsulated bacteria; 2SGJ: 2 h in the presence of simulated gastric juice; 2SPJ: 2 h in the presence of simulated pancreatic juice; 2SB–24SB: 2 h up to 24 h in simulated bile. Note: numbers represent hours of experiment duration.

that *luxS* play a major role on *L. rhamnosus* GG (LGG) bile resistance. Furthermore, this study showed that LGG wild type showed an increased capacity of producing EPS and therefore of forming biofilms in the presence of bile, showing a metabolic response against bile exposure. At the same time, a study of Whitehead et al. (36) with *L. reuteri* showed that growth in high concentrations of bile (0.3%–5%) reduced the growth rate between 3 and 4 times, being the exposure times tested shorter than ours.

So, we infer that 2 h was not enough for *L. rhamnosus* to implement adaptation mechanisms due to a reduced growth rate associated to a 3% bile exposure, but 24 h was needed to develop a defense mechanism through the production of EPS. Free cells would therefore form biofilms through EPS production, which provide a barrier against bile action, hence reducing cell damage and allowing recovering of viable cell numbers. A synergy seems to take place between LGG's EPS production and the capsules, making the capsules even more compact and impenetrable by the bile salts and resulting also in viable cell number recovering.

Finally, as there are different degrees of interactions between bacteria and the encapsulating material, it should be of interest to perform a screening to test the exopolysaccharide-strain biocompatibility, since different levels of protection between species were detected in literature, mainly after simulated gastrointestinal conditions exposure (23,29).

Overall, the protection offered by the specific microbial polymers used in this work to the lactobacilli strains under evaluation, mainly the polymeric mixture of xanthan:gellan gum (1%:0.75%), showed to be more resistant in extreme simulated bile conditions (i.e., lower intestinal gut). Thus, microbial polymers appear to be an interesting source of encapsulating material. EPS with suitable properties for microencapsulation (i.e., gelling agent, harmlessness) should be considered for prospective studies of probiotic encapsulation for oral delivery applications and clinical trials.

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