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Effective encapsulation of reuterin-producing *Limosilactobacillus reuteri* in alginate beads prepared with different mucilages/gums

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ABSTRACT

The mainly aim of this study was to use mucilaginous solutions obtained from tamarind, mutamba, cassia tora, psyllium and konjac powdered to encapsulate reuterin-producing *Limosilactobacillus reuteri* in alginate beads by extrusion technique. In the particles were determined the bacterial encapsulation efficiency, cell viability during storage and survival under simulated gastric and intestinal conditions. Moreover, the reuterin production, its entrapment into the beads and the influence on viability of encapsulated microorganism were evaluated. Scanning electron microscopy and Fourier Transform Infrared spectroscopy were employed to characterize the produced particles. The beads showed a relatively spherical shape with homogenous distribution of *L. reuteri*. The use of gums and mucilages combined with alginate improved the encapsulation efficiency (from 93.2 to 97.4%), the viability of encapsulated bacteria during refrigerated storage (especially in prolonged storage of 20, 30 and 60 days) and the survival after exposure to gastric and enteric environments (from 67.7 to 76.6%). The *L. reuteri* was able to produce reuterin via bioconversion of glycerol in the film-forming solutions, and the entrapment of the metabolite was improved using konjac, mutamba and tamarind mucilaginous solutions in the encapsulation process (45, 44.57 and 41.25%, respectively). Thus, our findings confirm the great potential of these hydrocolloids to different further purposes, enabling its application as support material for delivery of chemical or biological compounds.

1. Introduction

Accepted by the European Food Safety Authority [1] as a food supplement to improve gastrointestinal health, *Limosilactobacillus reuteri* is recognized for its ability to produce and accumulate, under anaerobic conditions, high contents of 3-hydroxypropionaldehyde (3-HPA) through bioconversion from glycerol [[2],[3]]. The 3-HPA belongs to a dynamic system, named as HPA system, which contains 3-HPA, its hydrated form 1,1,3-trihydroxypropane, and its dimer 2-(2-hydroxyethyl)-4-hydroxy-1,3-dioxane [4]. This system was patented with the name reuterin [5]. Its scientific interest is notable, since reuterin has antimicrobial properties, such as antifungal activity and broad spectrum of activity against Gram-positive and negative bacteria [[6],[3]] being related to the probiotic activity of *L. reuteri* [7].

Currently, the concept of probiotic is defined by Food and Agriculture Organization of the United Nations (FAO), supported by the World Health Organization (WHO) as bacteria and yeasts that, when administered in adequate amounts, confer health benefits to the host [8]. Due to the benefits conferred to human health, probiotics have been investigated in the prevention and treatment of various conditions [[9],[10],[11],[12]]. However, to promote beneficial effects probiotics must be stable to environmental conditions, enabling its adherence to the intestinal mucosa, colonization of the human gastrointestinal tract, production of antimicrobial compounds and maintenance of metabolic activity in the intestine [[13],[14]]. Even though the evident health-promoting properties related to the administration of probiotics, the use of these microorganisms is limited by extrinsic factors, such as oxygen concentration, presence of hydrogen peroxide, pH and temperature variations, etc. In this sense, bacterial encapsulation is a useful approach to protect encapsulated cells under adverse conditions [[15],[16]].

Several techniques are currently available to encapsulate probiotic cells, but these procedures should not be aggressive, ensuring sufficient viability of the encapsulated cells [[17],[16]]. Thus, due to low cost,

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easy application and possibility of use of several hydrocolloid materials, the extrusion technique, which is a relatively mild process is widely used [[18],[19],[20],[21]]. A diversity of hydrocolloid materials can be used to encapsulate bacterial cells by extrusion, for instance alginate, gums and mucilages. Alginates are commonly used in the probiotic encapsulation. However, its use results in the formation of porous particles, which are susceptible to disintegration [16]. In contrast, the combination of alginate with others hydrocolloid compounds, such as gums and mucilages is related with the improvement of the viability of encapsulated microorganisms [[18],[22],[21]].

Abundantly available in nature, gums and mucilages have been used in partial substitution of alginates to change the particle properties obtained, improving the protection and survival of encapsulated cells during storage, under food processing conditions and passage through simulated gastric and intestinal tracts [[23],[21],[24]]. Recently, Rodrigues et al. [16] reported traditional and novel materials extracted from plants for probiotic encapsulation, such as psyllium (*Plantago ovata* Forssk) mucilage, konjac (*Amorphophallus konjac*) gum, mutamba (*Guazuma ulmifolia*) mucilage, cassia tora (*Cassia tora* L. and *Cassia obtusifolia*) gum and tamarind (*Tamarindus indica* L.) gum. Despite its structural characteristics indicate great potential as emerging biopolymer for probiotic encapsulation [[25],[26],[7],[27],[28]], until now the available literature related is scarce. Besides, there have been no literature records regarding the use of mutamba, cassia tora and tamarind mucilages to encapsulate probiotic microorganisms.

This study evaluated the influence of mucilaginous solutions obtained from tamarind, mutamba, cassia tora, psyllium and konjac in alginate beads carriers of reuterin-producing *Limosilactobacillus reuteri* DSM 20016 obtained by extrusion technique. The encapsulation efficiency, cell viability during storage and survival under simulated gastrointestinal conditions of encapsulated bacteria was evaluated and compared among the samples. Moreover, the reuterin production, its entrapment into the beads and influence on viability of encapsulated *L. reuteri* was also determined. The particles were characterized by scanning electron microscopy and Fourier Transform Infrared spectroscopy.

2. Materials and methods

2.1. Microorganism and chemicals

Limosilactobacillus reuteri DSM 20016 was obtained from Tropical Culture Collection (André Tosello Foundation), classified in this collection by the number CCT 3433. The following substances were employed for preparing the beads: High viscosity sodium alginate (Dinâmica Química Contemporânea Ltda, Diadema, SP, Brazil); mutamba mucilage, extracted from the seed of mutamba (*Guazuma ulmifolia* Lam.) fruits collected between August and September 2018 in natural areas of the Cerrado Biome located in the municipality of Uberlândia (18°55′07″ South latitude, 48°16′38″ West longitude and 863 m altitude), Minas Gerais, Brazil; psyllium (*Plantago ovata* Forssk) mucilage (SARGOL 99/100), cassia tora (*Cassia tora* L.) gum (LB COL-200) and tamarind (*Tamarindus indica* L.) gum (TEMCOL 30), kindly provided from Sarda Biopolymers Pvt. Ltd. (Maharashtra, India); and konjac (*Amorphophallus konjac*) gum, supplied by Fooding Group Limited (Shanghai, China). Other chemicals and solvents used were: tryptophan, hydrochloric acid, calcium chloride, sodium citrate (Dinâmica Química Contemporânea Ltda, Diadema, SP, Brazil); ethanol 95% (Vetec Química Fina Ltda., RJ, Brazil), glycerol ≥99.5% (Anidrol Produtos para Laboratórios Ltda., SP, Brazil), and acrolein standard obtained from Riedel-de Haën (Seelze, Hannover, Germany). The pepsin (swine gastric mucosa), lipase and pancreatin (swine pancreas), besides bovine bile, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Microbial growth conditions

L. reuteri DSM 20016 were kept frozen at −18°C in a medium composed of glycerol (130 g/L), bacteriological peptone (4.3 g/L), yeast extract (2.6 g/L), and NaCl (4.3 g/L), and then was activated in de Man, Rogosa and Sharpe sterile broth (Merck, Germany). The culture was incubated at 37°C for 24 h under aerobic static conditions. Subsequently, *L. reuteri* biomass was obtained by centrifugation at 9800 ×g for 10 min (Sorvall Legend XTR, Thermo Scientific™, Germany) followed by its resuspension in sterile buffered peptone water (1 g/L at pH 6.5). Cell count was adjusted at 10^9 CFU/mL before the encapsulation.

2.3. Extraction of mutamba mucilage

The method used to extract the mucilage from mutamba seeds was adapted from Pereira et al. [28]. Precisely 10 g of seeds were weighed into centrifuge tubes containing 180 mL of ultrapure water (OS50LXE, Gehaka, SP, Brazil). The mixture was kept at room temperature for 20 h to ensure complete seed hydration. The mucilaginous layers were extracted using a 13 mm diameter, 19 kHz ultrasonic probe (Unique, Disruptor, 800 W, Indaiatuba, SP, Brazil) at a power of 475 W for 13 min. The mucilage was obtained by separating the mucilaginous liquid from the seeds with stainless steel sieves followed by centrifugation at 5000 ×g for 10 min (Sorvall Legend XTR, Thermo Scientific™, Germany). Subsequently, the mucilage obtained was lyophilized and kept frozen (−18°C) until use.

2.4. Encapsulation of reuterin-producing L. reuteri

Using a digital disperser (Ultra-turrax, IKA T25, Campinas, SP, Brazil) the sodium alginate was homogenized (6000 rpm) with or without the addition of 100 mmol/L of glycerol in 50 mL of distilled water or mucilaginous solution from mutamba, psyllium, cassia tora, tamarind and konjac, which were previously hydrated overnight at 5°C. The composition and nomenclature of alginate-mucilaginous solutions are presented in Table 1. Due to the high apparent viscosity, the concentration of psyllium and konjac mucilaginous solutions were adjusted to 5 g/L, which allowed the use of the proposed extrusion technique. The film-forming solutions were sterilized in an autoclave at 121°C for 15 minutes and after cooling (42°C) the biomass suspension of *L. reuteri* DSM 20016 was aseptically added to reach an initial cell count between 8.3 and 8.7 log CFU/mL. Then, *L. reuteri* was encapsulated by extrusion technique. The film-forming solutions was dripped using sterile needles (ø 0.7 mm) into a sterile $CaCl_2$ solution (20 g/L). The beads obtained was

Table 1
Composition and nomenclature of alginate-mucilaginous solutions blends used for encapsulate *L. reuteri*.

Composition			Nomenclature
alginate (20 g/L)	–	–	Control
		glycerol (100 mmol/L)	Control-G
	tamarind gum (10 g/L)	–	TG
		glycerol (100 mmol/L)	TG-G
	mutamba mucilage (10 g/L)	–	MM
		glycerol (100 mmol/L)	MM-G
	cassia tora gum (10 g/L)	–	CG
		glycerol (100 mmol/L)	CG-G
	psyllium mucilage (5 g/L)	–	PM
		glycerol (100 mmol/L)	PM-G
	konjac gum (5 g/L)	–	KG
		glycerol (100 mmol/L)	KG-G

kept for 30 minutes for complete crosslinking and gelling, being later removed by aseptic filtration, washed with sterile distilled water and stored at 4°C ± 2°C.

2.4.1. Scanning electron microscopy (SEM)

The wet particles were dried in an oven with air circulation (Tecnal, TE-394/1, Piracicaba, SP, Brazil) at 45°C for 36 h. Then, the surface and cross-sectional morphology of the *L. reuteri*-loaded particles were observed using a scanning electron microscope (SEM; Leo 440i, LEO Electron Microscopy, Cambridge, UK) with X-ray dispersive energy detector (EDX; 6070, LEO Electron Microscopy, Cambridge, UK). Prior to observation, the samples were coated with 200 Å-thick layer of gold using a sputter coater (K450, Kent, UK).

2.4.2. Fourier-transform infrared spectroscopy (FT-IR)

The FT-IR spectra of the particles and the powdered hydrocolloid materials were recorded with a IRPrestige-21 Fourier transform infrared spectrometer (Shimadzu, Kyoto, Japan) in the wavelength range of 4000–400 $^{cm-1}$. The KBr pellets were prepared with a sample to KBr ratio of 1:100 (w/w) (except for powdered konjac gum and KG, whose were prepared in 1.5:100 ratio) in stainless steel molds (13 mm of diameter) compressed into transparent discs using an SSP-10A hydraulic press at 80 kN for 10 min under vacuum (Shimadzu, Kyoto, Japan).

2.4.3. L. reuteri encapsulation efficiency (EE)

The EE was determined by counting of the *L. reuteri* viable cells before and after extrusion in calcium chloride. The number of *L. reuteri* viable cells present in the film-forming solutions or in the beads were determined by direct counting of the number of colony-forming units (log CFU/g) employing the pour plating method. Thus, 1 g of each sample was homogenized and diluted using a vortex mixer (Model K45-2810, Kasvi, Brazil) with periodic agitations (10 s every min) in 9 mL of sterile sodium citrate solution (30 g/L) with pH adjusted to 6.0 and temperature of 45°C for 15 min, followed by serial dilution and plating in de Man, Rogosa and Sharpe agar (Merck, Germany). Then, the samples were incubated at 37°C for up to 72 h under aerobic conditions. The data obtained were applied in Eq. (1), where Xt referred to the total number of viable cells encapsulated; Xi referred to the total number of live cells inoculated into film-forming solution.

$$EE(\%) = \frac{Xt}{Xi} \cdot 100 \quad (1)$$

2.4.4. L. reuteri survival in refrigerated storage

The produced beads were analyzed to determine the number of *L. reuteri* viable cells during storage (0, 3, 6, 9, 12, 15, 20, 30 e 60 days) at 4°C ± 2°C, according to direct counting of the number of colony-forming units (log CFU/g) proposed in Section 2.4.3.

2.4.5. L. reuteri survival under simulated gastrointestinal conditions

Prior to assay, a *L. reuteri* free cell culture (FC) was previously obtained according to the method proposed in Section 2.2. Then, the survival of FC and encapsulated *L. reuteri* was evaluated in different buffer solutions and at predetermined times. Thus, 3 g of each sample were placed in falcon tubes containing 30 mL of simulated gastric juice (0.85% saline solution with pH adjusted to 2.3–2.6 with 1 M HCl) containing pepsin and lipase, at a final concentration of 3 g/L and 0.9 mg/L, respectively. The tubes were incubated at 37°C for 2 h, simulating the gastric phase. In the next step – enteric phase I, the pH of the reaction medium was adjusted to 5.4–5.7 using an alkaline solution (150 mL of 1 M NaOH and 14 g of $NaH_2PO_4.2H_2O$ for 1 L of distilled water). Bile and porcine pancreatin were added to the reaction medium to reach a concentration of 10 g/L and 1 g/L, respectively. After a period of 2 h at 37°C, the pH of the samples was adjusted to 6.8–7.2 using the same alkaline solution used in the enteric phase I, maintaining the concentrations of bile and pancreatin. Then the samples were incubated at 37°C for another 2 h, simulating the enteric phase II [29]. The *L. reuteri* survival was performed with aliquots of sample dilutions (from 1 g of sample) after 0, 2, 4 and 6 h of *in vitro* digestion, which were plated in de Man, Rogosa and Sharpe agar (Merck, Germany) by the pour plate technique. The samples were incubated at 37°C for up to 72 h under aerobic conditions.

2.5. Reuterin production

The ability of *L. reuteri* DSM 20016 for the *in situ* reuterin production in alginate-mucilage/gum-based film-forming solutions using glycerol as substrate was tested (samples Control-G; PM-G; KG-G; MM-G; CG-G and TG-G described in Table 1). These film-forming solutions produced according to method described in Section 2.4 were subjected to anaerobic fermentation at 37°C for 24 h, according to Rodrigues et al. [30]. At the end of fermentation *L. reuteri* and reuterin-content produced present in the film-forming solutions were encapsulated by extrusion technique in calcium chloride cross-linking solution (20 g/L) according to Section 2.4.

2.5.1. Determination of in situ reuterin production

The reuterin quantification in the film-forming solutions and in the produced beads were carried out indirectly according to the colorimetric method proposed by Circle et al. [31], with adaptations. Initially, 1 g of each sample analyzed was diluted in 9 mL of sodium citrate (20 g/L). Posteriorly, 320 µL of the samples was homogenized with 300 µL of a 0.1 M tryptophan solution (dissolved in 0.05 M HCl) and 600 µL of ethanol (95%). The samples were incubated at 40°C for 50 min. Then, the absorbances were measured by spectrophotometry (DU 640, Beckman Coulter, CA, USA) at 560 nm. The reuterin content was determined by comparing the absorbance of the samples with an acrolein standard curve previously constructed in the range from 2 to 100 mmol/L, assuming that 1 M of dehydrated reuterin corresponded to 1 M of acrolein.

2.5.2. Reuterin entrapment

The reuterin entrapment was estimated via the comparison of reuterin-content present in the film-forming solutions to the reuterin detected after extrusion in calcium chloride and consequent formation of hydrocolloid particles. The data obtained were applied in Eq. (2), where Rt referred to the reuterin detected in the beads; Ri referred to the reuterin-content present in the film-forming solutions.

$$Reuterin\ Entrapment\ (\%) = \frac{Rt}{Ri} \cdot 100 \quad (2)$$

2.6. Statistical analysis

All samplings were made in triplicate, except the *L. reuteri* survival under simulated gastrointestinal conditions, which was performed in duplicate. The data were subjected to ANOVA and the means were compared by the Tukey test using the Statistica 10 software. The t-test was applied when the comparison of means between two sample groups was required. The results were considered significant at $p<0.05$.

3. Results and discussion

3.1. Scanning electron microscopy (SEM)

Details obtained by SEM to evaluate the structure, surface morphology and the microstructure of the beads are presented in Figs. 1 and 2. The particles produced by the extrusion technique showed a spherical or relatively spherical shape independently of the addition of gums or mucilages (Fig. 1 A, C, E and G). Homogenous distribution of *L. reuteri* was observed in the surface and cross-sectional morphology of the particles (Fig. 1 E and H). Similar results were reported by Karimi

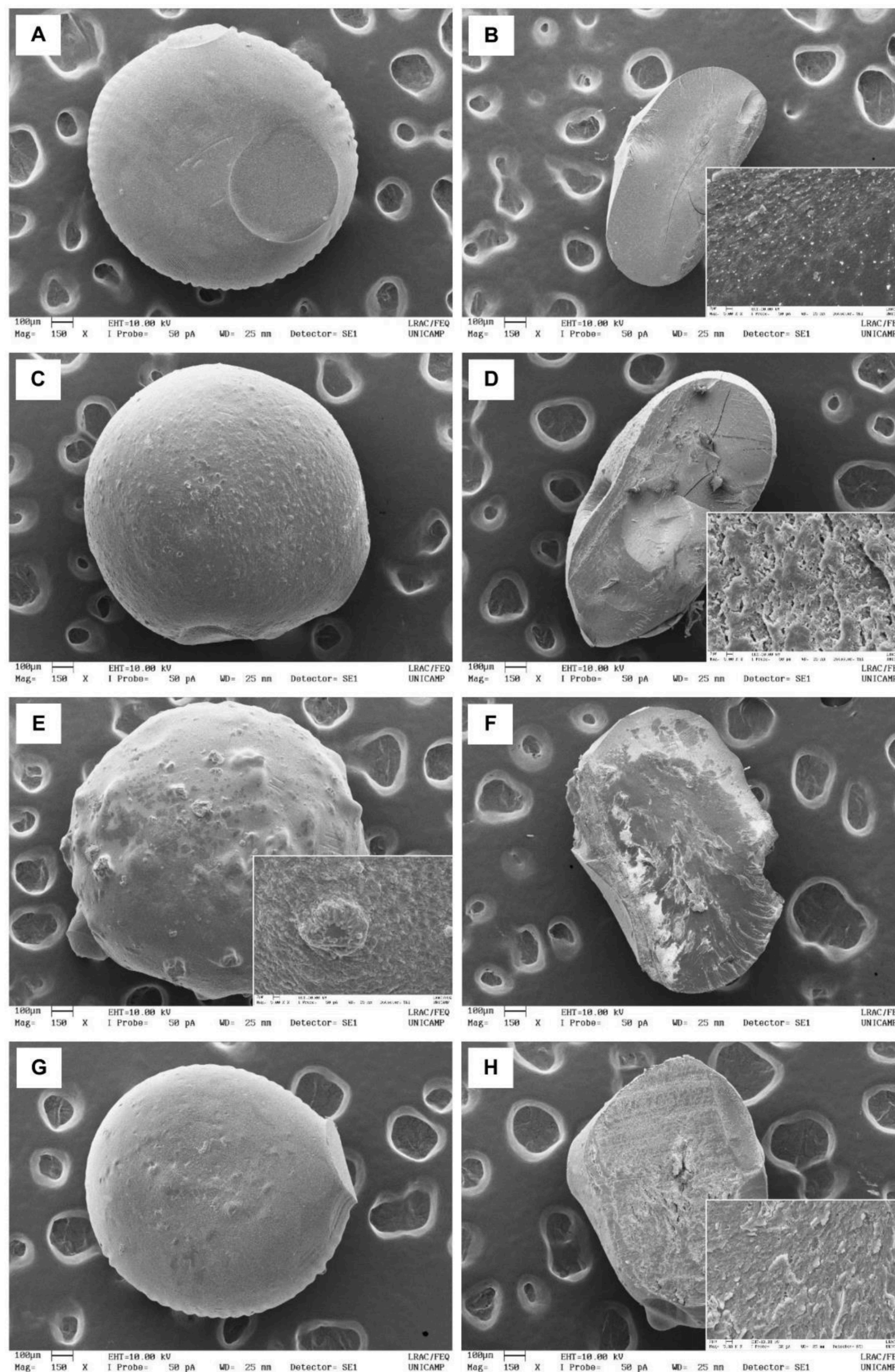

Fig. 1. Scanning electron microscopy (SEM) of the *L. reuteri*-loaded particles. Surfaces of the particles and cross-sectional morphology of Control (A & B), TG (C & D), CG (E & F), and KG (G & H) at 150× magnification. The images inserted in B, D, E and H were taken at 5000× magnification. *Refer to Table 1 for details of each of the samples.

et al. [32] that produced *L. reuteri* particles based on alginate. However, the addition of asafoetida and zedo gum make the beads surface smoother and improved the physical barrier to the encapsulated cells.

The particle surface of the Control sample was smoother then observed into the beads containing gum or mucilage, suggesting that fractions of these hydrocolloids gelled on the surface of the particles, slightly increasing the roughness (Fig. 1 A, C, E and G). This result is in accordance with data reported by Nasiri et al. [33], which encapsulated *Lactobacillus casei* in alginate microcapsules containing wild sage seed mucilage by emulsion technique. Their results demonstrated spherical beads with roughness surface due to wild sage seed mucilage incorporation. In this sense, the use of glycerol in the particle's composition changed the structure of the produced beads making them smoother (Fig. 2). In this sense, Jouki et al. [34] related the softer structure of

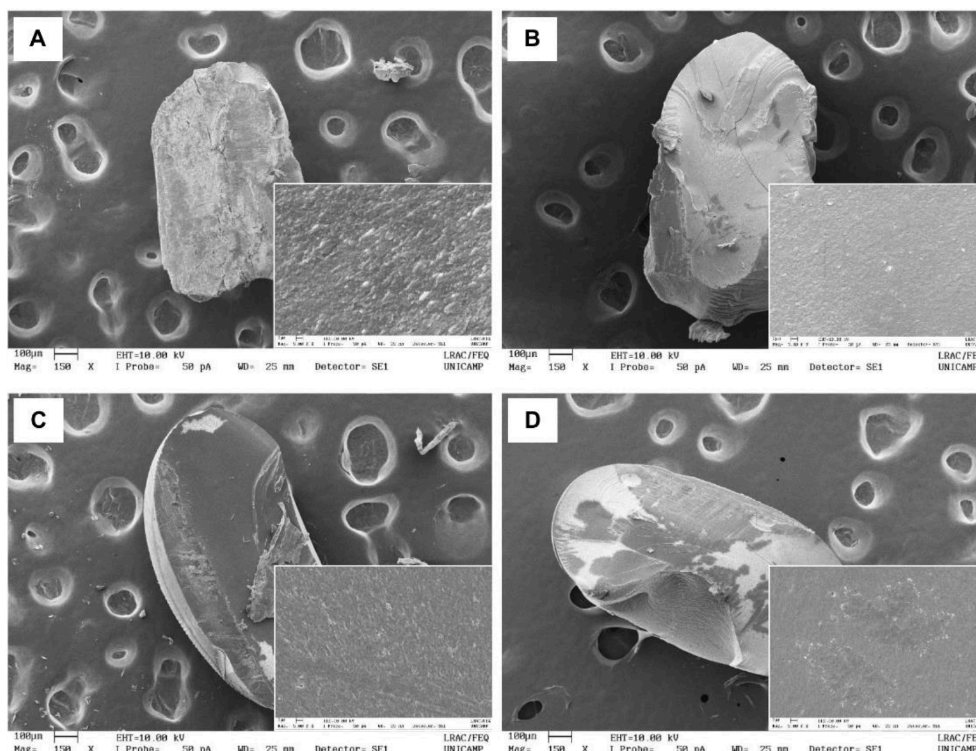

Fig. 2. Scanning electron microscopy (SEM) of the *L. reuteri*-loaded particles. Cross-sectional morphology of PM (A), PM-G (B), MM (C) and MM-G (D) at 150× magnification. The inserted images were taken at 5000× magnification. *Refer to Table 1 for details of each of the samples.

quince seed gum-alginate beads to plasticizer capacity of glycerol used in the production of microcapsules. In addition, to characterize the structure of the bead's polymeric network, FTIR assays were conducted and discussed in the next section.

3.2. Fourier-transform infrared spectroscopy (FT-IR)

In general, the FT-IR spectra of the powdered hydrocolloid materials (Fig. 3A) showed a characteristic band of O–H stretching and intra- and intermolecular hydrogen bonds between 3650 cm^{-1} and 3000 cm^{-1}, being a broad band centered at 3430 cm^{-1}. Another weaker peak was detected in the same samples in the region between 2950 cm^{-1} and 2800 cm^{-1}, centered at 2910 cm^{-1} due to C–H stretching vibrations. These two bands are characteristics of all polysaccharides [28].

The powdered alginate FT-IR spectra showed two characteristic bands at 1600 and 1400 cm^{-1} corresponding to a carbonyl group of carboxylic acid [35] and asymmetric and symmetric stretching vibrations of C–O bond of carboxylate salt ion [36], characteristic of an alginate structure. In addition, the spectral bands between 1320 and 1025 cm^{-1} suggest the presence of guluronic acid [37], with peaks between 1150 and 1045 cm^{-1} being previously related to C–O stretching vibrations of the pyranosyl ring [36]. At the same time, were detected asymmetric stretching vibrations between 900 and 800, whose usually correspond to mannuronic acid units [37].

The FT-IR spectra of powdered tamarind gum presented three bands at 1730, 1640 and 1540 cm^{-1}, which are regions with stretching vibrations related to proteins and carbohydrates. Being the 1730 and 1640 cm^{-1} wavenumbers related to C=O stretching characteristic of amide I (–CONH$_2$ groups), while 1540 cm^{-1} was associated to amide III ($-NH_3^+$ groups) [38]. The peak at 1440 cm^{-1} is related to C–C stretching vibrations, and the bands between 1400 and 1050 cm^{-1} are characteristic of xyloglucan backbone, being related to CH$_2$ bending (1370 cm^{-1}) and C–O bond stretching of the xyloglucan ring (1161 and 1050 cm^{-1}) [39].

Although until now there have been few records available, the FT-IR patterns of the lyophilized mutamba mucilage detected bands at 1732 and 1143 cm^{-1}, which are related with stretching vibrations of C=O and C–O–C present in the pyranose ring. In addition, the bands at 1600 and 1417 cm^{-1} its due to presence of the carboxylate ion from the uronic acids, as well as the peak observed at 1253 cm^{-1} may represent uronic acid contents. Asymmetrical vibrations were also detected between 1075 and 1045 cm^{-1}, whose are assigned to C–O–C stretching of glycosidic bonds and C–O–H bending [28]. In parallel, the FT-IR spectra of cassia tora gum showed an absorption peak at 1654 cm^{-1} indicating the presence of carbonyl groups [40], while stretching vibrations at 1022 cm^{-1} are attributed to C–H bending, indicating carbohydrate fractions in the mucilage [41].

The FT-IR spectrum of psyllium mucilage showed bands at 1726 cm^{-1} and 1649 cm^{-1}, whose are characteristics vibration of ester carbonyl group and carboxylic group [35]. The stretching vibration of C–O–C group was detected at 1049 cm^{-1}, suggesting the presence of arabinoxylans ([35]; Monge [42]). In parallel, the konjac gum FT-IR spectra detected bands at 1730 and 1651 cm^{-1} with stretching vibrations that are characteristics assigned to acetyl groups (C=O) and the presence of amide groups (–CONH–), respectively [43]. Bending vibration correspondent to symmetric carboxylate group and C–O at 1390 and 1174 cm^{-1}, respectively were detected, while the peak at 1014 cm^{-1} is related to C–O–C stretching bond [44].

The FT-IR spectra of alginate-mucilage/gum interactions were studied (Fig. 3B). In the presence of Ca^{2+} ions, the beads presented a similar FT-IR spectra with the powder results. Besides, the presence of lower intensity peaks in Control indicates the interaction of alginate with calcium ions. In the samples, the O–H groups had been detected in the region between 3600 cm^{-1} and 3200 cm^{-1}, however the peak was narrower, and the intensity was reduced. According to Daemi and Barikani [45] the hydroxyl and carboxylate groups present in the alginate participate to form chelating structure to the calcium ion, which decrease in hydrogen bonding between hydroxyl functional groups in calcium alginate. The peaks at 1627 and 1417 cm^{-1}, also narrower and lesser intensity, were assigned to the stretching vibrations of carbonyl group. In contrast, Ramos et al. [37] reported peaks of greater intensity

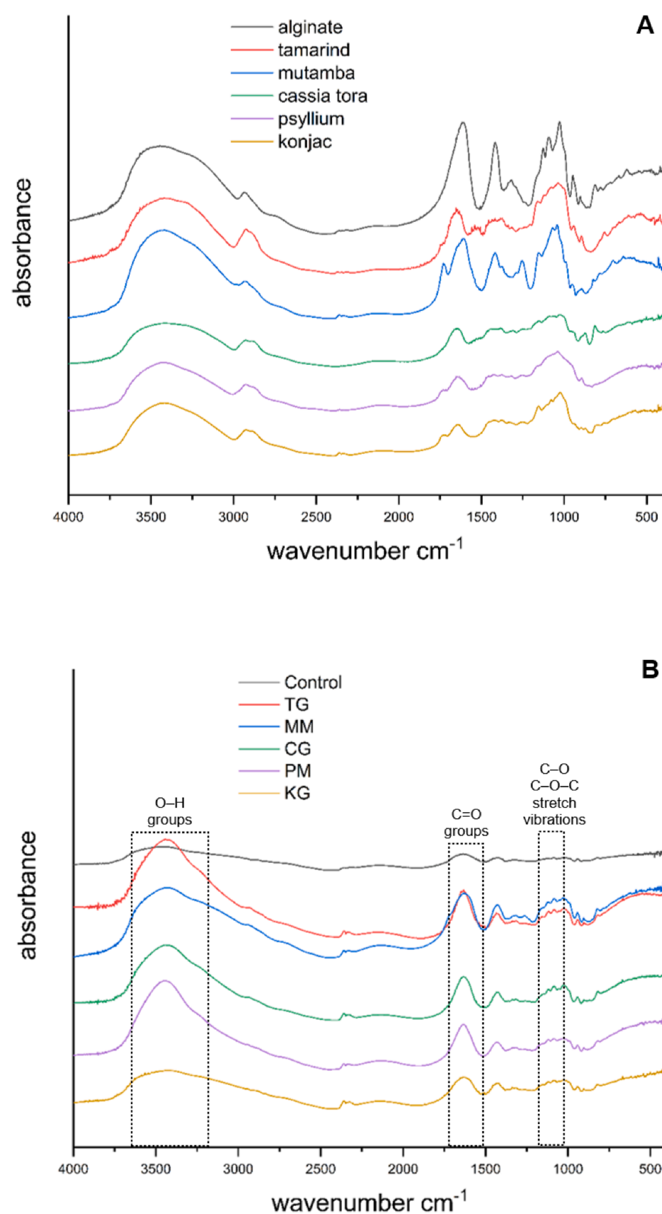

Fig. 3. FT-IR spectra of the powdered hydrocolloid materials (A) and of the alginate-mucilage/gum beads (B). *Refer to Table 1 for details of each of the samples.

in the same region in calcium alginate particles. The authors attributed this change to the substitution of sodium ions by calcium ions, which can result in a change in the charge density of carboxyl groups. In addition, the shifting of COO^{-} peaks to higher wavenumber are related to the satisfactory interaction between calcium and anionic hydrocolloid fractions [36]. The asymmetric bands in the region between 1150 and 1020 cm^{-1} were assigned to the stretching vibrations C–O and C–O–C, which are the characteristic of the natural hydrocolloids [46]. Overall, the FT-IR spectra of alginate-mucilage/gum beads showed characteristic peaks without any significant shift. Thus, no interaction between the hydrocolloids was confirmed.

3.3. *L. reuteri encapsulation efficiency*

In present study, the evaluation of EE according to the hydrocolloid materials used to produce the *L. reuteri* particles ranged from 85 to 97.4% (Table 2). In general, the encapsulation technique was sufficiently satisfactory to entrap the *L. reuteri* cells in the hydrocolloid matrices. There was no critical difference in EE when mucilages and gums were used with alginate. The highest EE was obtained in CG, PM, PM-G, KG and KG-G ($p<0.05$). In contrast, beads produced only alginate-based showed lower EE (Control and Control-G). Despite widely employed for microbial encapsulation, the use of alginate can result in the formation of porous particles. Therefore, the use of hydrocolloids extracted from plants in combination with alginate can improve particle properties and enhance the entrapment of the cells [16]. Besides exhibiting high colloidal stability, several mucilaginous solutions present anionic characteristics, which can increase the electrostatic interactions in the formation of the beads [47], improving the EE.

Table 2
L. reuteri encapsulation efficiency (EE) in alginate-mucilage/gum beads.

Samples	Log CFU.g^{-1} during encapsulation		EE (%)
	before[I]	after[II]	
Control	8.54 ± 0.080	7.80 ± 0.020	91.44[bc]
Control-G	5.50 ± 0.142	4.68 ± 0.036	85.09[c]
TG	8.61 ± 0.066	8.25 ± 0.058	95.93[ab]
TG-G	5.76 ± 0.020	5.37 ± 0.108	93.23[ab]
MM	8.51 ± 0.046	8.12 ± 0.034	95.42[ab]
MM-G	6.31 ± 0.270	5.90 ± 0.020	93.50[ab]
CG	8.45 ± 0.075	8.23 ± 0.191	97.40[a]
CG-G	5.94 ± 0.025	5.58 ± 0.095	93.94[ab]
PM	8.47 ± 0.026	8.15 ± 0.136	96.10[a]
PM-G	6.47 ± 0.198	6.18 ± 0.113	95.36[a]
KG	8.34 ± 0.068	8.04 ± 0.050	96.28[a]
KG-G	6.73 ± 0.115	6.46 ± 0.030	95.99[a]

[I]*L. reuteri* cells detected in the film-forming solution. [II]*L. reuteri* cells detected in the beads. *Refer to Table 1 for details of each of the samples. **Means followed by the same lowercase letter in the column do not differ by the Tukey test ($p>0.05$). ***Each data point represents the mean of assays performed in triplicate

Similar to our results, Kahieshesfandiari et al. [48] and Nami et al. [23] reported the encapsulation by extrusion of *Enterococcus faecium* ABRIINW.N7 and *Lactococcus lactis* ABRIINW-N19 in alginate-basil-fenugreek and alginate-persian gum, respectively. The produced particles showed an EE >98%. In contrast, Shah et al. [49] reported EE between 71 and 79% using β-glucan to encapsulate *Lactobacillus plantarum* NCDC 012, *Lactobacillus casei* NCDC 297, and *Lactobacillus brevis* NCDC 021 by emulsion technique. In parallel, Rajam and Anandharamakrishnan [50] reached an EE between 70 and 73% when encapsulating *Lactobacillus plantarum* MTCC 5422 using fructooligosaccharides by spray drying technique. As consequence, it is evident that both material and technique applied affect the entrapment of bacterial cells in the obtained particles.

3.4. *L. reuteri survival in refrigerated storage*

The *L. reuteri* survival in refrigerated storage was investigated. In fact, the most of probiotic content-products must be kept at refrigeration temperatures to maintain the microorganism survival, avoiding sharp decreases in its microbial population [51]. Fig. 4 shows that the viable cell count of encapsulated *L. reuteri* decreased during storage ($p<0.05$). However, the decrease was smaller in samples without glycerol and no subjected to anaerobic fermentation process before encapsulation. The glycerol bioconversion can result in reuterin production and its accumulation in the medium can cause *L. reuteri* cell death by either the inhibition of ribonucleotide reductase or the interaction and modification of thiol groups in active peptide structures, which can compromise the DNA synthesis and induce cells to oxidative stress, respectively [[52],[53],[6]]. In this sense, the *L. reuteri* beads, in which film-forming solutions were subjected to previous anaerobic fermentation, showed a strong decrease in the encapsulated bacterium viable cell count (Fig. 4B). In 12 days of refrigerated storage no viable *L. reuteri* cells were detected in Control-G, TG-G, MM-G and PM-G, while a low number of

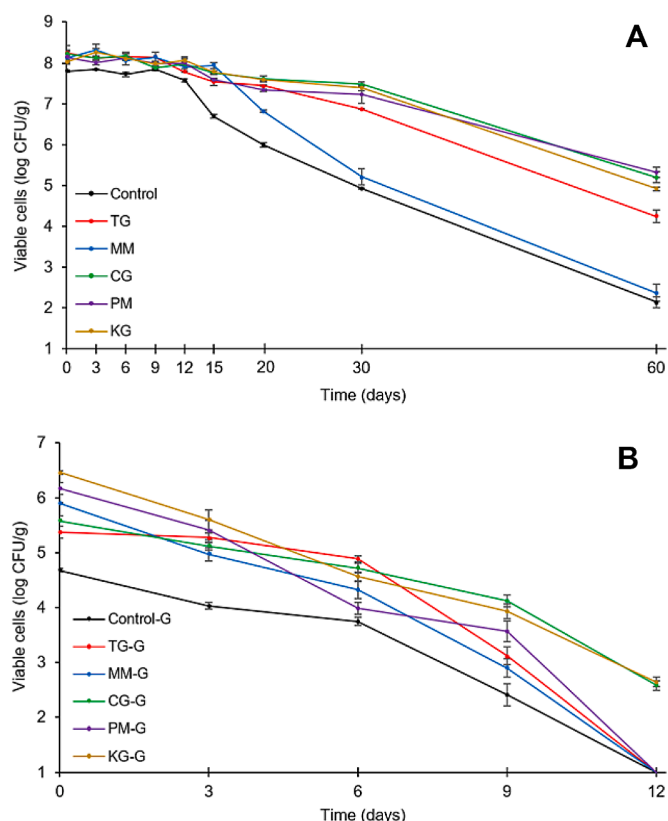

Fig. 4. *L. reuteri* survival in alginate-mucilage/gum beads during refrigerated storage. Samples with (B) or without (A) the induction of reuterin production. *Refer to Table 1 for details of each of the samples. **Each data point represents the mean of assays performed in triplicate.

viable cells ($p>0.05$) was detected in CG-G and KG-G (2.65 and 2.58 log CFU/g, respectively).

In contrast, the samples in which the *in situ* reuterin production was not induced showed viable cell counts after 60 days of refrigerated storage (Fig. 4A), although a significant reduction, compared to the initial count ($p<0.05$), was noticed. The total reduction in viable cell count after 60 days of refrigerated storage was averaged 2.99 log CFU/g in KG, PM, and CG, while the Control showed a sharp decrease of 5.66 log CFU/g ($p<0.05$). This indicates that the use of hydrocolloids, such as konjac gum, cassia tora gum or psyllium mucilage combined with alginate can improve the survival of encapsulated *L. reuteri* cells over time. It is important to emphasize that cassia tora mucilage have not been reported to encapsulate probiotics. This hydrocolloid extracted from dark-colored seeds of *Cassia tora* L. and *Cassia obtusifolia*, basically constituted of mannose and galactose [16], demonstrated to be an efficient support for encapsulating *L. reuteri*. In addition, no decrease in the number of *L. reuteri* viable cells in KG, PM and CG was detected for 12 days of refrigerated storage ($p>0.05$).

Mu et al. [7] employed konjac glucomannan hydrogels to encapsulate *Lactobacillus acidophilus* by the emulsion technique. The cell viability of the encapsulated bacteria was evaluated for 4 weeks at 4 and 25°C. The hydro microcapsules produced presented stability during storage, with decreases of 1.7 and 2.5 log CFU/g at 4 and 25°C, respectively. The authors emphasized the improvement of *L. acidophilus* viability when konjac oligosaccharides were applied as prebiotic, besides the influence of high temperatures in the particle protective effect, which can be weakens due to decomposed of polymer material inducing changes in the permeability of the beads. In parallel, Peredo et al. [54] reported high survival in storage conditions (4°C for 30 days) of *Lactobacillus plantarum* (Lp33 and Lp17) encapsulated in alginate-psyllium mucilage blend by extrusion. The total reduction in the number of viable cells of the encapsulated probiotics at the end of storage ranged from 0.75 to 0.77 log CFU/g. A similar trend was evidenced in the present study, the *L. reuteri* beads constituted of alginate-psyllium mucilage showed a total reduction in the number of viable cells of 0.91 and 2.82 CFU/g in 30 and 60 days of refrigerated storage, respectively. The psyllium mucilage is composed of arabinoxylans that consist of xylan structures with multiple side chains consisting of xylose and arabinose residues. Although it was not fully explored, the improvement of the alginate-psyllium mucilage particle properties and consequent bacteria protection can be related with the improvement of integrity of surface topography of the beads [16], or due to herbal-based polymers, such as psyllium present prebiotic characteristic which can favor encapsulated probiotic cells [55].

On the other hand, the survival of *L. reuteri* in beads consisting of alginate-mutamba mucilage (MM) showed a maintenance behavior of probiotic cell viability for 15 days of refrigerated storage ($p>0.05$), showing total reduction on 0.18 log CFU/g. However, after 20 days a sharp decrease was detected, reaching at the end of storage (60 days) on 5.74 log CFU/g; not differing from the Control ($p>0.05$). According to Pereira et al. [28], the mutamba mucilage present a profile of monosaccharides containing galactose, galacturonic and glucuronic acids, rhamnose, glucose and smaller amounts of arabinose/mannose. The surface charge density (ζ potential) confers a stable hydrocolloid mucilage system with an anionic character, which according to Manzoor et al. [56] may favor the ionic crosslinking of the polymer chains by divalent cations, like the calcium cations used in extrusion technique. Despite producing stable gels to temperature changes, they can present dryness. Until now, the mutamba mucilage has not been explored to encapsulate microorganisms, but Pereira et al. [28] reported the encapsulation of the volatile compounds from orange peel oil in emulsion systems. The mutamba mucilage could efficiently retain volatile compounds after 72 h, especially if compared with other biopolymers widely used by food industry (gum acacia, Hi-cap 100 and Snow-Flake E6131).

Tamarind gum, another hydrocolloid not yet explored to encapsulate bacteria, was tested. Constituted mainly by galactoxyloglucans containing glucose, galactose and xylose, the hydrocolloid from tamarind seed is related to its ability to form high viscosity solutions with thermal and chemical stability [16]. Moreover, due to its valuable potential functional and a low-cost, the tamarind gum should be applied in encapsulation procedures [57]. The same authors reported that the tamarind gum was successfully employed as a novel wall material for sesame oil encapsulation, protecting it against oxidation. In contrast, the tamarind gum present low solubility in cold water and biodegradability, which can limit its efficiency in forming microparticles, mainly caused by the droplet aggregation [58]. To overcome these disadvantages, Khounvilay et al. [59] suggested a carboxymethylation process for crude tamarind gum, which can confer it an anionic character. In our study, the results obtained during refrigerated storage showed that the beads constituted of alginate-tamarind gum (TG) were capable to maintain the number of viable cells of encapsulated *L. reuteri* for 9 days of storage ($p>0.05$); result also observed in Control. Even though the total decrease observed in the *L. reuteri* cell viability in TG was greater than KG, PM and CG ($p<0.05$), reaching 4.01 log CFU/g after 60 days of refrigerated storage, this decrease was smaller ($p<0.05$) when compared to MM and Control samples, especially in prolonged storage conditions (20, 30 and 60 days). In addition to maintain the cell viability during storage, microorganisms with probiotic claims must be stable under gastrointestinal conditions [16]. In this way, the *L. reuteri* survival in acid and enteric simulated environments are discussed in the next section.

3.5. *L. reuteri survival under simulated gastrointestinal conditions*

The encapsulation technique and the encapsulating materials used should be suitable for that the entrapped bacteria remain viable during

passage through gastric and enteric juices [16]. The survival of FC and encapsulated *L. reuteri* in different hydrocolloid blends before (0 h) and during exposure to gastric (2 h) and enteric (4 and 6 h) conditions is showed in Fig. 5A. It was observed that the total reduction in the number of *L. reuteri* viable cells after exposure to simulated gastric and enteric juices was higher for FC, which showed an expressive decrease by 6.72 log CFU/g on viable cell count ($p<0.05$). This demonstrates that the encapsulation process protected the encapsulated cells under gastric and enteric environments.

After 2 h of exposure to simulated gastric juice, the viability of FC showed sharped decrease ($p<0.05$) by 4.87 log CFU/g, representing a reduction of 49.39%. In contrast, the survival of encapsulated *L. reuteri* under the same conditions was improved. The use of gums and mucilage in the composition of the beads conferred even greater protection to *L. reuteri* cells compared to Control ($p<0.05$), which showed reduction of 26.38%, while TG, MM, CG, PM and KG showed an average reduction of 12.55, 14.67, 13.67, 12.96 and 13.6%, respectively. The sharped decline in the viability of bacterial cells subjected to gastric juices were generally attributed to the acidic environment and presence of digestive enzymes [[60],[61]]. Similar to our results, Nami et al. [23] reported the survival of *Lactococcus lactis* ABRIINW-N19 encapsulated in alginate-persian gum-inulin blend under simulated gastric juices. According to the authors, un-encapsulated cells presented a reduction rate (log CFU/g) of 6.52, while cells encapsulated cells only 1.46. In parallel, Lai et al. [61] reported the encapsulation of *Lactobacillus rhamnosus* GG in blend based in alginate-pectin-flaxseed mucilage by co-extrusion technique. After simulated gastric digestion, free cells showed greater reduction in the *L. rhamnosus* GG cell viability by 31.8%, while encapsulated cells presented reduction between 5.9 and 10.7%. Therefore, the use of gums and mucilages as wall material, besides improving the properties of the particles [47], it can increase the resistance of the encapsulated microorganisms due to possible synergistic effects between the entrapped bacteria and the encapsulating material [[55],[61],[21]].

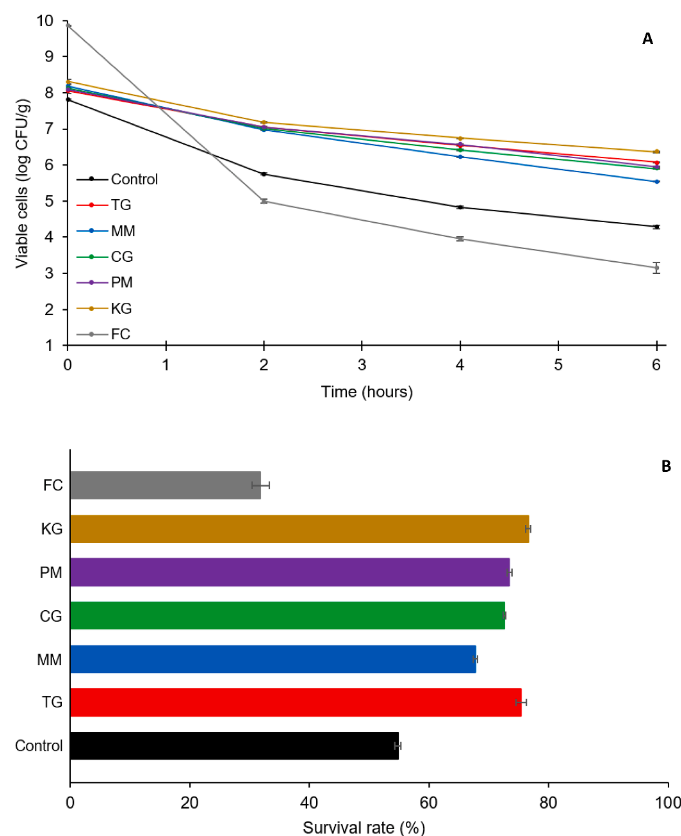

Fig. 5. Viable cell counts (A) and survival rate (B) of free (FC) and encapsulated *L. reuteri* in alginate-mucilage/gum beads before (0 h) and after exposure to simulated gastric (2 h) and enteric (4 and 6 h) conditions. *Refer to Table 1 for details of each of the samples. **Each data point represents the mean of assays performed in duplicate.

After exposure to simulated gastric phase, free and encapsulated *L. reuteri* were subject to simulated enteric stages (I and II) to complement the 6-h *in vitro* assay. Although milder than the behavior observed in gastric phase, all samples also showed reduction in *L. reuteri* viable cell counts after both enteric stages, however the decrease was sharper ($p<0.05$) in FC (1.85 log CFU/g), representing decrease of 37.07%, evidencing the harmful effects of bile salts on *L. reuteri* cells. In contrast, under the same conditions, higher survival cells rates were obtained in KG, TG, PM and CG, in which the decrease after exposure to simulated enteric juices were of 11.42, 13.78, 15.6 and 15.83%, respectively. Besides, the protection conferred to the encapsulated microorganisms in these hydrocolloid blends was higher ($p<0.05$) than obtained in the Control, which presented a reduction of 25.57% in *L. reuteri* viable cell count after the enteric phases. Although alginate gels are widely used to encapsulate and protect microorganisms against intestinal fluids [[62],[63]], the protective effects of the beads depend on size, porosity, and surface properties [16]. The addition of gums and mucilages to alginate beads can improve the effectiveness of encapsulation process for protecting probiotics in intestinal conditions, mainly because the particles remain in contact with intestinal juices for a relatively long time [24].

Based on *L. reuteri* survival rate after simulated gastrointestinal conditions (Fig. 5B), KG, PM, CG, and TG provided higher protection, which ranged from 72.6 to 76.6% ($p<0.05$), while the Control and FC showed only 54.8 and 31.84%, respectively. Konjac glucomannan hydrogel combined with alginate were used to encapsulate *Lactobacillus acidophilus*. Its use improved the stability of the particles and the bacterium survival, mainly by increasing the tightness of the wall material and performing prebiotic activity, which can improve the cell viability by ensuring protection under gastric phase and progressively release it under enteric juices [64]. In parallel, psyllium mucilage, was related with effective improvement of the alginate particle properties, in which integrity was improved [25], besides it was associated with prebiotic characteristics, which can benefit probiotic bacteria, conditions that can enhances the cell survival under low pH and in the presence of bile salts [55], Furthermore, the tamarind and cassia tora gums have been used in pharmaceutical applications, such as drug delivery and sustained release agent [[65],[66]] and until now there were no literature records regarding their use to encapsulate microorganisms. In this sense, our study confirmed great potential of these gums to microorganism encapsulate purposes. Moreover, these hydrocolloid blends were also tested as support for the *in situ*-produced reuterin by *L. reuteri* cells. The main findings are discussed in the next section.

3.6. The reuterin production and entrapment

The Table 3 showed the reuterin-content present in the film-forming

Table 3
Reuterin production (mmol/L) in the film-forming solutions and its detection after the entrapment into the beads.

Samples	Reuterin-content		Reuterin entrapment (%)
	solution	bead	
Control-G	58.52 ± 3.533	20.24 ± 0.855	34.63 [c]
TG-G	50.00 ± 1.959	20.61 ± 0.565	41.25[ab]
MM-G	60.12 ± 1.864	26.79 ± 1.190	44.57 [a]
CG-G	51.23 ± 0.932	19.75 ± 0.770	38.56[bc]
PM-G	50.98 ± 1.826	20.24 ± 0.213	39.73 [b]
KG-G	50.25 ± 1.496	22.59 ± 0.641	45.00 [a]

*Refer to Table 1 for details of each of the samples. **Means followed by the same lowercase letter in the column do not differ by the Tukey test ($p>0.05$). ***Each data point represents the mean of assays performed in triplicate.

solutions and the reuterin detected after the encapsulation and consequent formation of the beads. In all tested conditions, the *L. reuteri* cells were able to produce reuterin. However, the highest reuterin production in film-forming solutions were obtained in MM-G and Control-G reaching 60.12 and 58.52 mmol/L, respectively ($p>0.05$). In parallel, Angiolillo et al. [67] reported the reuterin quantification in alginate solutions containing *L. reuteri* and glycerol. The reuterin content ranged from 1.92 to 11.42 mmol/L according to alginate concentration. The authors suggested that the presence of vitamin B_{12} in the alginate composition, which is cofactor of the glycerol dehydratase favored the glycerol-reuterin conversion. Therefore, the increase of alginate concentration in the film-forming solution improved the reuterin production.

Compared to samples MM-G and Control-G, the reuterin production in TG-G, CG-G, PM-G and KG-G was slightly smaller, ranging between 50 and 50.25 mmol/L ($p<0.05$). Chen et al. [68] reported the reuterin production by *L. reuteri* DPC16 in alginate-chitosan microcapsules. The authors emphasized a markedly lower reuterin content when the particles contained chitosan in their composition. Thus, the addition of other hydrocolloids to the alginate matrix may change the physical and chemical properties of the beads, which may interfere in the glycerol diffusion into the particles, reducing its availability to immobilized *L. reuteri*, decreasing the bioconversion of glycerol into reuterin.

The encapsulation process can result in decrease of reuterin content [67]. In this study, the evaluation of reuterin entrapment into the beads ranged between 34.63 and 45% (Table 3), being that the entrapment was higher in KG-G, MM-G and TG-G ($p<0.05$). Although the high *in situ* reuterin production, the reuterin entrapment in Control-G was the lowest (34.63%) reported in this study, however this result did not differ from CG-G ($p>0.05$), which present reuterin entrapment of 38.56%. In parallel, Mishra et al. [69] encapsulated the reuterin produced by *L. reuteri* BPL-36 using alginate and guar gum by extrusion technique. The reuterin encapsulation efficiency varied between 36.35 and 37.97%. Similarly, Narsaiah et al. [70] optimized the encapsulation of nisin in alginate-guar gum particles, reaching nisin entrapment of 36.65%. The authors suggested that the addition of another hydrocolloid to structure of alginate beads may increase the density of the gel and favor the metabolite entrapment, which can be related with the results obtained in this study.

Currently, the use of reuterin in food preservation technologies gaining ground [71]. Recently, it was reported the first development of a pectin coating with reuterin as a food preserver [72]. In parallel, it is common the use of hydrocolloid-based edible films carried of lactic acid bacteria with the same objective [[73],[74]]. Therefore, the combination of alginate and others hydrocolloids reported in this study establish conditions that enable its application for different purposes, such as edible film/coatings for extend the shelf life of foods and/or support material for delivery of chemical or biological compounds.

4. Conclusion

L. reuteri DSM 20016 was successfully encapsulated in different alginate beads added of mucilaginous solutions obtained from tamarind, mutamba, cassia tora, psyllium, and konjac by extrusion technique. The beads showed a relatively spherical shape independently of the addition of gums or mucilages, besides homogenous distribution of *L. reuteri*. The use of gums and mucilages with alginate improved the EE and the survival of encapsulated bacteria during refrigerated storage, being that konjac gum, cassia tora gum and psyllium mucilage improved the *L. reuteri* survival especially after prolonged storage (20, 30 and 60 days). In contrast, the samples in which reuterin production was induced showed a strong decrease in the encapsulated cells count and after 12 days of storage no viable cells were detected.

The encapsulation process protected the encapsulated cells under gastric and enteric environments. However, the effects were dependent on the presence of gums or mucilages in the composition of the beads, being that the highest survival rates were obtained in KG, TG, PM, and CG. *L. reuteri* was capable to produce reuterin from glycerol in all in film-forming solutions. The highest production was obtained in Control-G and MM-G. In contrast, the metabolite entrapment was improved when mucilaginous materials from konjac, mutamba or tamarind were used. Thus, our findings confirm the great potential of the hydrocolloids tested as support material for biological or/and chemicals compounds. Enabling its applications for different further purposes, such as for delivery of probiotic microorganisms or for use of these reuterin content-mucilaginous solutions as edible film/coating for foods, a valuable approach to food safety.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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