



Membrane Emulsification as an Emerging Method for *Lacticaseibacillus rhamnosus* GG® Encapsulation

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Abstract

Techniques capable of producing small-sized probiotic microcapsules with high encapsulation yields are of industrial and scientific interest. In this study, an innovative membrane emulsification system was investigated in the production of microcapsules containing *Lacticaseibacillus rhamnosus* GG® (Lr), sodium alginate (ALG), and whey protein (WPI), rice protein (RPC), or pea protein (PPC) as encapsulating agents. The microcapsules were characterized by particle size distribution, optical microscopy, encapsulation yield, morphology, water activity, hygroscopicity, thermal properties, Fourier-transform infrared spectroscopy (FTIR), and probiotic survival during in vitro simulation of gastrointestinal conditions. The innovative encapsulation technique resulted in microcapsules with diameters varying between 18 and 29 µm, and encapsulation yields > 93%. Combining alginate and whey, rice, or pea protein improved encapsulation efficiency and thermal properties. The encapsulation provided resistance to gastrointestinal fluids, resulting in high probiotic viability at the end of the intestinal phase (> 7.18 log CFU g⁻¹). The proposed encapsulation technology represents an attractive alternative to developing probiotic microcapsules for future food applications.

Keywords Probiotic · Microencapsulation · Alginate · Whey protein · Rice protein · Pea protein

Introduction

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have defined probiotics as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (FAO/OMS, 2001; Hill et al., 2014). The global probiotics market was valued at USD 58.17 billion in 2021 and is expected to expand at a compound annual growth rate (CAGR) of 7.5% from 2021 to 2030 (Grand View Research,

2021). The health promotion provided by these microorganisms has been the main driving force of this niche market. Recent studies and clinical evidence have shown that the *L. rhamnosus* GG strain has promoted health and reduced the risk of some diseases, such as the antiviral effect against the SARS-CoV-2 virus (Tang et al., 2021), control and reduction of serum cholesterol (Lu et al., 2022) and diabetes (Gu et al., 2022), antiobesity activity (Huang et al., 2021a), and reduced risk of colon cancer (Celebioglu et al., 2021).

Probiotics must reach the human gut in adequate amounts to colonize it and exert some benefits on human health (Hill et al., 2014; Misra et al., 2022). However, most probiotics have a considerable loss of viability after passing through the digestive tract due to their low stomach pH tolerance and high concentration of bile salts in the small intestine (Premjit & Mitra, 2021). In addition, when incorporated into commercial products, intrinsic or process factors such as low pH, high water activity, high shear stress during food manufacturing, or high cooking temperatures can negatively affect the integrity of the probiotic strain (Arepally et al., 2022; Camelo-Silva et al., 2022; Sharma et al., 2022). In this sense, microencapsulation has been disseminated as a

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technology that protects probiotic cells within a polymeric matrix. This method is defined as the process in which cells are retained within an encapsulating membrane to reduce or minimize the loss of probiotic bacteria (Verruck et al., 2019). Microencapsulation techniques, including extrusion, emulsification, spray-drying, and freeze-drying, have been accepted to protect probiotic cells within a polymeric matrix (Fangmeier et al., 2019; Manassi et al., 2022). Nevertheless, these techniques produce microcapsules of varying sizes, generally larger than recommended ($< 100 \mu\text{m}$) to avoid sensory rejection due to the “gritty sensation” during product consumption (Bampi et al., 2016; Heidebach et al., 2012).

The membrane emulsification technique is a relatively new method with great potential to overcome the limitations of conventional techniques. The main advantages are the production of uniform microcapsules with controlled size (control by membrane pore size), low shear stress, low energy expenditure, high flexibility, and operation under mild temperature conditions (Piacentini et al., 2014). In addition, controlling the microcapsule’s final size and size distribution is a valuable advantage for food applications. Most of the published studies present pilot or laboratory scale experiments, reporting that membrane emulsification is technically feasible for encapsulating several compounds such as vitamin B5 (Konovalova et al., 2023), probiotics (Camelo-Silva et al., 2022), essential oils (Reis et al., 2022), polyphenols (Junjing Wang et al., 2020), and drugs (Lagrega et al., 2020). In 2014, Spyropoulos et al. indicated that most of the commercial applications of membrane emulsification were in the pharmaceutical area. More recently, Zhi et al. (2022) highlighted that attention has been given to food applications, given the higher number of studies and patents in this field. Even though the investigations show the technical feasibility of producing such encapsulated materials, they do not evaluate environmental and economic aspects, which could help design industrial-scale production. To our knowledge, there is only one documented case regarding a commercial product obtained through membrane emulsification: the “Yes light,” a low-fat butter substitute produced by Moringa Milk Industry in Japan (Spyropoulos et al., 2014). On the other hand, finding new applications for applying the technology in the food industry may induce companies to support research and scale-up practices.

So far, a single study on membrane emulsification for encapsulating a probiotic bacteria strain (Song et al., 2003) can be found. The authors used a microporous glass (SPG) membrane with a pore diameter between 4 and 6.4 μm to encapsulate *Lactocaseibacillus casei* YIT 9018. The dry microcapsules obtained had a size between 31–52 μm and were resistant to gastric fluid and bile ($< 10^6 \log \text{CFU g}^{-1}$). Yeast encapsulation using membrane emulsification has also been reported. Morelli et al. (2017) encapsulated *Saccharomyces cerevisiae* cells using a flat disc metallic membrane with a pore diameter of 30 μm .

The size of the droplets (wet suspension) containing *S. cerevisiae* cells varied between 60 and 340 μm . Still, more studies are needed to elucidate the response of each strain to the membrane emulsification method parameters and encapsulating agents.

Different encapsulating materials, known as carrier agents, have been used in microencapsulation. Sodium alginate (ALG) is widely used due to its low cost, biocompatibility, food-grade, and targeted delivery of probiotics (soluble in basic media, e.g., in the intestine) (Marcial-Coba et al., 2019). However, microcapsules made of ALG tend to be porous, favoring the permeation of substances harmful to the probiotic (e.g., stomach acidity). Proteins have been combined with ALG to increase the stability of these microencapsulated microorganisms during adverse conditions, for example, in simulated gastrointestinal conditions, or to increase encapsulation yield (Apiwattanasiri et al., 2022; Gao et al., 2022). Premjit and Mitra (2021) encapsulated the probiotic strain *Leuconostoc lactis* (NCDC 200) by electrospraying technique using an emulsion system based on soy protein isolate (SPI) (12–15% w/v) and sunflower oil (SO) (0–5% w/v). The authors observed that a simultaneous increase in the concentration of SPI and SO led to a significant increase in encapsulation yield. Using vegetable proteins such as rice (RPC) and pea (PPC) seems to be an attractive and innovative strategy. The available literature on using these proteins as probiotic encapsulating agents is still scarce. In October 2022, we performed an advanced search in the Scopus database using the keywords “probiotic,” “encapsulat*,” “rice protein,” and “pea protein.” Only one study on rice protein was published in 2021, while eight studies on pea protein were published between 2010 and 2021. They are known for their hypoallergenic potential and high nutritional value, with a high digestibility rate ranging from 92 to 99% for pea protein concentrate and 88% for rice protein concentrate (Fabian & Ju, 2011). Another important finding is that lactic acid bacteria can hydrolyze these proteins and generate peptides and amino acids to be used as substrates more efficiently than the native protein (Allahdad et al., 2022).

The membrane emulsification technique was not previously used to encapsulate *L. rhamnosus* GG; therefore, this study is a pioneer in the area. This work proposes to develop an innovative protection system combining ALG with whey protein isolate, rice, and pea proteins to produce probiotic microcapsules, using membrane emulsification as a first step in microencapsulation.

Material and Methods

Materials

A freeze-dried probiotic culture composed of *L. rhamnosus* GG (Lr) (Chr. Hansen, Hønsholm, Denmark) was used as the active material for the microcapsules. Sodium alginate

(ALG) (Êxodo Científica, Brazil), rice protein concentrate (RPC) (80.00 g protein 100 g⁻¹) (VeganWay, Brazil), pea protein concentrate (PPC) (80.00 g protein 100 g⁻¹) (VeganWay, Brazil), whey protein isolate (WPI) (95.00 g protein 100 g⁻¹) (Essentia Pharma, Brazil), refined soybean oil (Coamo, Brazil), CaCl₂·2H₂O (dihydrated calcium chloride), and NaCl (calcium chloride) (Neon, Brazil) were used for microcapsule preparation. De Man, Rogosa, and Sharpe (MRS) broth and MRS Agar (Kasvi, Brazil) were used for bacterial growth. The emulsifier sorbitan monooleate (SPAN 80), bacteria gram-stain kit, enzymes α -amylase (28.75 U mg⁻¹ protein), pepsin from porcine gastric mucosa (400 U mg⁻¹ protein), pancreatin from porcine pancreas (digestive power: 8 × USP specifications), and the bovine bile salts were supplied by Sigma-Aldrich, Brazil. The viability of the probiotic bacteria was assessed with the BacTiter-Glo™ Viability Kit (Promega, USA). All chemicals were of analytical grade or with equivalent purity.

Probiotic Suspension Preparation

For the preparation of the stock solution, freeze-dried probiotic cells of *L. rhamnosus* GG were rehydrated in sterile skimmed milk (25 g L⁻¹) and glycerol (20 g L⁻¹) and stored in sterile falcon vials at -20 ± 2 °C (Camelo-Silva et al., 2022b). The stock solution was added to the MRS broth and incubated at 37 ± 1 °C

for 48 h. After the incubation period, probiotic cells were harvested by centrifugation (1000 × g) for 10 min (Centrifuge-SL 700 from Solab, Brazil) at 25 ± 1 °C and washed twice with sodium chloride solution (0.9 g 100 mL⁻¹). *L. rhamnosus* GG cell pellets were kept at 4 ± 1 °C until encapsulation.

Microencapsulation Process

The probiotic microcapsules were prepared by membrane emulsification, following the method proposed by Morelli et al. (2017) and Vinner et al. (2019), with modifications. A scheme with the steps of the procedure is shown in Fig. 1.

Four emulsions were produced and denominated Lr-ALG, Lr-ALG-WPI, Lr-ALG-RPC, and Lr-ALG-PPC. Before encapsulation, RPC and PPC proteins were solubilized in water with the pH corrected to 7.0 (0.5 M NaOH) and stirred at room temperature overnight (Jiapei Wang et al., 2014). Briefly, the dispersed phase of the Lr-ALG sample was prepared by mixing 1.0% (w/v) sodium alginate and the probiotic suspension (~ 9 log CFU mL⁻¹). The dispersed phases of the Lr-ALG-WPI, Lr-ALG-RPC, and Lr-ALG-PPC samples were prepared with 1.0% (w/v) of sodium alginate, probiotic suspension (~ 9 log CFU mL⁻¹), and 0.5% (w/v) protein (WPI, PRC, or PPC, respectively). The continuous phase consisted of soybean oil (3:1) with 3% (v/v) of SPAN 80.

The microcapsules were obtained using a membrane emulsification system coupled to a specially designed module with

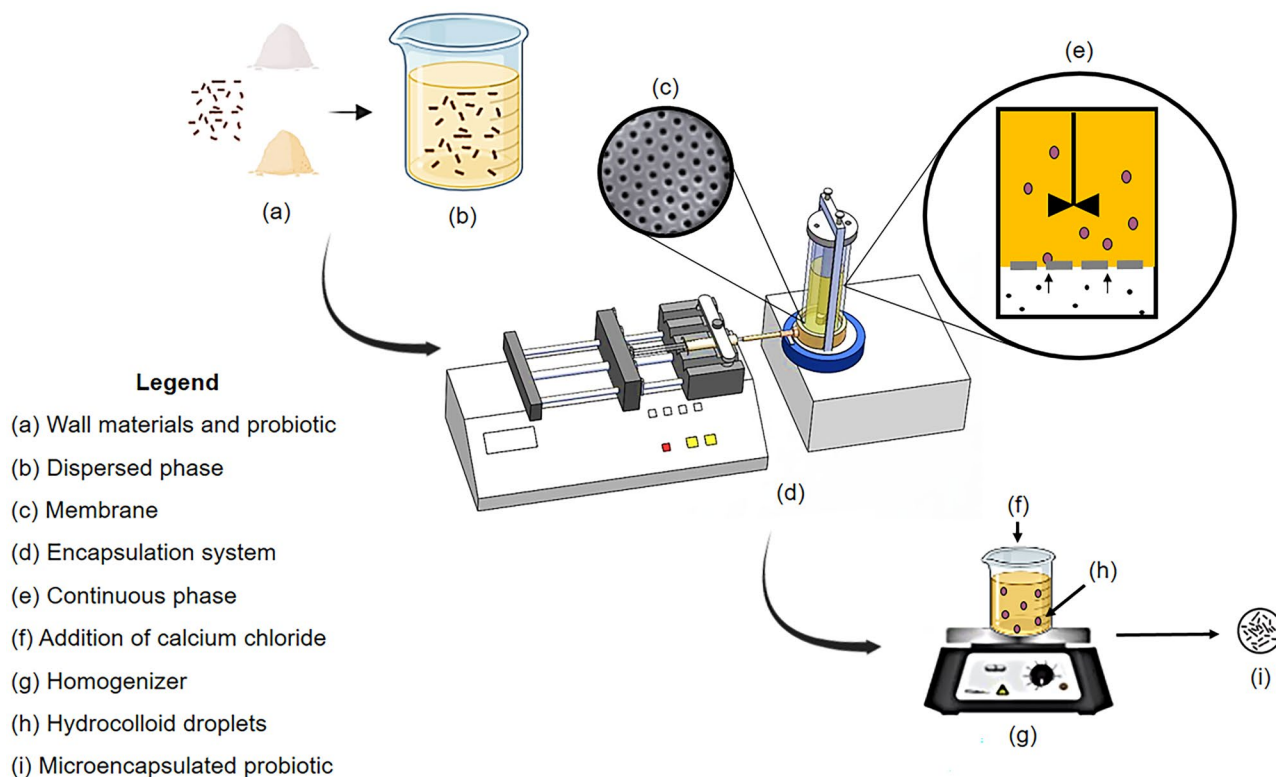


Fig. 1 Process for the preparation of probiotic microcapsules using membrane emulsification

a porous (~20 µm) stainless steel filter (181 cm² of effective area). The membrane was soaked in the continuous phase and treated for 30 min in an ultrasonic bath before the microencapsulation process to avoid its wetting by the dispersed phase. The shear stress for droplet detachment was provided by a bladed rotor (25 mm long and 6 mm high) that generates a radial flow pattern. The distance between the bladed rotor and the membrane surface was 5 mm, and the rotation speed was 250 rpm. The membrane (c) was placed in the module, and the continuous phase of the emulsion was poured into the container. Then, the dispersed phase (b) was gently fed into the cell by an infusion pump (Insight, Brazil), forcing it to permeate through the membrane pores. The dispersed phase injection rate was adjusted to 1 mL min⁻¹, and the emulsification experiments were conducted at room temperature.

When emulsification was complete, a 1.5% (w/v) solution of calcium chloride dihydrate was gently added to the emulsion (stirring at 250 rpm) and allowed to stand for 30 min. Then, the emulsion was centrifuged (1000×g for 10 min), and the supernatant was discarded. Gelled microcapsules were separated by vacuum filtration, washed with sodium chloride solution (0.9 g 100 mL⁻¹), and analyzed in terms of optical microscopy, size distribution, and encapsulation yield.

Characterization of Gelled Microcapsules

Microstructure and Microcapsule Size

The microstructure of the gelled microcapsules containing *L. rhamnosus* GG and free *L. rhamnosus* GG (fixed by gram stain) was determined from images obtained under a bright-field microscope (Olympus BX41, Olympus Optical Ltd., Japan) connected with a digital camera (Q-imaging). The determination of the mean microcapsule size and size distribution was performed by dynamic light scattering (DLS) (LUMiSizer, LUM GmbH, Germany) (Alves et al., 2021). The mean microcapsule size was calculated from a normal Log model using Origin 8.5 software (OriginLab, USA). The width of the size distribution was expressed by the Span value calculated according to Eq. 1.

$$\text{Span} = \frac{D_{90} - D_{10}}{D_{50}} \quad (1)$$

where D_{10} , D_{50} , and D_{90} correspond to the microcapsule diameters obtained at 10%, 50%, and 90% of the cumulative size distribution curve.

Encapsulation Yield (EY)

The encapsulation yield was determined according to Ji et al. (2019), with modifications. One gram of the gelled probiotic

microcapsules was resuspended in 9 mL of PBS (phosphate buffer saline) at pH 7.4, 0.1 mol L⁻¹, and stirred with a vortex (Norte Científica, Brazil) for five 1-min cycles, with 1-min rest intervals between cycles. Then, 100 µL of the sample was mixed with 50 µL of BacTiter-Glo™ reagent and submitted to a microplate reader (GloMax® Explorer System model GM3500, Promega, USA) to quantify the number of viable cells released from the microcapsules. The results obtained from the microplate reader were correlated with a calibration curve constructed based on plate counts. Results were expressed as log colony-forming units per gram (log CFU g⁻¹). The encapsulation yield (%) was calculated using Eq. 2.

$$\text{EY}(\%) = \frac{N}{N_0} \times 100 \quad (2)$$

where N_0 and N are the number of viable cells (CFU g⁻¹) before and after encapsulation, respectively.

Dried-Gelled Microcapsules and Characterization

The gelled probiotic microcapsules were frozen at -18 ± 2 °C for 4 h in a domestic freezer (Electrolux, Brazil) and then at -80 ± 2 °C for 24 h in a vertical freezer (Glacier, USA) on the same day they were produced. They were dried in a freeze-dryer (Liotop L101, Brazil), setting the pressure to 0.200–0.300 µHg and condenser temperature at -54 °C for 24 h.

Morphology of *L. rhamnosus* GG Microcapsules

For this analysis, the samples were dried using critical point dryer equipment (model EM CPD 030/LEICA) (Silveira et al., 2022). The morphology of the dried probiotic microcapsules was observed with a scanning electron microscope (model JSM 6390 LV, Jeol, Japan). The microcapsules powder was spread over the carbon tape and covered with a thin layer of gold and then analyzed at an accelerating voltage of 10 kV and micrographs at magnifications between 100 and 2000×.

Water Activity (A_w)

Water activity was measured at 25 ± 1 °C using a water activity analyzer (Aqua lab, Decagon Devices, USA).

Hygroscopicity (HG)

The hygroscopicity of the probiotic microcapsules was determined according to the method described by Bhusari et al. (2014) and Caparino et al. (2012). Briefly, 1 g of the freeze-dried capsules was placed in Petri dishes in a glass desiccator containing a saturated solution of sodium chloride (75.3% RH at 25 ± 1 °C) for 7 days. HG (%) was calculated using Eq. 3.

$$HG(\%) = \frac{\frac{\Delta_m}{(M+M_i)}}{1 + \frac{\Delta_m}{M}} \quad (3)$$

where Δ_m (g) is the increase in weight of the microcapsules after equilibrium, M (g) is the initial mass of the microcapsules, and M_i (% wb) is the free water content of the microcapsules before exposure to the humid air environment.

Thermal Analysis

The melting temperature of probiotic microcapsules and wall materials was obtained by differential scanning calorimetry (DSC) (Shimadzu DSC-60, Japan). A standard reference of indium was used for the preliminary calibration of equipment. Approximately 8.0–10.0 mg of the samples was placed in aluminum pans with lids and scanned between 30 and 220 °C at a heating rate of 10 °C min⁻¹ under a nitrogen flow of 50 mL min⁻¹. An empty crucible was used as a reference (Ashwar et al., 2018).

Interaction Between Microcapsule Components

The dried probiotic microcapsules, encapsulating materials, soybean oil, and *L. rhamnosus* GG were analyzed by Fourier transform infrared spectroscopy (FTIR) (IRPrestige-21, Shimadzu Scientific Instruments Inc., USA). The samples were homogenized in KBr (potassium bromide) pellets, and the spectra were acquired from 400 to 4000 cm⁻¹ with a resolution of 4 cm⁻¹. Measurements were performed at room temperature.

Resistance of Free and Microencapsulated *L. rhamnosus* GG During In Vitro Digestion

Probiotic cell viability was evaluated in simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) according to INFOGEST, a standardized protocol for static digestion (Table 1) (Brodkorb et al., 2019).

Five grams of free or microencapsulated *L. rhamnosus* GG was homogenized with SSF stock solution 1:1 (w/w). The α -amylase was added in the concentration of 75 U mL⁻¹, and the prepared oral bolus was kept at 37 °C for 2 min at 200 rpm. Then, the oral bolus was mixed with SGF 1:1 (w/w) and porcine pepsin (2000 U mL⁻¹), and the pH was adjusted to 3.0 with HCl 1 mol L⁻¹. Then, it was incubated at 37 °C with constant shaking at 50 rpm for 2 h. Finally, the gastric chyme was mixed with SIF 1:1 (w/w) pancreatin solution (trypsin activity 100 U mL⁻¹) and 10 mM of bile salts and incubated at 37 °C for a further 2 h at 50 rpm. The samples were centrifuged, and the viable cell count was performed according to item 2.4.2. *L. rhamnosus* GG performance under in vitro gastrointestinal steps was

also evaluated by the recovery rate (%) (Eq. 4) (Verruck et al., 2017).

$$\text{Recovery rate}(\%) = \left(\frac{N_1}{N_0} \right) \times 100 \quad (4)$$

where N_1 is the *L. rhamnosus* GG viable cells count (log CFU g⁻¹) after exposure to each in vitro gastrointestinal step, and N_0 is *L. rhamnosus* GG viable cells count (log CFU g⁻¹) before in vitro gastrointestinal exposure.

Statistical Analysis

The mean and standard deviation (SD) was calculated from data obtained in triplicate. The one-way analysis of variance (ANOVA) was conducted using the STATISTICA version 13.3 software (TIBCO Software Inc., USA). Differences between means were tested using Tukey at a significance level of 0.05.

Results and Discussion

Microstructure and Microcapsule Size

Figure 2 shows the microstructure of the probiotic *L. rhamnosus* GG (a) and the gelled microcapsules. The surface of the microcapsules in Fig. 2b presents a thin oily film, indicating that the encapsulation technique was adequate for producing a W/O emulsion. Furthermore, the gelled microcapsules are spherical and with a polydisperse size distribution. Dark spots can be found inside some microcapsules (Fig. 2c, d, e, and f), suggesting the existence of the probiotic suspension dispersed within the gelled microcapsules.

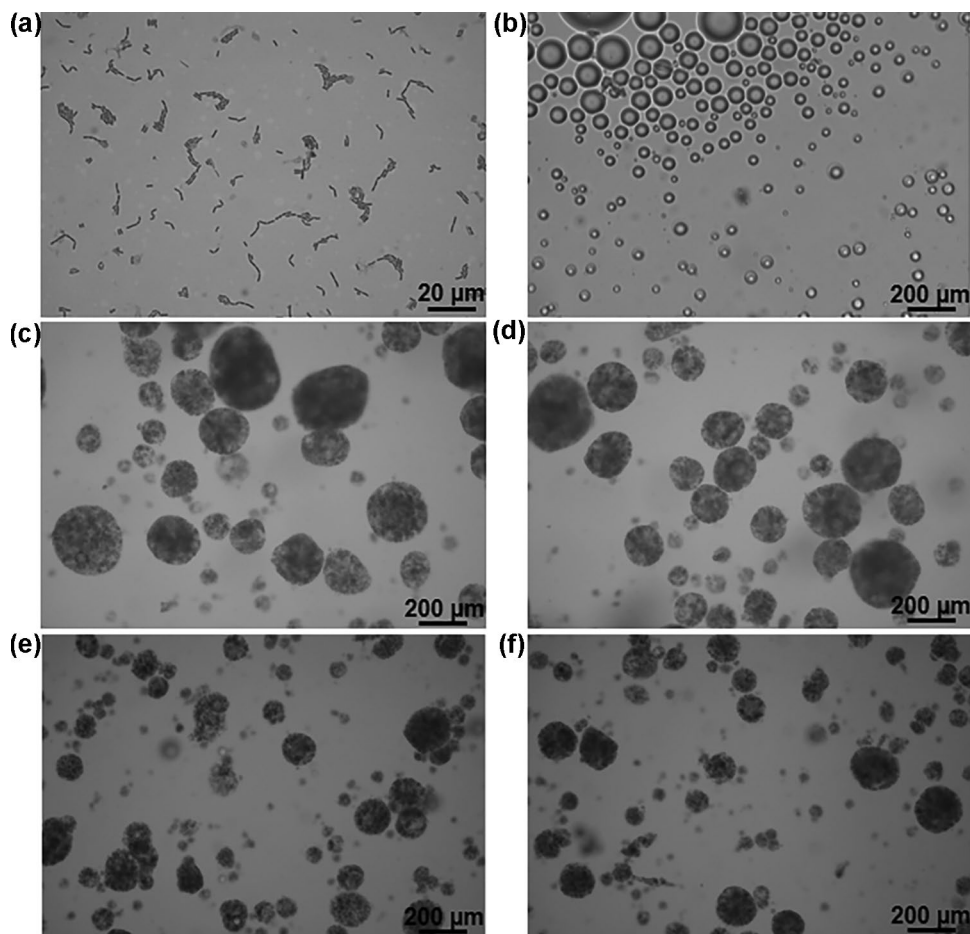
The gelled microcapsule size distribution curves are shown in Supplementary Fig. S1, while gelled microcapsule size and span are presented in Table 2. The distribution curves for gelled microcapsules were narrow, and the

Table 1 Composition of solutions used to prepare simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) (Brodkorb et al., 2019)

Component	Solution concentration (mM)		
	SSF	SGF	SIF
KCl	15.10	6.90	6.80
KH ₂ PO ₄	3.70	0.90	0.80
NaHCO ₃	13.60	25.00	85.00
NaCl	-	47.20	38.40
MgCl ₂ (H ₂ O) ₆	0.15	0.12	0.33
(NH ₄) ₂ CO ₃	0.06	0.50	-
HCl	1.10	15.60	8.40
CaCl ₂ (H ₂ O) ₂	1.50	0.15	0.60
pH	7.00	3.00	7.00

—: none

Fig. 2 Optical micrographs of probiotic bacteria and gelled microcapsules. **a** *L. rhamnosus* GG, **b** alginate microcapsules without probiotic, **c** Lr-ALG, **d** Lr-ALG-WPI, **e** Lr-ALG-RPC, and **f** Lr-ALG-PPC



microcapsule size (Table 2) was close to the membrane pore size. Particles larger than 20 μm (nominal membrane pore size) can result from the coalescence of droplets as soon as they leave the membrane pore (because of the proximity of adjacent pores) and also from the membrane pore size distribution. It is well known that the average droplet diameter can be from 2 to 10 times larger than the membrane pore size (Williams et al., 1998; Reis et al., 2022). The control microcapsules (alginate microcapsule without probiotic) had an

average size of 19.5 μm , indicating that adding the probiotic did not alter the gelled microcapsule size. In turn, the span ranged from 1.52 to 2.77, proving the potential of the membrane emulsification technique in producing microcapsules with a narrow width of the size distribution (Kaade et al., 2020). According to Spyropoulos et al. (2011), membrane emulsification produces emulsion droplets individually/one at a time, which allows their size distribution and size to be carefully controlled. The microcapsule size distribution

Table 2 Characteristics of probiotic microcapsules

	Probiotic microcapsules			
	Lr-ALG	Lr-ALG-WPI	Lr-ALG-RPC	Lr-ALG-PPC
Encapsulation yield (%)	93.16 ^b ± 0.20	95.07 ^a ± 0.40	95.22 ^a ± 1.20	95.71 ^a ± 0.40
Microcapsule size (μm)	24.00 ± 0.51	28.76 ± 0.95	18.26 ± 0.80	21.60 ± 0.91
Span	1.52 ± 0.02	2.77 ± 0.03	1.89 ± 0.01	2.31 ± 0.05
Water activity	0.47 ^c ± 0.01	0.33 ^a ± 0.01	0.47 ^c ± 0.01	0.38 ^b ± 0.01
Hygroscopicity (%)	1.00 ^a ± 0.07	2.39 ^c ± 0.12	1.24 ^{a,b} ± 0.08	1.35 ^b ± 0.10

Results expressed as mean ± standard deviation

^{a-c}Within a line, different superscript lowercase letters denote significant differences ($p < 0.05$) between the samples *Lr-ALG*, *L. rhamnosus* GG microcapsules with alginate; *Lr-ALG-WPI*, *L. rhamnosus* GG microcapsules with alginate-whey protein; *Lr-ALG-RPC*, *L. rhamnosus* GG microcapsules with alginate-rice protein; *Lr-ALG-PPC*, *L. rhamnosus* GG microcapsules with alginate-pea protein

behavior found in this study is in agreement with what was reported in works that used a similar dispersion cell for membrane emulsification (Consoli et al., 2020; Ilić et al., 2017; Imbrogno et al., 2015; Morelli et al., 2017; Piacentini et al., 2013; Richards et al., 2021).

Encapsulation Yield (EY)

The encapsulation yield of *L. rhamnosus* GG (Table 2) is related to the number of viable cells trapped in the microcapsules and the probiotic strains' survival rate after the encapsulation process. The EY was higher than 93% for the four microcapsules prepared. The high encapsulation yield obtained in this study may be due to the mild conditions employed during the membrane emulsification process, such as mild temperature and the non-use of organic solvents. Jiang and Charcosset (2022) and Consoli et al. (2020) obtained similar yields (between 94.5 and 97.7%) using membrane emulsification to encapsulate curcumin and resveratrol, respectively. On the other hand, we observe that the combination of alginate and protein (WPI, RPC, or PPC) improved ($p < 0.05$) the entrapment of probiotic cells in the microcapsules and promoted the achievement of encapsulation yields higher than 95%. Our results are consistent with those of Morsy et al. (2022), who found that the formulation containing ALG 2% (w/v) and WPI 2% (w/v) was more effective in trapping (EY: 98%) *L. rhamnosus* GG. Obradović et al. (2022) reported that the combination of ALG 1% (w/v) and WPI 15% (w/v) improved the encapsulation yield (82.46%) of a blend of lactic acid bacteria probiotics after freeze-drying. Another study revealed that freeze-dried microcapsules containing a mixture of alginate 2% (w/v), inulin 1% (w/v), and concentrated soy protein 10% (w/v) improved the encapsulation yield (90.43%) of *L. casei* ATCC10171 (Kouamé et al., 2023). This behavior occurs because of the superior gelling properties of the proteins. The hydroxyl groups present in the chemical structure of alginate can react with the amino groups of the side chain of the peptides, resulting in cross-linking (Massounga Bora et al., 2021). These bonds form a tough and rigid layer preventing the early migration of bacterial cells to the outside of the microcapsule. Using proteins in the microcapsules showed a more remarkable effect on protecting *L. rhamnosus* GG in simulated gastrointestinal conditions (“Resistance of free and microencapsulated *L. rhamnosus* GG during simulated gastrointestinal digestion”). Finally, our results suggest that RPC and PPC are comparable to the WPI considering encapsulation capacity (verified by the similar yield obtained), supporting the promising trend of using vegetable source materials in the food industry.

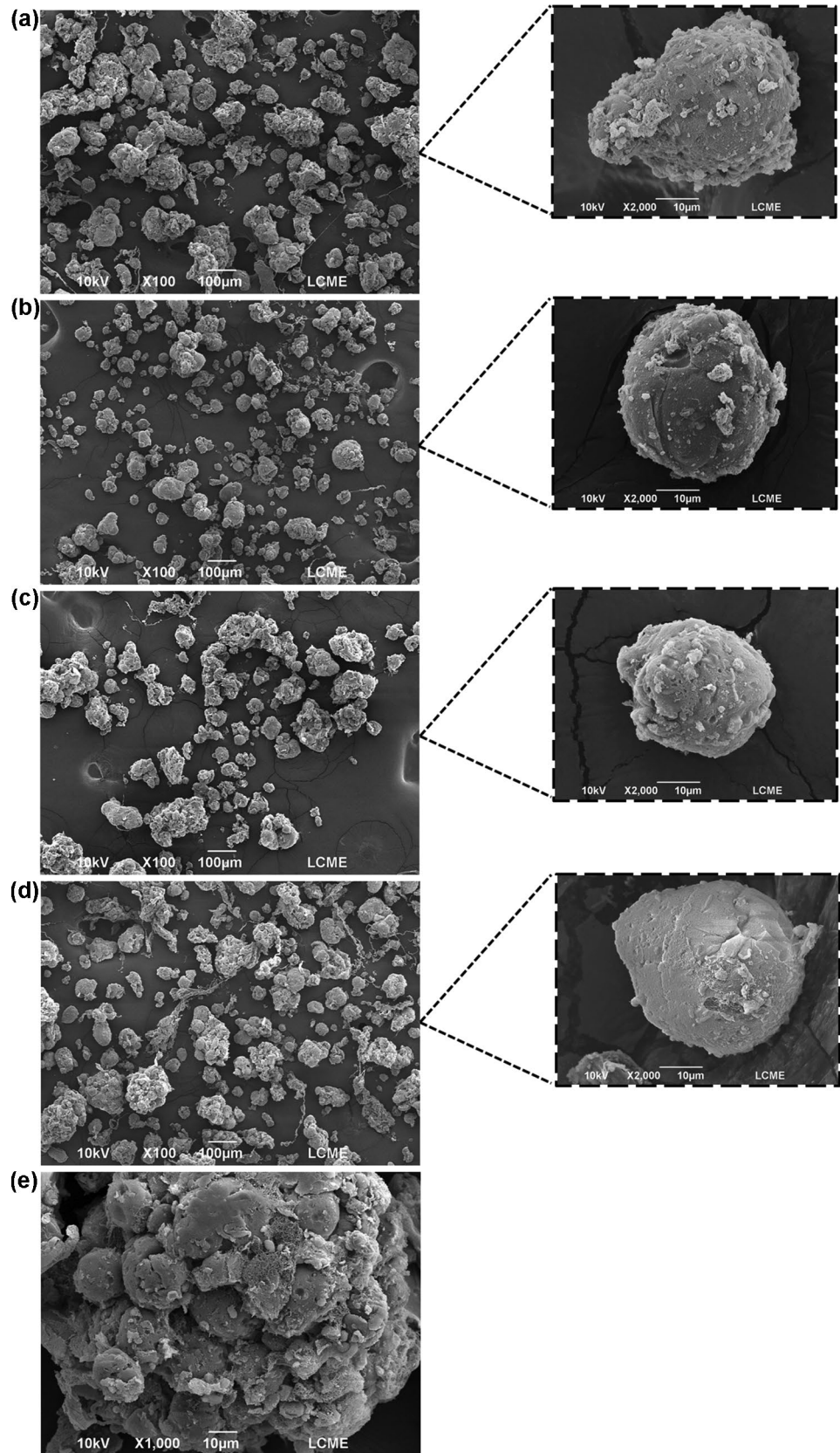
Morphology, Water Activity, and Hygroscopicity of the Dried Microcapsules Containing *L. rhamnosus* GG

Figure 3 shows the morphology of the dried probiotic microcapsules. All samples showed the same surface characteristics, indicating that the type of protein did not influence the morphology of the dried microcapsules. The microcapsules were irregular spheres with rough surfaces. They presented no apparent porosity. As also observed for the encapsulation yield, low porosity is essential to ensure better protection and retention of the encapsulated material, avoiding gas exchange with the environment or the early permeation of substances harmful to probiotics. Furthermore, the microcapsules formed agglomerates (Fig. 4e), corroborating the results of Holkem et al. (2016), who produced alginate microcapsules with *Bifidobacterium animalis* subsp. *lactis* BB-12 dried by freeze-dried and with those by Rama et al. (2020), who encapsulated *Lacticaseibacillus paracasei* ATR6 with cheese whey in a spray-dryer.

The moisture content of freeze-dried probiotic microcapsules is a critical factor that influences the stability of the bacteria during storage (Holkem et al., 2016). The water activity (in the range of 0.33–0.47) of the microcapsules (Table 2) was affected ($p < 0.05$) by the type of protein used, following the order WPI < PPC < RPC. This difference may be associated with plant proteins' higher water-holding capacity (WRC). Rafe et al. (2016) observed that gels made with pea protein-whey protein had reduced WRC compared to those made with pea protein alone. Similar results were obtained by Ashwar et al. (2018), who evaluated the viability and physicochemical properties of rice starch microcapsules containing *Levilactobacillus brevis* (MTCC 01), *Lacticaseibacillus casei* (MTCC 297), and *Lactiplantibacillus plantarum* (MTCC 021), obtained by emulsification followed by freeze-drying. According to Silva et al. (2018), a_w around 0.3 is considered satisfactory for dried probiotic microcapsules since conditions with $a_w < 0.2$ may favor lipid oxidation, consequently reducing the number of viable cells. On the other hand, when $a_w > 0.4$, water absorption is enhanced, which may accelerate the probiotic viability loss. The water activity observed in the Lr-ALG-WPI (0.33) favors the application of these microcapsules in low-moisture food matrices and can increase the stability of probiotic powders during prolonged storage. It is worth emphasizing that the Lr-ALG-PPC sample also presented an a_w within the recommended limit, demonstrating the potentiality of pea protein to produce probiotic powders with low water activity.

Hygroscopicity (HG) is closely related to the survival of the probiotic during storage. As shown in Table 2, although the

Fig. 3 Morphology (SEM) of dried probiotic microcapsules. **a** Lr-ALG, **b** Lr-ALG-WPI, **c** Lr-ALG-RPC, and **d** Lr-ALG-PPC



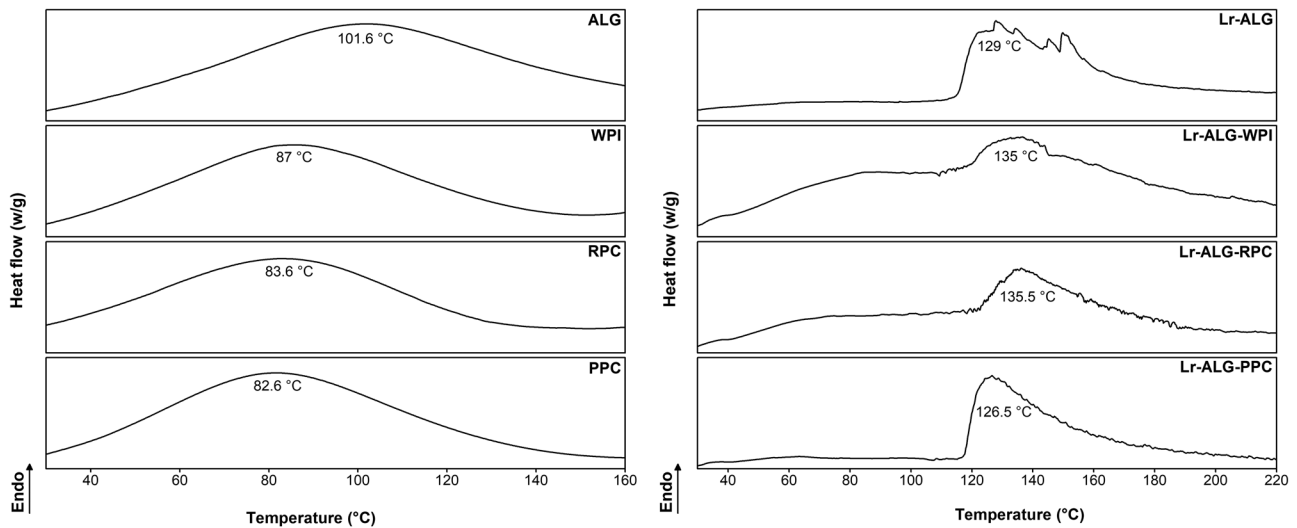


Fig. 4 Thermograms of pure wall materials and microcapsules containing *L. rhamnosus* GG

wall material influenced the HG values ($p < 0.05$), the highest HG obtained was 2.39% (Lr-ALG-WPI), still below the values found in the literature for probiotic powders: (9.55–13.97%) (Barajas-Álvarez et al., 2022), (7.6–8.7%) (Hernández-López et al., 2018), (11.19–13.58%) (Rajam & Anandharamakrishnan, 2015), and (10.00–19.40%) (Soukoulis et al., 2014). The HG values depend on the number of hydrophilic groups present in the structure of the microcapsule wall material, which can bind to the H_2O molecules present in the surrounding air and absorb water (Šipailienė & Petraitytė, 2018). Although several hydrophilic groups exist in the structure of the proteins used in this study, mainly carboxyl and amine groups, the effect was not considerable. Holkem et al. (2016) attributed the low HG value (2.0%) of alginate capsules containing *Bifidobacterium animalis* subsp. *lactis* BB-12 to the formation of a film with an oily residue on the particle surface. This film reduced the exposed area (cavities and pores) and resulted in environmental lower moisture absorption.

Thermal Analysis

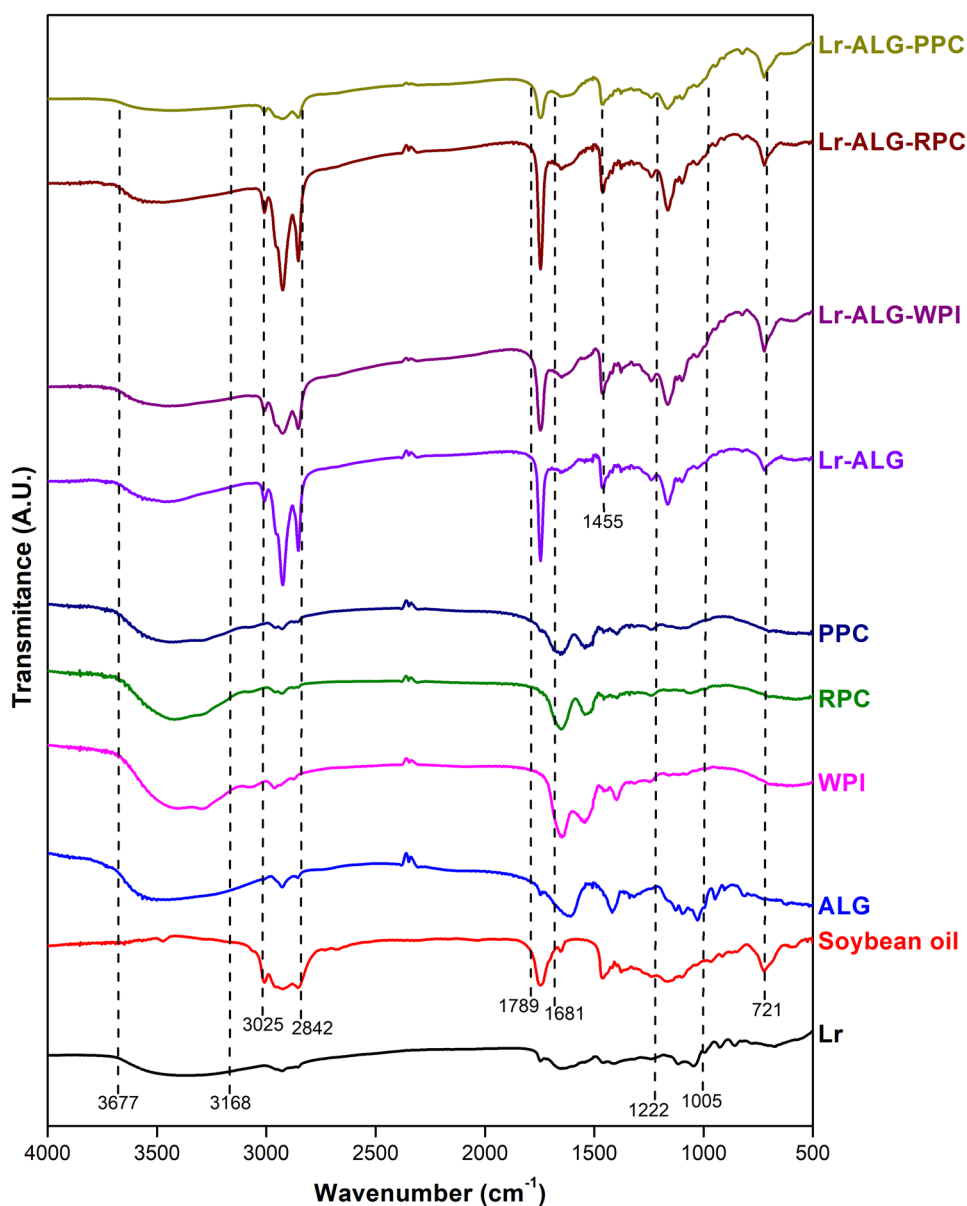
Thermograms of pure wall materials and microcapsules containing *L. rhamnosus* GG are shown in Fig. 4. The endothermic peak at 101.6 °C for pure sodium alginate (ALG) is consistent with other works and may indicate water loss from the matrix (Han et al., 2020). The WPI presented an endothermic event at 87 °C, possibly due to the heat-induced transitions in whey proteins (mainly β -lactoglobulin and α -lactalbumin) (Han et al., 2020). The endothermic peaks at 83.6 °C and 82.6 °C for RPC and PPC, respectively, indicate the thermal denaturation of the peptide fractions of these proteins (Perrechil et al., 2021; Tanger et al., 2020).

After encapsulation, the melting temperature increased from 101.6 °C (pure sodium alginate) to 129 °C (cross-linked sodium alginate, Lr-ALG). This temperature rise may be associated with the ionic cross-linking of Ca^{+2} in the alginate layer (egg box model). Generally, an increase in the melting temperature indicates a strengthening of the interfacial structure, giving higher stability to microencapsulated probiotics when subjected to high temperatures (Beldarrain-Iznaga et al., 2020). Furthermore, mixing WPI, RPC, or PPC with sodium alginate shifted the endothermic peaks of all probiotic microcapsules to temperatures > 120 °C. The increase in endothermic peaks in the microencapsulated material compared to the pure materials suggests that the alginate-protein interaction can form a firm and dense network due to strong electrostatic and hydrophobic interactions, producing a heat-resistant film. Thus, more energy was necessary to break the bonds between the chains and decompose them. These results corroborate previous studies that related the increase of the thermal stability of complex particles to the protein-polysaccharide complexes (Ghobadi et al., 2021; Luo et al., 2020). These results are relevant and could provide probiotic bacteria with higher thermal resistance, allowing the probiotication of foods that undergo heat treatments, such as bakery products and pasteurized or blanched foods.

Interaction Between Microcapsule Components

FTIR spectroscopy allowed for analyzing the main interactions between the materials used in probiotic microcapsule manufacture (Fig. 5). The WPI, RPC, and PPC proteins displayed the same characteristic bands (Fig. 5) but with different intensities. Typical absorption peaks of the spectrum

Fig. 5 Fourier transform infrared spectroscopy (FTIR) of encapsulating agents, *L. rhamnosus* GG, and probiotic microcapsules



of WPI, RPC, and PPC proteins were between 3168 and 3677 cm^{-1} (NH stretching and hydrogen bonding from amide A) and between 1222 and 1681 cm^{-1} (C=O stretching/hydrogen bonding and COO⁻ from amide I, CN stretching and NH bending modes from amide II, and CN stretching and NH bending from and amide III). Peaks from 2842 to 3025 cm^{-1} correspond to the axial deformation of carbon-hydrogen (-CH) bonds. Similar results have been reported for WPI (Shi et al., 2023; Tao et al., 2023), RPC (Jia et al., 2022; Perrechil et al., 2021), and PPC (Vergara et al., 2023; Wang et al., 2022). Regarding the spectra of raw materials and microcapsules, the functional groups in the microcapsules closely resembled the functional groups of proteins, ALG, soybean oil, and Lr. Previous studies have suggested that the main interactions between protein-polysaccharides

are electrostatic and that absorption peaks are often displaced. For example, Luo et al. (2022) observed that the characteristic peak of amide I in WPI moved from 1633 to 1621 cm^{-1} , indicating some electrostatic interactions formed between ALG and WPI. Carpentier et al. (2021) reported that PPC amide I, II, and III peaks changed from 1631, 1523, and 1232 cm^{-1} to 1638, 1536, and 1233 cm^{-1} , respectively, after complexation with tragacanth gum. They attributed this shift to conformational changes in the α -helix structures toward the β -sheet configuration.

A broad peak between 3677 and 3168 cm^{-1} occurred in all samples except soybean oil. This event represents the stretching vibration of intramolecular primary and secondary hydroxyl groups bound (Dehkordi et al., 2020). In addition, a smoother peak was observed between 1222 and

1005 cm^{-1} in the probiotic microcapsules and the Lr sample. This finding was also observed by Hosseini et al. (2022), who attributed this result to the probiotic *L. rhamnosus* cells inside the liposomes. According to Huang et al. (2021b), this vibration can indicate the phosphodiester backbone of nucleic acid (P=O group) and components of the cell wall (C–O–C group) of *L. rhamnosus*. Amplification of the spectral profile of the bands (3025 to 2842 cm^{-1}) was also observed in all probiotic microcapsules. The broadening of the spectral profile of these bands is generally attributed to a decrease in the conformational order of the lipid acyl chains and an increase in their dynamics. This widening is attributed to the insertion of protein chains between the oil acyl chains (Herrero et al., 2011).

Proteins have both hydrophobic and hydrophilic polypeptide side chains in their structure. The hydrophobic side chains tend to be preferentially located towards the oily layer of the microcapsules. In contrast, the hydrophilic side chains tend to be located towards the encapsulating materials inside the capsule. This preferential orientation of hydrophobic side chains in the oil involves interactions between structurally different acyl and protein chains, which cause the soybean oil lipid chain's disordering upon the addition of vegetable proteins (Pintado et al., 2015). An intense peak between 1789 and 1681 cm^{-1} was also detected in all probiotic microcapsules. This event is attributed to the stretching vibration of a carbonyl ester functional group (CO double bond) of soybean oil (Pizzo et al., 2022), typical in microcapsules produced in combination with oils (Premjit & Mitra, 2021). Furthermore, a band at 721 cm^{-1} indicates the overlapping of the CH_2 rocking and out-of-plane vibrations of cis-disubstituted olefins, characteristic of long-chain fatty acids such as soybean oil (Pereira et al., 2022). Finally, the peak at 1455 cm^{-1} in all probiotic microcapsules is due to the asymmetric and symmetrical stretching carboxyl groups ($-\text{COO}-$) of sodium alginate, as observed by Dehkordi et al. (2020) and Beldarrain-Iznaga et al. (2020).

Resistance of Free and Microencapsulated *L. rhamnosus* GG During Simulated Gastrointestinal Digestion

The viable cell count and recovery rate of free and microencapsulated *L. rhamnosus* GG after oral, gastric, and intestinal phases exposure are shown in Table 3 and Fig. 6, respectively.

Compared to the initial count, when exposed to simulated mouth conditions (SSF), a decrease ($p < 0.05$) in viable cell count was detected for Lr-ALG, Lr-ALG-PPC microcapsules, and free bacteria. The number of viable cells for Lr-ALG-WPI and Lr-ALG-RPC microcapsules remained unchanged ($p > 0.05$). This behavior is attributed to the higher initial cell concentration for the Lr-ALG-WPI and Lr-ALG-RPC samples.

At the end of the simulated gastric phase (SGF), the number of viable cells and the recovery rate of free and encapsulated *L. rhamnosus* GG decreased ($p < 0.05$). This behavior is related to the interfacial structure formed in each microcapsule. Non-encapsulated cells decreased by 3.73 log CFU^{-1} , while Lr-ALG, Lr-ALG-WPI, Lr-ALG-RPC, and Lr-ALG-PPC microcapsules decreased by 1.80, 0.69, 0.84, and 1.55 log CFU g^{-1} , respectively. These results are consistent with those of Zhang et al. (2015). They attributed the low decrease in the number of viable cells of *L. salivarius* in the gastric phase to improved survivability after encapsulation. For microcapsules, the greatest reduction was observed for Lr-ALG ($p < 0.05$), suggesting that the combination of alginate with whey, rice, or pea proteins provided enhanced protection for probiotics against gastric fluids (Zhang et al., 2015). The improved probiotic protection capacity for Lr-ALG-WPI, Lr-ALG-RPC, and Lr-ALG-PPC is due to the buffering capacity of alginate and the insolubility of the proteins (protein isoelectric point-pI). ALG at acidic pH can sequester protons and convert them to insoluble alginic acid,

Table 3 Viable *L. rhamnosus* GG cell count after exposure to simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF)

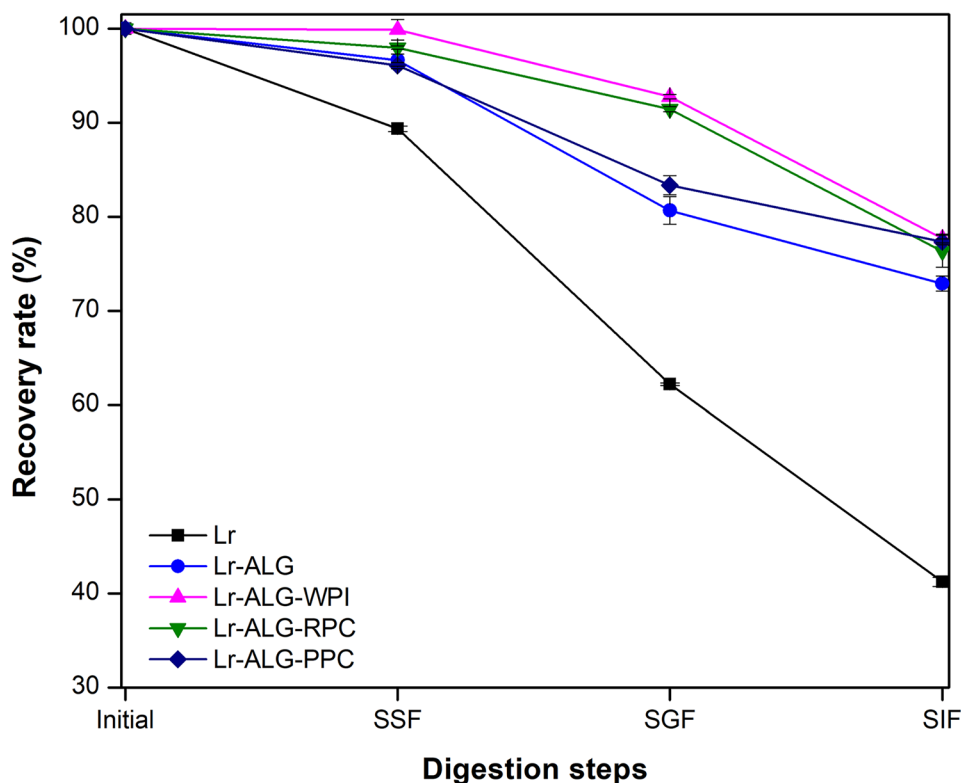
Samples	Viable cell count (log CFU g^{-1})			
	Initial	SSF	SGF	SIF
Free cells	9.91 ^{a,A} ± 0.02	8.85 ^{c,B} ± 0.03	6.16 ^{d,C} ± 0.01	4.08 ^{d,D} ± 0.05
Lr-ALG	9.28 ^{d,A} ± 0.02	8.96 ^{c,B} ± 0.06	7.48 ^{c,C} ± 0.14	6.76 ^{c,D} ± 0.07
Lr-ALG-WPI	9.43 ^{c,A} ± 0.05	9.42 ^{b,A} ± 0.10	8.74 ^{a,B} ± 0.02	7.32 ^{a,b,C} ± 0.04
Lr-ALG-RPC	9.77 ^{b,A} ± 0.02	9.57 ^{b,A} ± 0.02	8.93 ^{a,B} ± 0.03	7.46 ^{a,C} ± 0.17
Lr-ALG-PPC	9.29 ^{d,A} ± 0.06	8.93 ^{c,B} ± 0.03	7.74 ^{b,C} ± 0.10	7.18 ^{b,D} ± 0.03

Results expressed as mean ± standard deviation

^{a-d}Within a column, different superscript lowercase letters denote significant differences ($p < 0.05$) among between samples for the same simulated gastrointestinal step. ^{A-D}Within a line, different superscript uppercase letters denote significant differences ($p < 0.05$) among the different steps of the simulated gastrointestinal conditions for each sample

Lr-ALG, *L. rhamnosus* GG microcapsule with alginate; Lr-ALG-WPI, *L. rhamnosus* GG microcapsule with alginate-whey protein; Lr-ALG-RPC, *L. rhamnosus* GG microcapsule with alginate-rice protein; Lr-ALG-PPC, *L. rhamnosus* GG microcapsule with alginate-pea protein

Fig. 6 Performance of *L. rhamnosus* GG in simulated in vitro gastrointestinal steps. SSF (simulated mouth conditions), SGF (Simulated gastric phase), and SIF (simulated intestinal phase). *Lines are used for better visualization



thus promoting a pH buffering effect that improves the survival of encapsulated probiotics (Tan et al., 2022). The pI of the proteins studied in the present study is in the pH range of 4.6 to 4.8 (Kornet et al., 2021; Lan et al., 2018; Zhang et al., 2021). When WPI, RPC, and PPC are in a medium below their pI, they will have a positive net charge, interacting with anionic polysaccharides (e.g., ALG) to form a complex (Mun et al., 2020). These insoluble complexes/clusters can create a protective barrier, preventing pepsin and gastric fluids from reaching cells. Although gastric juice (pH 3.0) and pepsin are highly harmful to the probiotic, viable cell counts and recovery rate remained above $6 \log \text{CFU g}^{-1}$ and 60% after this step for all samples, respectively. The maintenance of high viable counts and recovery rate for the microcapsules at this stage may also be related to the presence of the oily film on the surface of the particles. The soybean oil layer limits the diffusion of H^+ , organic acids, water, and oxygen through the capsule membrane and minimizes cell death. These results corroborate those of hygroscopicity since the oily film present on the surface of the microcapsules reduced the absorption of ambient water by the microcapsules and contributed to low HG values.

At the end of the intestinal phase, all microcapsules showed adequate levels ($> 6 \log \text{CFU g}^{-1}$) to confer some benefit to the health of the host and followed the order of probiotic viability $\text{Lr-ALG-RPC} = \text{Lr-ALG-WPI} > \text{Lr-ALG-PPC} > \text{Lr-ALG}$. However, viable counts and recovery rates

for all the samples decreased significantly ($p < 0.05$). A drastic reduction of $5.81 \log \text{CFU g}^{-1}$ was seen for free cells, while moderate decreases were noted for microcapsules. The microcapsules that contained proteins in their formulation resulted in viable cell counts $> 7.18 \log \text{CFU g}^{-1}$ and recovery rate $> 76.32\%$. The microcapsules produced only with alginate (Lr-ALG) presented a count of $6.76 \log \text{CFU g}^{-1}$ and a recovery rate of 72.88%. The reduction in viable cell counts and recovery rate for these microcapsules is due to the lipolysis of the oily film on the surface of the microcapsules caused by pancreatic lipases. This process causes the demulsification of the microcapsules, which finally exposes the phospholipids of the cell membrane of *L. rhamnosus* to the action of bile salts (Zhang et al., 2015). Bile salts have a detergent effect and dissolve bacterial membranes causing growth inhibition and even cell death (Madureira et al., 2011). The higher viability for the Lr-ALG-WPI, Lr-ALG-RPC, and Lr-ALG-PPC samples is attributed to the protection the proteins provided. Our results are consistent with those of Obradović et al. (2022), who found that a formulation containing ALG 1% (w/v) and WPI 15% (w/v) dried in a spray dryer reduced only $0.36 \log \text{CFU mL}^{-1}$ for a mixture of probiotic lactic bacteria under simulated intestinal conditions. According to the authors, the positive effect of WPI on the protection of probiotic cells can be explained by the exposure of sulfhydryl and hydrophobic groups due to denaturation, which leads to a decrease in cell diffusion in the

medium. As observed in the DSC analysis, the combination of ALG with WPI, RPC, or PPC can form a dense network due to electrostatic and hydrophobic interactions, limiting the diffusion of bile salts in the microcapsules and delaying the interaction with probiotic bacteria. This effect allows the bacteria to colonize the small and large intestines and thus provide health benefits to the host (Martins et al., 2018).

Conclusions

A membrane emulsification system was used to produce microcapsules containing *Lactocaseibacillus rhamnosus* GG[®]. Combining alginate with whey, rice, or pea protein did not affect the microcapsules' average size. The latter was comparable to the membrane pores' diameter or morphology. Furthermore, proteins improved encapsulation efficiency and thermal properties and provided higher resistance to gastrointestinal fluids. In general, all microcapsules prepared showed promising results. Using vegetable proteins, especially rice, resulted in microcapsules with similar characteristics to those produced with whey. The high viability of microencapsulated *L. rhamnosus* in the final stage of digestion suggested that the encapsulation strategy employed is a promising alternative to include probiotics in some food matrices and improve their viability during processing, gastrointestinal conditions, and storage. Finally, our outstanding achievement was in particle size distribution and encapsulation efficiency. We obtained microcapsules between 18.26 and 28.76 μm with encapsulation yields > 93%, confirming the potential of the membrane emulsification method in the microencapsulation of probiotic strains. Future work could investigate the stability of these microcapsules during prolonged storage and in gastrointestinal conditions in vivo.

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Author Contribution Callebe Camelo-Silva: conceptualization, data curation, formal analysis, investigation, methodology, software, writing—original

draft. Lais Leite Figueredo: data curation, methodology. Karina Cesca: methodology, writing—review & editing. Silvani Verruck: conceptualization, data curation, formal analysis, methodology, writing—review & editing. Alan Ambrosi: conceptualization, data curation, formal analysis, methodology, writing—review & editing. Marco Di Luccio: conceptualization, formal analysis, methodology, funding acquisition, project administration, resources, supervision, writing—review & editing.

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Data Availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of Interest The authors declare no competing interests.

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