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RESEARCH

Physiological and Technological Properties of Probiotic *Lacticaseibacillus rhamnosus* GG Encapsulated with Alginate-Chitosan Mixture and Its Incorporation in Whole Milk

Ana Júlia Alves Aguiar Athayde[1,2] · Lúcia Raquel Ramos Berger[3] · Thatyane Mariano Rodrigues de Albuquerque[4,7] · Karoliny Brito Sampaio[4] · Karina Félix Dias Fernandes[4] · Heloisa Maria Almeida do Nascimento[4] · Sônia Paula Alexandrino de Oliveira[4] · Laênia Angélica Andrade Lopes[5] · Carlos Eduardo Vasconcelos de Oliveira[6] · Maria Lúcia da Conceição[4] · Evandro Leite de Souza[4] · Thayza Christina Montenegro Stamford[1,2]

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Abstract

This study developed and evaluated chitosan-sodium alginate capsules containing the probiotic *Lacticaseibacillus rhamnosus* GG using extrusion and emulsification techniques. The encapsulated *L. rhamnosus* GG cells were also evaluated for technological and probiotic-related physiological functionalities, as well as when incorporated in UHT and powdered milk. Extrusion ($86.01 \pm 1.26\%$) and emulsification ($74.43 \pm 1.41\%$) encapsulation techniques showed high encapsulation efficiency and high survival rates of *L. rhamnosus* GG during 28 days of refrigeration and room temperature storage, especially emulsification capsules (> 81%). The encapsulated *L. rhamnosus* GG cells showed high survival rates during exposure to simulated gastrointestinal conditions (72.65 ± 1.09–$114.15 \pm 0.44\%$). *L. rhamnosus* GG encapsulated by extrusion and emulsification performed satisfactorily in probiotic-related physiological (pH and bile salts tolerance) and technological properties (positive proteolytic activity, diacetyl and exopolysaccharides production, high NaCl tolerance (> 91%), besides having high heat tolerance (> 76%). *L. rhamnosus* GG in extrusion and emulsification capsules had high survival rates (> 89%) and did not significantly affect physicochemical parameters in Ultra-High Temperature (UHT) and powdered milk during storage. The results demonstrate that *L. rhamnosus* GG can be successfully encapsulated with alginate-chitosan as a protective material through extrusion and emulsification techniques. UHT and powdered milk could serve as appropriate delivery systems to increase the intake of this encapsulated probiotic by consumers.

Keywords Encapsulation · Survival · Digestion · Delivery · Storage · Powdered milk · UHT milk

Introduction

In recent decades, the interest in functional foods has greatly increased worldwide, especially foods carrying probiotics [1]. Probiotics are living microorganisms that confer health benefits to consumers when administered in sufficient doses [2]. Protecting and preserving probiotic live cells in the delivery food matrix is crucial to reaching their claimed health-related benefits [3]. Furthermore, severe pH conditions in the stomach and the presence of bile salts in the

✉ Thatyane Mariano Rodrigues de Albuquerque
thatyane.albuquerque3@academico.ufpb.br

1 Post-Graduate Program in Nutrition, Health Sciences Center, Federal University of Pernambuco, Recife, PE, Brazil

2 Laboratory of Applied Microbiology, Medical Science Center, Federal University of Pernambuco, Recife, PE, Brazil

3 Laboratory of Agricultural and Environmental Microbiology, Department of Biology, Federal Rural University of Pernambuco, Recife, PE, Brazil

4 Laboratory of Food Microbiology, Department of Nutrition, Federal University of Paraíba, João Pessoa, PB, Brazil

5 Department of Nutrition, College of São Francisco of Paraíba, Campina Grande, PB, Brazil

6 Laboratory of Bromatology, Department of Nutrition, Federal University of Paraíba, João Pessoa, PB, Brazil

7 Departamento de Nutrição, Centro de Ciências da Saúde, Universidade Federal da Paraíba, Campus I – Cidade Universitária, João Pessoa, PB CEP: 58051-900, Brazil

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small intestine are the main barriers limiting the arrival of probiotic cells to the ileum and colon, where these microorganisms interact with the host-residing intestinal microbiota [4]. Dairy products have been the most common food matrix to deliver probiotics, although studies assessing powdered milk as a probiotic vehicle are still scarce [5, 6].

The increase in the stability of probiotics has been a challenge in functionalizing foods with these microorganisms [7, 8]. Encapsulation could be an effective technology to overcome this limitation since it could protect probiotic cells from adverse conditions imposed by the intrinsic characteristics of the delivering foods and during passage through the gastrointestinal tract [9]. The encapsulation of probiotics using natural or synthetic polymers could have the advantages of being highly targeted, presenting low cytotoxicity, and adequate stability [10–12]. The selection of wall materials to produce the capsules and the technique used in the manufacture are of paramount importance and strictly affect the final morphology and functional properties of the produced capsules [13].

Probiotic encapsulation could help extend the typical short storability of some probiotic-supplemented products and maintain cell viability and functionality during passage through the gastrointestinal tract [14], besides avoiding contact with harsh conditions, like acidic environments, that could increase thermal stress to probiotic cells [15]. Chitosan has been typically ineffective as a coating material to increase probiotic cell survival when used alone [16]. However, other coating materials could be combined with chitosan to encapsulate probiotic bacteria, such as whey protein, poly-lysine, sodium caseinate, and sodium alginate [17].

The chitosan coating (as a multi-component compound) on negatively charged capsules of calcium alginate could increase the physical and chemical stability of the produced capsules, besides decreasing the destructive effects of calcium ion chelating agents on the capsule structure [18, 19]. D-mannuronic acid and L-glucuronic acid, linked by glycosidic bonds, comprise the alginate molecule, a polymer widely used to encapsulate probiotics [17]. Calcium alginate capsules can be produced using emulsion and extrusion techniques [15] and combining calcium alginate with polymers from different sources could produce a uniform and homogeneous mixture [18].

Lacticaseibacillus rhamnosus GG is among the lactic acid bacteria strains most tested for probiotic application, showing a high potential to adhere to and pass through the gastrointestinal tract, besides having antimicrobial activity, resistance to lysozyme, phenol, and antibiotics, and antioxidant activity [13, 20–22]. However, studies evaluating chitosan and sodium alginate mixture to encapsulate *L. rhamnosus* GG and incorporating encapsulated cells in delivery food are still scarce. This study aimed to develop chitosan-sodium alginate capsules containing *L. rhamnosus* GG using extrusion and emulsification techniques. The encapsulated *L. rhamnosus* GG cells were evaluated regarding technological and probiotic-related physiological functionalities, as well as when incorporated in whole Ultra-High Temperature (UHT) and powdered milk.

Materials and Methods

Probiotic Cultivation Conditions

Lacticaseibacillus rhamnosus GG (ATCC 53103) was obtained from Chr. Hansen A/S (Denmark). Before use in the assays, the strain was inoculated into 9 mL of de Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Melbourne, Australia) and incubated at 37 ± 0.5 °C for 24 h under anaerobiosis (Anaerobic System Anaerogen, Oxoid, Hampshire, UK). The cells were collected with centrifugation (4000 × *g*, 10 min, 4 °C), washed twice with sterilized distilled water, and suspended in 4 mL of sterile distilled water for subsequent use as control-free cells and to produce the capsules [23]. The inoculum suspension had a cell concentration of approximately 9 log CFU/mL when plated on MRS agar (Oxoid).

Encapsulation of *L. rhamnosus* GG Cells

Extrusion Technique

The suspended *L. rhamnosus* GG cells were mixed with 40 mL of sterile sodium alginate solution (2% w/v) (D 3247 AJAX Chemicals Ltd., Sydney, Australia). The obtained cell suspension was placed in a sterile syringe and injected through a 0.11-mm needle (2 cm of distance from needle to solution) into sterile 0.05 mol/L calcium chloride ($CaCl_2$) containing 0.1 g/100 g Span 80®. After 30 min of gelification in 0.05 mol/L $CaCl_2$, the capsules were washed with sterile distilled water and filtered (Whatman Grade 4, GE Healthcare, Chicago, IL, USA). Low-molecular-weight chitosan (0.5 g) (Sigma-Aldrich, St. Louis, MA, USA) was dissolved in 100 mL of lactic acid solution (1% v/v), with pH adjusted to 5.7–6.0 using 1 mol/L NaOH, and sterilized with autoclavation (121 °C, 15 min, 1 atm) [23]. The capsules were placed in the chitosan solution for 40 min under magnetic stirring (56 × *g*). Afterward, the capsules were washed with sterile distilled water, filtered, and stored under refrigeration (4 ± 0.5 °C) in a sterile 0.05 mol/L $CaCl_2$ solution [23]. The capsule size was approximately 2 mm, measured using a caliper.

Emulsification Technique

The suspended *L. rhamnosus* GG cells were mixed with 40 mL of sterile sodium alginate solution (2% w/v) (D 3247 AJAX Chemicals Ltd.) and added 2 mL of a sterile $CaCO_3$ (500 mM) solution. After homogenization, the mixture was dispersed into soybean oil (200 mL) under magnetic stirring (56 × *g*), and after 15 min of emulsification, 40 mL of soybean oil containing glacial acetic acid (7 mL) was added to the emulsion using a sterile syringe, injected through a 0.11-mm needle into the mixture, and magnetic stirring (56 × *g*) continued to permit $CaCO_3$ solubilization. Afterward, 300 mL of 0.05 mol/L $CaCl_2$ solution was added to the flask wall and kept under magnetic stirring (56 × *g*) for 10 min. The capsules were settled down, the top layer of the oily phase was removed by aspiration, and the capsules were harvested with two subsequent washings to remove remnant oil, filtered (Whatman), and placed in the chitosan solution (as described in the "Extrusion Technique" section) for 40 min under magnetic stirring (56 × *g*). Afterward, the capsules were washed, filtered, and stored under refrigeration (4 ± 0.5 °C) in a sterile 0.05 mol/L $CaCl_2$ solution [24]. The capsule size was approximately 0.5 mm, measured using a caliper.

Encapsulation Efficiency of *L. rhamnosus* GG Cells

Encapsulation efficiency (EE) was determined as the fraction of *L. rhamnosus* GG viable cells in each capsule compared to the viable cells in the feed bacterial cell dispersion.

The EE (%) was determined using Eq. 1:

$$\text{EE}\ (\%) = (N/N_0) \times 100 \tag{1}$$

N is the count of encapsulated viable cells, and N_0 is the count of viable cells before the encapsulation.

One gram of the capsule was dispersed in 10 mL of sodium citrate solution (3% w/v), the suspension was shaken in a vortex until the complete capsule dissolved, and aliquots (0.1 mL) were dispersed in sterile peptone water (peptone 0.1 g/100 mL, 0.9 mL) for serial dilutions. Aliquots (0.1 mL) of each dilution were plated on MRS agar by the microdrop technique and incubated under anaerobiosis (Anaerogen) at 37 ± 0.5 °C for 48 h. The viable cell counts (log CFU/mL) were determined at the end of the incubation [25].

Survival of *L. rhamnosus* GG in the Capsules During Storage

The survival of *L. rhamnosus* GG in the extrusion and emulsification capsules was determined at 7, 14, 21, and 28 days of refrigeration (4 ± 0.5 °C) and room temperature (25 ± 0.5 °C) storage, following suspension preparation, plating, and incubation described in the "Encapsulation Efficiency of *L. rhamnosus* GG Cells" section. The viable cells of *L. rhamnosus* GG in the capsules were counted, and the results were expressed as survival rates (%) determined using Eq. 2:

$$\text{Survival rates}\ (\%) = (\log N/\log N_0) \times 100 \tag{2}$$

where log N is the viable cell count (log CFU/mL) of *L. rhamnosus* GG in capsules at each monitored storage time interval, and log N_0 is the viable cell count (log CFU/mL) of *L. rhamnosus* GG after the encapsulation.

The detection limit of the tests for enumerating *L. rhamnosus* GG viable cells was 1 log CFU/mL.

Evaluation of Probiotic-Related Physiological Properties of *L. rhamnosus* GG

pH and Bile Salts Tolerance

L. rhamnosus GG suspension (1% v/v, control-free cells as described in the "Probiotic Cultivation Conditions" section) and *L. rhamnosus* GG capsules (1% w/v) were inoculated into phosphate buffer solution (PBS, 10 mL) with pH adjusted to 7.2, 5, 3, and 2 using 1 M HCl or supplemented with bile salts (0.1%, 0.15%, 0.2%, 0.3%, or 1%, w/v) (Sigma-Aldrich) under stirring (150 rpm) [26]. After 3 h of incubation under the tested conditions, an aliquot (0.1 mL) of the suspension was used to determine the survival rates of free and encapsulated cells of *L. rhamnosus* GG, as described in the "Survival of *L. rhamnosus* GG in the Capsules During Storage" section.

Exposure of *L. rhamnosus* GG to Simulated Gastrointestinal Conditions

L. rhamnosus GG suspension (1%, v/v, control-free cells as described in the "Probiotic Cultivation Conditions" section) and *L. rhamnosus* GG capsules (1% w/v) were exposed to simulated gastrointestinal conditions in UHT whole milk (10 mL; Camponesa, Lagoa da Prata, MG, Brazil). The simulation occurred continuously in sterile bottles to mimic esophageal-stomach, duodenum, and ileum conditions. Mechanical agitation simulated peristaltic movements, and experiments were done in an incubator at 37 ± 0.5 °C with rotation adjustment in each phase. The esophageal-stomach condition was simulated with 25 mg of pepsin diluted in 1 mL of 0.1 M HCl, added at a rate of 0.05 mL/mL, with a gradual decrease of pH with 1 M HCl (pH 5.5/10 min, pH 3.8/20 min, and pH 2.0/60 min) under stirring (130 rpm). Duodenal conditions were simulated with 2 g pancreatin/L of 0.1 M $NaHCO_3$ and 12 g bovine bile salts/L of 0.1 M $NaHCO_3$, pH adjusted for 5 with 0.1 M $NaHCO_3$, and

exposure time of 30 min under stirring (45 rpm). Ileal conditions were simulated with pH adjusted to 6.5 with 0.1 M $NaHCO_3$, and the exposure time was 60 min under stirring (45 rpm) [26]. All enzymes and components were obtained from Sigma-Aldrich. At each digestion phase, an aliquot (0.1 mL) of the suspension was used to determine the viable cells of *L. rhamnosus* GG in the capsules. The results were expressed as survival rates of free and encapsulated cells of *L. rhamnosus* GG, as described in the "Survival of *L. rhamnosus* GG in the Capsules During Storage" section.

Technological Properties of *L. rhamnosus* GG

Proteolytic and Lipolytic Activity

Proteolytic activity was evaluated using an aliquot (10 µL) of *L. rhamnosus* GG culture (control, i.e., free cells) grown anaerobically in MRS broth (20–24 h, 37 ± 0.5 °C, Anaerogen), as well as of dissolved capsules previously released by homogenizing 1 g of each capsule with sodium citrate solution (3% w/v, 1 mL) using a vortex until visible complete dissolution. Aliquots (10 µL) were streaked onto plate count agar (HiMedia, Mumbai, India) supplemented with (10% w/v) skimmed milk (Camponesa) and incubated under anaerobiosis (37 ± 0.5 °C, 72 h). The appearance of a clear zone surrounding the colonies was indicative of proteolytic activity.

For evaluating the lipolytic activity, an aliquot (10 µL) of *L. rhamnosus* GG (culture control, i.e., free cells) and dissolved capsules were plated on tributyrin agar (Sigma-Aldrich) and incubated under anaerobiosis (37 ± 0.5 °C, 72 h). The appearance of a clear zone surrounding the colonies was indicative of lipolytic activity [26].

Exopolysaccharide and Diacetyl Production

L. rhamnosus GG culture (1%, v/v, control, i.e., free cells) and *L. rhamnosus* GG capsules (1% w/v of extrusion and emulsification capsules) were added in 10 mL of MRS broth containing 2% glucose (Sigma-Aldrich) and incubated under anaerobiosis (37 ± 0.5 °C, 72 h, Anaerogen). The cells were centrifuged (4000 rpm, 10 min, 4 °C), mixed at a rate of 1:2 with 95% (v/v) cold ethanol (Fmaia, Belo Horizonte, MG, Brazil), and maintained at 4 ± 0.5 °C for 24 h to induce the exopolysaccharide (EPS) precipitation. EPS precipitates were qualitatively evaluated as positive (+) or negative (−) for EPS production [27].

L. rhamnosus GG culture (1%, v/v, control, free cells) and *L. rhamnosus* GG capsules (1% w/v of extrusion and emulsification capsules) were added to 10 mL of UHT whole milk (Camponesa). After 24 h of incubation (37 ± 0.5 °C), 0.5 mL of 1% (w/v) α-naphthol (Sigma-Aldrich) and 16% (w/v) KOH (Sigma-Aldrich) were mixed with bacterial culture (1 mL) in UHT whole milk and incubated (37 ± 0.5 °C, 10 min). The formation of a red ring at the top of the mixture indicated diacetyl production [26, 27]. The diacetyl production was classified as weak (+), medium (++), and strong (+++) based on the intensity of the red ring [26].

Heat Temperature and Salt Tolerance

The resistance of free (control cells as described in the "Probiotic Cultivation Conditions" section) and encapsulated *L. rhamnosus* GG cells to high temperatures (55, 65, 75, 85, and 95 °C for 30 s) was evaluated using sterile distilled water as a suspending medium. For the free cells, 1 mL of the suspension was collected, and for the extrusion and emulsification capsules, 1 g was collected and transferred to test tubes, each containing 9 mL of sterile distilled water. The samples were subjected to water bath thermal treatments (Dist DI950 M, Florianópolis SC, Brazil). Afterward, the samples were cooled (1 min in an ice-cold vessel) to room temperature (25 ± 0.5 °C) [28], and an aliquot (0.1 mL) was used to determine the survival rates of *L. rhamnosus* GG in capsules, as described in the "Survival of *L. rhamnosus* GG in the Capsules During Storage" section.

Salt (sodium chloride, NaCl) tolerance was evaluated using *L. rhamnosus* GG free cells (1%, v/v, control) and *L. rhamnosus* GG capsules (1% w/v), which were transferred to tubes containing 10 mL of MRS broth supplemented with different NaCl concentrations (0, 1, 3, and 5%, w/v), and incubated under anaerobiosis (37 ± 0.5 °C, 24 h, Anaerogen) [26]. At the end of the incubation period, the survival rates of *L. rhamnosus* GG in the capsules were determined, as described in the "Survival of *L. rhamnosus* GG in the Capsules During Storage" section.

Survival of Encapsulated *L. rhamnosus* GG in Whole UHT and Powdered Milk

L. rhamnosus GG capsules (1% w/v of extrusion and emulsification capsules) were inoculated in whole UHT milk (100 mL) and powdered milk (100 g) (Camponesa) and stored for 28 days under refrigeration (4 ± 0.5 °C). On days 0, 7, 14, 21, and 28 of storage, an aliquot (1 mL and 1 g, respectively) of the inoculated UHT and powdered milk was collected to determine survival rates of *L. rhamnosus* GG, as described in the "Survival of *L. rhamnosus* GG in the Capsules During Storage" section.

At the same storage intervals, some physicochemical parameters of UHT and powdered milk were determined. The pH values were determined using a potentiometer with a combined glass electrode for pH determination (Model Q400AS, Diadema, SP, Brazil). The titratable acidity (expressed as mmol H^+/100 g) was determined by titration with 0.1 N NaOH. The soluble solids (°Brix) were

determined using a digital refractometer (Hanna Instruments, model HI 96801, São Paulo, SP, Brazil) at 25 ± 0.5 °C and the moisture content was determined using standard procedures [29].

Statistical Analysis

The assays were performed in triplicate on three independent occasions, and the results were expressed as average ± standard deviation. Initially, the data were assessed via descriptive analysis (average and standard deviation) to obtain the description of the variables. Subsequently, the data were submitted to inferential analyses to determine significant differences ($p \leq 0.05$) using Student's *t*-test or ANOVA followed by post hoc Tukey's test. The statistical analysis was performed using the software SPSS 22 (Statistical Package for Social Sciences).

Results and Discussion

Encapsulation Efficiency and Survival Rates of Encapsulated *L. rhamnosus* GG During Storage

Figure 1 shows the encapsulation efficiency of alginate-chitosan capsules prepared using extrusion and emulsification techniques was around 86.01 ± 1.26% and 74.43 ± 1.41%, respectively. Encapsulation efficiency refers to the concentration of probiotic viable cells within the capsules compared to the initial probiotic concentration in the suspension used to prepare them. Various factors could impact encapsulation efficiency, including the concentration of sodium alginate and $CaCl_2$ used in the capsule formulation, the encapsulated microorganism, and the method and particle size used to formulate the capsules [30].

The primary advantage of using polymers to encapsulate bioactive components through the extrusion and emulsification techniques could be entrapping bacterial cells within the matrix during the capsule formation. It could prevent unintended bacterial cell release into the supplemented product, a common occurrence with other encapsulation techniques, including freeze-drying, spray-drying, and fluidized bed drying [18, 31]. Moreover, the extrusion and emulsification techniques could offer the benefit of employing mild conditions during capsule formation, aiding in maintaining high bacterial cell survival rates [32].

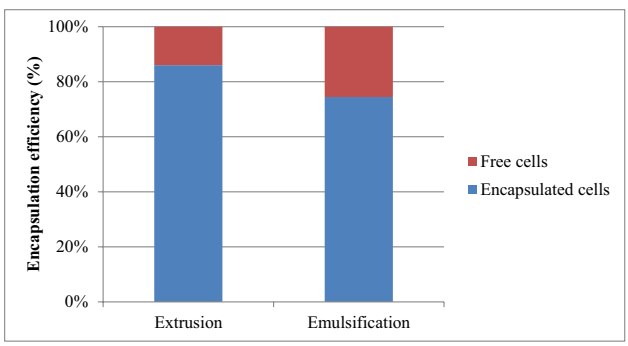

Fig. 1 Encapsulation efficiency (%; average ± standard deviation; $n = 3$) of *Lacticaseibacillus rhamnosus* GG encapsulated with extrusion and emulsification techniques

Similar encapsulation efficiency for *L. rhamnosus* GG cells (84–93%) was reported in early studies using spray-drying and gum Arabic blended with agave fructans, maltodextrin, inulin, and trehalose as wall materials [13]. Sodium alginate used as an encapsulation material in this study yielded satisfactory encapsulation efficiency, although the extrusion technique has shown greater efficiency in encapsulation than emulsification technique to encapsulate *L. rhamnosus* GG.

Table 1 shows the survival rates of *L. rhamnosus* GG in the extrusion and emulsification capsules during 28 days of room and refrigeration temperature storage. The survival rates of *L. rhamnosus* GG in the extrusion capsule (82.20 ± 0.98–85.03 ± 1.37%) were higher than in the emulsification capsule (69.80 ± 0.84–71.05 ± 0.24%) on day 7 of room and refrigeration temperature storage ($p \leq 0.05$). The survival rate decreased on day 14 of room temperature storage ($p \leq 0.05$), especially in the extrusion capsule (< 1.00 ± 0.00%). The survival rate of *L. rhamnosus* GG in the extrusion capsule was low until 28 days of room temperature storage (< 1.00 ± 0.00%). The survival rates of *L. rhamnosus* GG in the emulsification capsule were higher on days 21 and 28 of room temperature storage (75.48 ± 1.03% and 81.77 ± 1.09%, respectively) than in the extrusion capsule ($p \leq 0.05$).

Bacterial metabolic activity is typically high above 22 °C, which can lead to reduced viability and cell death over time [32], agreeing with the results showing low survival rates of *L. rhamnosus* GG in the extrusion capsules under room temperature storage. However, it does not agree with the results showing high survival rates of *L. rhamnosus* GG in the emulsification capsules under room temperature storage. The sustained survival rates of *L. rhamnosus* GG in the emulsification capsules indicate the efficacy of the emulsification technique in protecting bacterial cells from adverse and stressful conditions over time [13].

The survival rates of *L. rhamnosus* GG in the extrusion capsule on day 7 of refrigerated storage (85.03 ± 1.37%) were higher than on days 14, 21, and 28 (62.20 ± 0.71–77.47 ± 0.66%) and higher than extrusion capsule under room temperature storage ($p \leq 0.05$). The survival rate of *L. rhamnosus* GG in the emulsification capsule under refrigerated storage increased over time (69.80 ± 0.84% to 89.82 ± 0.45%), with a higher survival rate on day 28 day of

Table 1 Encapsulation efficiency (%) and survival rate (%; average ± standard deviation; $n = 3$) of *L. rhamnosus* GG encapsulated with extrusion and emulsification techniques during room temperature (25 ± 0.5 °C) and refrigeration storage (4 ± 0.5 °C)

Encapsulation method	Days of storage/survival rate (%)			
	7	14	21	28
Extrusion 25 °C	82.20 ± 0.98^{Ba}	$< 1.00 \pm 0.00^{Cb}$	$< 1.00 \pm 0.00^{Cb}$	$< 1.00 \pm 0.00^{Db}$
Extrusion 4 °C	85.03 ± 1.37^{Aa}	77.47 ± 0.66^{Ab}	62.20 ± 0.71^{Bd}	69.28 ± 0.40^{Cc}
Emulsification 25 °C	71.05 ± 0.24^{Cc}	68.01 ± 1.41^{Bd}	75.48 ± 1.03^{Ab}	81.77 ± 1.09^{Ba}
Emulsification 4 °C	69.80 ± 0.84^{Dc}	76.73 ± 1.03^{Ab}	77.54 ± 0.76^{Ab}	89.82 ± 0.45^{Aa}

[a–c]Different superscript small letters in the same row for the same encapsulation methodology at different storage time intervals denote difference ($p \leq 0.05$), based on the Tukey test

[A–D]Different superscript capital letters in the same collum for the same storage time interval denote differences between the encapsulation technique ($p \leq 0.05$), based on the Tukey test

storage. The higher survival rates of *L. rhamnosus* GG in the emulsification capsules indicate superior performance of the emulsification technique in protecting probiotic cells during storage rather than the extrusion technique [33]. The prolonged survival of *L. rhamnosus* GG cells in the emulsification capsule under refrigeration storage could be linked to the reduced bacterial metabolic activity induced by low temperatures, as well as the increased capsule stability over time [32].

Probiotic-Related Physiological Functionality Properties of Encapsulated *L. rhamnosus* GG

pH and Bile Salts Tolerance

Table 2 shows the survival rates of *L. rhamnosus* GG in the extrusion and emulsification capsules when exposed to different pH values (7.2, 5, 3, and 2) and bile salt concentrations (0.1, 0.15, 0.2, 0.3, 1%). After 3 h of exposure to pH 7.2 and 5, *L. rhamnosus* GG had a higher survival rate in the extrusion capsule (99.86 ± 1.11–99.72 ± 2.15%) ($p \leq 0.05$) than in the emulsification capsule (52.23 ± 1.28–77.69 ± 1.11%) and control (61.01 ± 1.03–71.26 ± 1.18%). After 3 h of exposure to pH 3, the survival rate of *L. rhamnosus* GG sharply decreased in the extrusion and emulsification capsules (< 1.00 ± 0.00%) ($p \leq 0.05$). However, after 3 h of exposure to pH 2, the highest survival rates of *L. rhamnosus* GG were detected in the emulsification capsule (99.90 ± 0.24%), followed by extrusion capsule (94.28 ± 1.23%) and control (40.42 ± 1.31%). Notably, the encapsulated *L. rhamnosus* GG cells had increased tolerance to low pH, indicating that encapsulation protected these cells from pH variations. Previous studies reported the potential use of sodium alginate and chitosan to encapsulate lactobacilli, with efficacy in protecting bacterial cells from acidic environments [34–36].

After exposure to 0.1, 0.15, and 0.2% bile salts, *L. rhamnosus* GG had the highest survival rates in the extrusion capsule (96.05 ± 1.18–99.81 ± 2.33%) ($p \leq 0.05$), followed by emulsification capsule (79.79 ± 2.17–91.60 ± 1.50%) and control (57.47 ± 1.44–65.51 ± 1.07%). After exposure to 0.3 and 1% bile salts, *L. rhamnosus* GG had the highest survival rate in the emulsification capsule (76.38 ± 1.25% and 40.03 ± 1.25%, respectively) ($p \leq 0.05$), followed by extrusion capsule (64.49 ± 2.06% and 16.32 ± 1.74%, respectively) and control (20.11 ± 2.04% and 9.57 ± 1.54%, respectively).

Bile and acid tolerance are important indicators of the survival capacity of probiotic strains during the gastrointestinal tract passage to reach the human colon [37]. Overall, the

Table 2 Survival rate (%; average ± standard deviation; $n = 3$) of *L. rhamnosus* GG free ells (control) and encapsulated by extrusion and emulsification techniques after 3 h of exposure different pH values and bile salt concentrations

pH values	Encapsulation method/survival rate (%)		
	Control	Extrusion	Emulsification
pH 7.2	71.26 ± 1.18^{Ac}	99.72 ± 2.15^{Aa}	77.69 ± 1.11^{Bb}
pH 5	61.01 ± 1.03^{Bb}	99.86 ± 1.11^{Aa}	52.23 ± 1.28^{Cc}
pH 3	56.41 ± 1.58^{Ca}	$< 1.00 \pm 0.00^{Cb}$	$< 1.00 \pm 0.00^{Db}$
pH 2	40.42 ± 1.31^{Dc}	94.28 ± 1.23^{Bb}	99.90 ± 0.24^{Aa}
Bile salt concentrations	Control	Extrusion	Emulsification
0.1%	58.71 ± 1.50^{Bc}	99.81 ± 2.33^{Aa}	91.60 ± 1.50^{Ab}
0.15%	65.51 ± 1.07^{Ac}	99.59 ± 1.20^{Aa}	86.48 ± 1.20^{Bb}
0.2%	57.47 ± 1.44^{Bc}	96.05 ± 1.18^{Ba}	79.79 ± 2.17^{Cb}
0.3%	20.11 ± 2.04^{Cc}	64.49 ± 2.06^{Cb}	76.38 ± 1.25^{Ca}
1%	9.57 ± 1.54^{Dc}	16.32 ± 1.74^{Db}	40.03 ± 1.25^{Da}

[a–c]Different superscript small letters in the same row denote differences between encapsulation methods and control ($p \leq 0.05$) for the same time interval in the same pH or in same bile salt concentration, based on Tukey's test

[A–C]Different superscript capital letters in the same column denote differences ($p \leq 0.05$) between different pH values or different bile salt concentration, in the same encapsulation method or control, based on Tukey's test

higher the bile salt concentration, the lower the *L. rhamnosus* GG survival rates [28, 37]. However, *L. rhamnosus* GG showed higher survival rates in the extrusion and emulsification capsules when exposed to all tested bile salt concentrations compared to the control. These results agree with early studies showing that calcium alginate and chitosan protect encapsulated bacterial cells from damage induced by bile salts [28, 38].

Exposure to Simulated Gastrointestinal Conditions

Figure 2 shows the survival rates of *L. rhamnosus* GG encapsulated with extrusion and emulsification techniques when exposed to different phases of the simulated gastrointestinal digestion. During the initial phases of the simulated gastrointestinal digestion, where the pH was 5.5 and 3.8 (esophageal-stomach), the survival rates of *L. rhamnosus* GG in the extrusion and emulsification capsules (83.01 ± 2.5–108.46 ± 1.1%) did not differ ($p > 0.05$) or were higher ($p \leq 0.05$) than in control (91.87 ± 0.88–95.62 ± 0.87%).

At the most acidic pH of the esophageal-stomach phase (pH 2.0), *L. rhamnosus* GG had the greatest survival rate in the emulsification capsule (106.04 ± 0.35%), followed by the extrusion capsule (99.35 ± 0.49%) and control (86.87 ± 0.88%). In the duodenal phase (pH 5.0), the survival rate of *L. rhamnosus* GG decreased ($p \leq 0.05$) in the emulsification capsule (81.91 ± 1.5%) and increased ($p \leq 0.05$) in the extrusion capsule and control (114.15 ± 0.44% and 91.87 ± 1.02%, respectively). At the end of the simulated gastrointestinal digestion (ileal phase, pH 6.5), *L. rhamnosus* GG had a survival rate similar of around 73.38 ± 0.71% and 72.65 ± 1.09% in the extrusion and emulsification capsules, respectively.

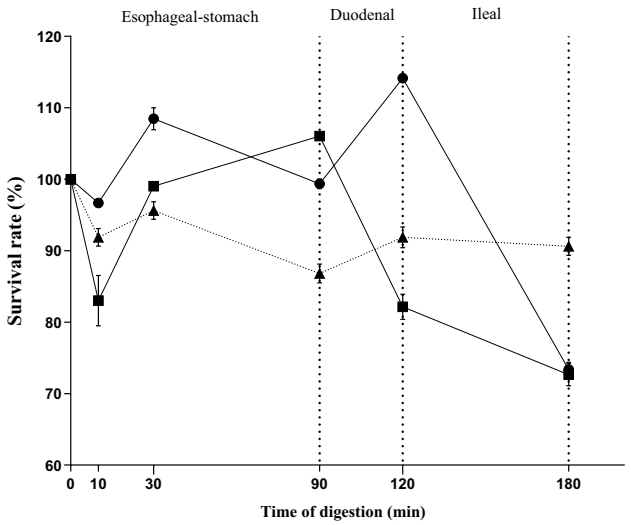

Fig. 2 Survival rate (%; average ± standard deviation; $n = 3$) of *Lacticaseibacillus rhamnosus* GG free cells (control; ▲) and encapsulated with extrusion (●) and emulsification (■) techniques when exposed to simulated gastrointestinal conditions

L. rhamnosus free cells typically have a sharp viability loss when exposed to simulated gastrointestinal conditions [13, 19]. Encapsulating probiotic microorganisms could increase the protection against adverse conditions due to pH variations and the action of bile salts and enzymes when exposed to the stomach and intestinal fluids [39]. Even with differences between the formulated capsules, *L. rhamnosus* GG cells had high survival rates (above 70%) in the extrusion and emulsification capsules, indicating that encapsulated cells could keep their probiotic functionality due to surviving passage through the gastrointestinal tract [21].

Alginate and chitosan as wall materials to encapsulate bacterial cells have been linked to high resistance to gastric fluids because of their typical cationic behavior in media [32, 40]. Furthermore, the mucoadhesive properties of natural polymers, such as alginate and chitosan, used to produce encapsulated *L. rhamnosus* GG cells could help high populations of live bacterial cells to reach the colon [18], where these cells could adhere to the intestinal epithelial cells and perform the desired claimed beneficial effects on the host [21, 41, 42].

Technological Properties of Encapsulated *L. rhamnosus* GG

Proteolytic and Lipolytic Activity

L. rhamnosus GG cells in the extrusion and emulsification capsules and control had positive results for proteolytic activity and negative results for lipolytic activity (Table 3). Proteolysis causes enzymatic protein degradation to produce medium- or low-molecular-weight peptides and free amino acids, affecting flavor and food texture [43]. *L. rhamnosus* GG cells commonly show high proteolytic activity when tested as encapsulated or free cells [44].

Lactic acid bacteria commonly have weak or no lipolytic activity, although this property varies among bacterial species [45]. Enterococci commonly have higher lipolytic activity than other lactic acid bacteria species, while *Lactococcus* and *Lactobacillus* commonly have weak lipolytic activity [46]. The weak lipolytic activity prevents or reduces the possibility of developing rancidity or off-flavors [47], and this feature may make *L. rhamnosus* GG cells encapsulated using extrusion and emulsification capsules techniques candidates for use in dairy products [48].

Exopolysaccharide and Diacetyl Production

L. rhamnosus GG in the extrusion and emulsification capsules and control produced EPS but had a low capacity for

Table 3 Technological properties of *L. rhamnosus* GG free cells (control) and encapsulated by extrusion and emulsification techniques

Encapsulation method	Proteolytic activity	Lipolytic activity	Diacetyl production	EPS production
Control	+	-	+	+
Extrusion	+	-	+	+
Emulsification	+	-	+	+

NaCl tolerance (survival rate, %)	0%	1%	3%	5%
Control	100 ± 0.01^{Aa}	94.26 ± 1.62^{Cb}	93.45 ± 1.79^{Bbc}	91.27 ± 1.63^{Ac}
Extrusion	100 ± 0.01^{Aa}	97.26 ± 0.85^{Bb}	94.17 ± 1.65^{Bc}	91.55 ± 2.43^{Ac}
Emulsification	100 ± 0.01^{Aa}	99.52 ± 1.75^{Aa}	99.39 ± 2.35^{Aa}	91.45 ± 1.62^{Ab}

Heat treatment (survival rate, %)	28 °C	55 °C	65 °C	75 °C	85 °C	95 °C
Control	100 ± 0.01^{Aa}	74.97 ± 1.44^{Cb}	57.27 ± 1.17^{Cc}	54.86 ± 1.11^{Cd}	44.85 ± 1.40^{Ce}	38.74 ± 1.17^{Cf}
Extrusion	100 ± 0.01^{Aa}	101.78 ± 1.35^{Ba}	100.68 ± 1.2^{Ba}	97.11 ± 1.18^{Bb}	87.89 ± 1.13^{Bc}	76.89 ± 1.26^{Bd}
Emulsification	100 ± 0.01^{Ac}	109.23 ± 1.50^{Aa}	106.73 ± 1.25^{Aa}	103.13 ± 1.20^{Ab}	95.15 ± 1.17^{Ad}	89.67 ± 1.49^{Ae}

[a–d]Different superscript small letters in the same row for the same encapsulation methodology at different NaCl concentrations or temperatures denote difference ($p \leq 0.05$), based on the Tukey test

[A–C]Different superscript capital letters in the same collum for the same NaCl concentration or for the same temperature denote differences between the encapsulation methods ($p \leq 0.05$), based on the Tukey test

diacetyl production (Table 3). Microbial EPS, a secondary metabolite produced by some probiotic strains, could exert several health-related benefits, such as antitumor [49], antibacterial, antioxidant [50, 51], immunomodulatory [52], and intestinal microbiota modulatory effects [53]. EPS produced by *L. rhamnosus* GG effectively mitigates oxidative damage and apoptosis in intestinal epithelial cells [54] and ameliorates intestinal inflammation [55].

L. rhamnosus GG cells can use the available substrate to produce diacetyl and acetoin as by-products. Moreover, *L. rhamnosus* GG increases diacetyl in different food substrates, such as oats and coconut [56]. Diacetyl, a volatile compound from citrate metabolism, can impart distinct characteristics to fermented products, particularly dairy products [57]. The natural diacetyl aroma is associated with creamy and buttery flavor in dairy products (e.g., butter, cheese, and fermented milk), affecting consumer acceptance, besides having antimicrobial effects that could be exploited by the food industry [58].

Heat and NaCl Tolerance

Table 3 shows the survival rates of *L. rhamnosus* GG in the extrusion and emulsion capsules and control when exposed to different temperatures (28, 55, 65, 75, 85, and 95 °C) and NaCl concentrations (1, 3, and 5%). *L. rhamnosus* GG in the extrusion and emulsification capsules had high survival rates (≥ 100%) when exposed to 28, 55, and 65 °C, which did not differ ($p > 0.05$) or were higher ($p \leq 0.05$) than control (100 ± 0.01, 74.97 ± 1.44%, and 57.27 ± 1.17%, respectively). *L. rhamnosus* GG in the emulsification capsule had a survival rate 103.13 ± 1.20% when exposed to 75 °C, while the survival rates decreased to 97.11 ± 1.18% and 54.86 ± 1.11% in the extrusion capsule and control, respectively. Similarly, *L. rhamnosus* GG had a higher survival rate ($p \leq 0.05$) in the emulsification capsule when exposed to 85 and 95 °C (95.15 ± 1.17% and 89.67 ± 1.49%, respectively), followed by the extrusion capsule (87.89 ± 1.13% and 76.89 ± 1.26%, respectively) and control (44.85 ± 1.40% and 38.74 ± 1.17%, respectively).

Heating is one of the main stresses to bacterial cells during food processing. Various bacterial cell functions are greatly disturbed when exposed to high temperatures, which cannot be countered by the cellular response system, leading to viability loss and cell death [59]. The higher stability of encapsulated *L. rhamnosus* GG cells should be a favorable technological attribute to their incorporation in foods exposed to heating during processing [60]. An early study reported a higher tolerance of *L. rhamnosus* GG cells encapsulated with whey protein and gum Arabic [61]. The increased tolerance of some lactic acid bacteria to high temperatures has been linked to increased EPS production, helping bacterial cells survive harsh environmental conditions [62].

The survival rates of *L. rhamnosus* GG in the extrusion and emulsification capsules exposed to 1% NaCl (97.26 ± 0.85% and 99.52 ± 1.75%, respectively) were higher than the control (94.26 ± 1.62%) ($p \leq 0.05$). Only *L. rhamnosus* GG in the extrusion capsule decreased the survival rate (94.17 ± 1.65%) when exposed to 3% NaCl compared to 1%

NaCl ($p \leq 0.05$). However, *L. rhamnosus* GG in the extrusion and emulsification capsules and control had high survival rates (approximately 91%) when exposed to 5% NaCl, representing a high NaCl tolerance, which is a positive feature for using probiotics in salted processed foods [63].

Performance of Encapsulated *L. rhamnosus* GG in UHT and Powdered Milk

Table 4 shows the survival rates of *L. rhamnosus* GG in the extrusion and emulsification capsules incorporated in whole UHT and powdered milk during 28 days of refrigeration storage. *L. rhamnosus* GG in the extrusion capsule had an overall higher survival rate in UHT milk than in powdered milk during storage ($p \leq 0.05$), while emulsification capsules

Table 4 Survival rate (%; average ± standard deviation; $n = 3$) of *L. rhamnosus* GG encapsulated with extrusion and emulsification techniques in UHT and powdered milk and physicochemical parameters during 28 days of refrigeration storage (4 ± 0.5 °C)

Extrusion	Days of storage/survival rate (%)				
	0	7	14	21	28
UHT milk	100 ± 0.01^{Ab}	114.88 ± 1.07^{Aa}	116.18 ± 1.06^{Aa}	113.29 ± 1.06^{Aa}	112.28 ± 2.18^{Aa}
Powdered milk	100 ± 0.01^{Ab}	91.91 ± 1.12^{Bc}	91.91 ± 1.03^{Bc}	106.06 ± 1.40^{Ba}	89.35 ± 0.32^{Bd}
Emulsification					
UHT milk	100 ± 0.01^{Ac}	105.22 ± 1.29^{Bb}	109.20 ± 2.10^{Ba}	108.52 ± 2.08^{Aab}	105.90 ± 1.16^{Ab}
Powdered milk	100 ± 0.01^{Ad}	121.86 ± 2.03^{Aa}	114.98 ± 2.07^{Ab}	105.81 ± 2.16^{Ac}	101.37 ± 1.0^{Bcd}
Physicochemical parameters/days of storage					
Extrusion	0	7	14	21	28
	Titratable acidity (mmol H^+/100 g)				
UHT milk	1.9 ± 0.06^{Bd}	1.9 ± 0.10^{Ad}	2.2 ± 0.03^{Ac}	2.4 ± 0.06^{Ab}	2.6 ± 0.13^{Aa}
Powdered milk	2.6 ± 0.13^{Aa}	2.0 ± 0.03^{Ab}	1.8 ± 0.03^{Bc}	1.8 ± 0.20^{Bc}	1.6 ± 0.01^{Bd}
Emulsification					
UHT milk	1.7 ± 0.10^{Bd}	1.7 ± 0.03^{Bd}	2.0 ± 0.06^{Ac}	2.2 ± 0.13^{Ab}	2.6 ± 0.06^{Aa}
Powdered milk	2.5 ± 0.13^{Aa}	2.1 ± 0.06^{Ab}	2.0 ± 0.13^{Ac}	1.7 ± 0.10^{Bd}	1.6 ± 0.06^{Be}
pH					
Extrusion					
UHT milk	6.7 ± 0.02^{Ab}	6.9 ± 0.03^{Aa}	6.5 ± 0.01^{Ac}	6.4 ± 0.01^{Bd}	6.3 ± 0.01^{Be}
Powdered milk	6.5 ± 0.07^{Bc}	6.9 ± 0.03^{Aa}	6.6 ± 0.10^{Ab}	6.6 ± 0.01^{Ab}	6.5 ± 0.01^{Ac}
Emulsification					
UHT milk	6.7 ± 0.03^{Ab}	7.0 ± 0.10^{Aa}	6.6 ± 0.01^{Ac}	6.5 ± 0.01^{Bc}	6.4 ± 0.01^{Bd}
Powdered milk	6.5 ± 0.07^{Bc}	6.9 ± 0.01^{Aa}	6.6 ± 0.04^{Ab}	6.6 ± 0.01^{Ab}	6.6 ± 0.01^{Ab}
Soluble solids (°Brix)					
Extrusion					
UHT milk	12.7 ± 0.21^{Ba}	12.6 ± 0.07^{Ab}	12.4 ± 0.21^{Ac}	12.4 ± 0.07^{Ad}	12.3 ± 0.07^{Ae}
Powdered milk	14.6 ± 0.07^{Aa}	10.5 ± 0.07^{Bb}	8.3 ± 0.01^{Bc}	7.5 ± 0.07^{Bd}	7.1 ± 0.14^{Be}
Emulsification					
UHT milk	12.8 ± 0.14^{Ba}	12.6 ± 0.07^{Ab}	12.45 ± 0.02^{Ac}	12.4 ± 0.07^{Ac}	12.3 ± 0.01^{Ad}
Powdered milk	14.6 ± 0.07^{Aa}	8.5 ± 0.28^{Bb}	7.5 ± 0.14^{Bc}	7.4 ± 0.08^{Bd}	7.4 ± 0.07^{Bd}
Moisture (%)					
Extrusion					
UHT milk	88.2 ± 0.01^{Ac}	88.1 ± 0.10^{Ac}	88.2 ± 0.01^{Ac}	88.4 ± 0.09^{Ab}	88.6 ± 0.02^{Aa}
Powdered milk	1.0 ± 0.36^{Be}	2.3 ± 0.01^{Bd}	3.4 ± 0.01^{Bc}	3.6 ± 0.01^{Bb}	4.1 ± 0.01^{Ba}
Emulsification					
UHT milk	89.3 ± 1.41^{Aa}	87.7 ± 0.01^{Ac}	88.4 ± 0.01^{Ab}	88.5 ± 0.01^{Ab}	88.4 ± 0.04^{Ab}
Powdered milk	0.96 ± 0.36^{Ba}	2.2 ± 0.01^{Bd}	3.6 ± 0.01^{Bc}	3.8 ± 0.01^{Bc}	4.4 ± 0.41^{Bb}

[a–c]Different superscript small letters in the same row for the same encapsulation methodology at different storage time intervals denote difference ($p \leq 0.05$), based on the Tukey test

[A–C]Different superscript capital letters in the same collum for the same storage time interval and the same encapsulation method denote differences between the powered and UHT milk ($p \leq 0.05$), based on the Tukey test

had a higher ($p \leq 0.05$) or similar survival rate ($p > 0.05$) in powdered milk compared to UHT milk.

On days 7 to 14 of storage, the survival rate of *L. rhamnosus* GG in the extrusion (114.88 ± 1.07% to 116.18 ± 1.06%) and emulsification (105.22 ± 1.29% to 109.20 ± 2.10%) capsules increased in UHT milk ($p \leq 0.05$). Powdered milk had highest survival rate (121.86 ± 2.03–114.98 ± 2.07%) of *L. rhamnosus* GG in the emulsification capsule ($p \leq 0.05$), while the survival rate of *L. rhamnosus* GG in the extrusion capsule was approximately 91% ($p \leq 0.05$).

On day 21 of storage, *L. rhamnosus* GG in the extrusion capsule had the highest survival rate (113.29 ± 1.06%) in UHT milk ($p \leq 0.05$), while *L. rhamnosus* GG in the emulsification capsule had the lowest survival rate in powdered milk (105.81 ± 2.16%) ($p \leq 0.05$). On day 28 of storage, *L. rhamnosus* GG in the extrusion and emulsification capsules decreased the survival rate in powdered milk (89.35 ± 0.32% and 101.37 ± 1.0%, respectively) ($p \leq 0.05$). The highest survival rate on day 28 of storage was detected in UHT milk for the extrusion capsule (112.28 ± 2.18%), followed by the emulsification capsule (105.90 ± 1.16%).

An important difficulty in incorporating probiotics in foods is the bacterial viability loss during storage [22]. The results showed overall high survival rates of *L. rhamnosus* GG in the extrusion and emulsification capsules in UHT and powdered milk during 28 days of refrigeration storage, which could be linked to the capsule size, tested probiotic, and/or post-acidification processes during storage [39]. These factors could affect the diffusion capacity of components within and outside the capsule due to the alginate porosity, suggesting that the nutrient passage could be used by the encapsulated bacterial cells to keep their viability during storage [22].

The survival rates of *L. rhamnosus* GG in the extrusion and emulsification capsules on day 28 of refrigeration storage were higher than previously reported for encapsulated *L. rhamnosus* cells in apple juice and yogurt during 22 and 30 days of refrigeration storage, respectively [22]. The extrusion and emulsification capsules could provide a favorable environment for *L. rhamnosus* GG cells and a physical barrier against environmental conditions affecting their viability and survival [13, 64].

The physicochemical characteristics of milk can be changed due to probiotic supplementation [65]. Table 4 shows the results of the measured physicochemical parameters of UHT and powdered milk supplemented with *L. rhamnosus* GG in the extrusion and emulsification capsules during 28 days of refrigeration storage. The titratable acidity increased during storage in UHT milk supplemented with *L. rhamnosus* GG in the extrusion and emulsification capsules (1.9 ± 0.06 to 2.6 ± 0.13 and 1.7 ± 0.10 to 2.6 ± 0.06°Brix, respectively) ($p \leq 0.05$), while decreased in powdered milk (2.6 ± 0.13 for 1.6 ± 0.01 and 2.5 ± 0.13 to 1.6 ± 0.06°Brix, respectively) ($p \leq 0.05$).

The pH values increased on day 7 of storage in UHT and powdered milk supplemented with *L. rhamnosus* GG in the extrusion and emulsification capsules but decreased on day 14 of storage ($p \leq 0.05$). Overall, the pH values did not differ between UHT and powdered milk supplemented with *L. rhamnosus* GG in extrusion or emulsification capsule at the same storage period but differed during storage. The increase in titratable acidity and reduction in pH values during storage in UHT and powdered milk supplemented with *L. rhamnosus* GG in the extrusion and emulsification capsules agree with results of previous studies with milk supplemented with encapsulated lactic acid bacteria [32, 65], which could be linked to increased lactic acid concentrations due to lactose degradation [66].

The total soluble solids decreased ($p \leq 0.05$) and did not change ($p > 0.05$) during storage in UHT and powdered milk supplemented with *L. rhamnosus* GG in the extrusion and emulsification capsules, respectively. As soluble solids decrease, the moisture content commonly increases in foods [65, 66]. The moisture did not change during storage in UHT milk supplemented with *L. rhamnosus* GG in the extrusion and emulsification capsules (88.1 ± 0.01–89.3 ± 1.41%) ($p > 0.05$) but increased in powdered milk regardless of the supplemented capsules (1.0 ± 0.36 to 4.4 ± 0.41%) ($p \leq 0.05$). Powdered milk commonly has moisture content varying from approximately 3 to 5% [67]. Moisture can affect bacterial cell viability and survival in powdered foods even when in encapsulated cells [17]; however, low moisture is recommended for maintaining stability during prolonged storage of probiotic powder foods [68].

Conclusion

Chitosan-sodium alginate capsules containing *L. rhamnosus* GG were successfully developed with extrusion and emulsification techniques. Extrusion and emulsification encapsulation techniques showed high encapsulation efficiency and high survival rates of *L. rhamnosus* GG during refrigeration and room temperature storage and during exposure to simulated gastrointestinal conditions. Still, *L. rhamnosus* GG in the extrusion and emulsification capsules performed satisfactorily in probiotic-related physiological and technological properties. *L. rhamnosus* GG in the extrusion and emulsification capsules had high survival rates and did not significantly affect physicochemical parameters in UHT and powdered milk during storage. These results indicated that *L. rhamnosus* GG can be effectively encapsulated with an alginate-chitosan mixture as wall materials using extrusion and emulsification techniques, as well as UHT and powdered milk could be suitable delivery matrices to enhance

the consumption of this encapsulated probiotic among consumers.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing Interests The authors declare no competing interests.

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