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ORIGINAL PAPER

Impact of the microencapsulation method and wall material on the survival of *Lactobacillus acidophilus* under simulated gastrointestinal conditions

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Abstract

The study aimed to assess the effect of microencapsulation methods and wall material composition on the survival of *Lactobacillus acidophilus*, both immediately after encapsulation and following exposure to simulated gastrointestinal conditions. *Lactobacillus acidophilus* was encapsulated by ionic gelation and spray drying using various concentrations of wall materials. Physicochemical and morphological characterizations were performed to analyze changes in wall materials and differences in survival rates. Furthermore, microcapsules were subjected to an in vitro digestion method to assess the probiotic survival rate. For ionic gelation, the optimal wall material composition was 50% sodium alginate, 25% Arabic gum and 25% whey protein isolate, yielding a post-encapsulation survival rate of 94%. For spray drying, the most effective formulation consisted of 80% Arabic gum and 20% whey protein isolate, which also resulted in a 94% survival rate. However, after in vitro simulated gastrointestinal digestion, the survival of *Lactobacillus acidophilus* was significantly higher in spray-dried microcapsules (36%) compared to those produced via ionic gelation (3%). The novelty of this study lies in preparing capsules containing *Lactobacillus acidophilus* to be delivered in the intestinal tract for greater health benefits. The higher concentration of reducing sugars relative to peptides indicated that glycolysis was the predominant degradation pathway during digestion. These findings suggest that spray drying offers enhanced protection for *Lactobacillus acidophilus*, likely due to the formation of a more stable and resistant encapsulation matrix. Consequently, spray drying represents a promising technique for applications in functional food development, supporting improved probiotic viability and potential health benefits.

Keywords Microencapsulation · *Lactobacillus acidophilus* · Ionic gelation · Spray drying · Survivability · *In vitro* digestion method

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Introduction

The International Scientific Association for Probiotics and Prebiotics (ISAPP) has defined probiotics as "live microorganisms that, when administered in adequate amounts, (at least 10^6–10^7 CFU/g), confer a health benefit on the host" [1]. Probiotics offer multiple health benefits, such as the control of enteric infections, regulation of serum cholesterol concentrations, management of inflammatory bowel disease, reduction of colon cancer cells, enhancement of lactose intolerance, and stimulation of the immune system through improved digestion [2–4]. Among probiotics, *Lactobacillus acidophilus* is widely used due to its beneficial impacts on human health [5]. *L. acidophilus* is a Gram-positive, homofermentative, catalase-negative, and thermophilic microorganism with a rod morphology, and capable of growing at 30–45 °C and pH 4.0–5.0 [6].

After the COVID-19 pandemic, both consumer and industry interest in functional foods that contribute to gut microbiota health has increased. However, probiotic viability is challenged by several factors, such as temperature, oxygen, osmotic stress, pH fluctuations, and harsh gastrointestinal transit, which can limit their ability to reach and colonize the intestine [7]. Consequently, the development of novel oral probiotic formulations and advanced delivery systems that enhance bacterial survival through the gastrointestinal tract has become a growing research focus [8]. Microencapsulation is a promising technology for protecting bacteria from environmental stresses by entrapping them within a biopolymeric matrix [4, 5, 9]. In the microencapsulation During microencapsulation, microcapsules, microspheres or microparticles are formed depending on the distribution of the bioactive ingredient in the polymeric matrix [10]. Various microencapsulation techniques are employed, including extrusion, emulsion, complex coacervation, lyophilization, fluidized bed drying, ionic gelation, and spray drying [11].

Spray drying is one of the most widely used encapsulation technologies in the food industry due to its cost-effectiveness, low energy consumption, continuous operation, and high productivity, making it suitable for large-scale manufacturing [6]. Common wall materials used in spray drying include maltodextrin, Arabic gum, xanthan gum, skimmed milk, whey protein concentrate, and whey protein isolate [3, 12]. This method yields microcapsules with low moisture content, extended shelf life, and ease of handling during transportation [3, 6]. For instance, Colín-Cruz et al. [12] demonstrated that encapsulation efficiency depends on the type of wall material, with whey protein concentrate (93.3%) and Arabic gum (89.4%) yielding the highest encapsulation efficiency. Arepally et al. [3] reported a viability of 7.87 CFU/g and 89.15% survival when *Lactobacillus acidophilus* NCDC 016 was encapsulated by spray drying using 7.5% Arabic gum and 20% maltodextrin.

Ionic gelation is another common technique for probiotic encapsulation. Sodium alginate is widely used as an encapsulating material in this method, often combined with pectin, gellan gum, and whey protein isolate to enhance capsule stability [13, 14]. This method is simple and requires no specialized equipment or organic solvents. However, it poses challenges for industrial scalability and often results in capsules with high moisture content, which may reduce stability [15]. Combining alginate with other biopolymers can mitigate these limitations. Olivarez-Romero et al. [13] reported improved gastric survival of *Lactobacillus acidophilus* LA-5 when encapsulated in sodium alginate by emulsification and external gelation. Similarly, Dehkordi et al. [14] achieved 8.35 log CFU/mL viability and 93.69% survival for *Lactobacillus acidophilus* PTCC 1643 using an alginate-whey protein isolate combination via emulsification and internal gelation.

Polysaccharides and proteins remain the primary materials used for encapsulation. Biopolymers such as maltodextrin, xanthan gum, chitosan, carrageenan, pectin, gelatine, alginate, Arabic gum, and whey proteins, are commonly used [2, 11]. In this study, sodium alginate, whey protein isolate, and Arabic gum were selected as wall materials due to their beneficial properties. Sodium alginate forms a protective hydrogel matrix that enhances *L. acidophilus* survival under gastric conditions; whey protein isolate improves structural integrity and shields against environmental stress; and Arabic gum stabilizes the encapsulated *L. acidophilus* while also providing prebiotic benefits [16, 17]. Recent studies suggest that optimizing wall material combinations can enhance probiotic survival [3, 6, 12, 18], as shown by the improved protection of *L. acidophilus* NCDC 016 when Arabic gum and maltodextrin were combined in spray drying, resulting in 89.15% encapsulation efficiency compared to 69.17% with maltodextrin alone [3].

Recent studies have explored novel biopolymer formulations, innovative encapsulation techniques, and synergistic combinations of prebiotics and probiotics to enhance encapsulation efficiency and probiotic stability [19]. Studies indicate that hybrid biopolymer matrices, incorporating both polysaccharides and proteins, significantly improve probiotic protection against harsh conditions [20]. Additionally, advancements in nanoencapsulation and multi-layered microcapsules are emerging as promising approaches to further enhance probiotic viability during digestion and storage [21].

Ensuring the survivability of microorganisms used as probiotics is crucial, as they must remain viable until reaching the target site in the gastrointestinal tract to exert health benefits. Simulation of in vitro digestion is helpful in trying

to replicate human digestive conditions in a precise, adaptable and reproducible way [22]. The in vitro digestion method is commonly employed to assess probiotic viability under simulated gastrointestinal conditions [23–25]. In vitro gastrointestinal digestion models provide a standardized, ethical, and cost-effective alternative to in vivo studies, allowing researchers to investigate food digestion dynamics under controlled conditions. The standardized static in vitro digestion method (INFOGEST) offers a reproducible approach to simulate human gastrointestinal processes, incorporating physiologically relevant parameters such as pH, enzyme activity, and digestion times [23]. Furthermore, correlations between in vitro and in vivo data have demonstrated that static models can reliably predict nutrient bioaccessibility and digestive behaviors, making them valuable tools for preliminary screening before conducting more complex and resource-intensive in vivo trials [26]. By minimizing ethical concerns related to human and animal testing, in vitro models enable high-throughput analysis of food matrices while maintaining scientific relevance and applicability [27].

While previous research on probiotic encapsulation has covered novel materials, innovative methods, prebiotic integration, and food applications [4, 6, 12, 17, 21, 28–31], few studies have directly analyzed how different encapsulation methods and wall materials influence probiotic survival after simulated gastrointestinal digestion [32, 33]. This gap underscores the need to identify the most effective microencapsulation techniques for preserving probiotic viability under physiological conditions. In this sense, we hypothesized that both the encapsulation method (ionic gelation or spray drying) and the type of wall material would significantly influence the survival of *Lactobacillus acidophilus* following encapsulation and under in vitro simulated gastrointestinal conditions. Therefore, the aim of this research was (a) to prepare capsules by ionic gelation or spray drying; (b) to carry out the physicochemical and morphological characterization of the obtained capsules; (c) to evaluate the survival of encapsulated *Lactobacillus acidophilus* under simulated gastrointestinal conditions (oral, gastric, and intestinal stage); and (d) to correlate the survival of *Lactobacillus acidophilus* with the physical and morphological characteristics of the capsules, the different encapsulation methods and the encapsulating materials.

Materials and methods

Material

Lactobacillus acidophilus LA-5® was provided by Chr. Hansen (Hoersholm, Denmark). Sodium alginate (viscosity 400–600 mPas) and calcium chloride ($CaCl_2$), agar, and Man, Rogosa and Sharpe (MRS) broth were purchased from Sigma-Aldrich (St. Louis, Mo, USA). The biopolymeric materials used for microencapsulation were Arabic gum TIC Pretested® food grade E 414 (Ingredion México S.A. de C.V., Tlalnepantla de Baz, México) and 9010 Instantized Whey Protein Isolate with a protein content of 92% (dry basis) (Hilmar Ingredients, Hilmar Cheese Company, Inc., USA). Fehling A and Fehling B ready-to-use solutions were supplied by Hycel de México S.A. de C.V. (Naucalpan, México). All other analytical grade chemicals were obtained from Sigma-Aldrich Corp. (St. Louis, Mo, USA).

Bacterial strain and growth conditions

A sample (25 g) of freeze-dried lactic culture was rehydrated in 500 mL of MRS broth and incubated anaerobically at 37.2 °C ± 1 °C for 48 h [12], to achieve a final culture concentration of at least 1×10^8 CFU/mL. Then, the culture was centrifuged using an Eppendorf centrifuge model 5430 R (Hamburg, Germany) at 3500 rpm for 15 min. The cells in the precipitate were recovered, washed with 0.9% sterile saline solution, and resuspended to a final volume of 250 mL. This procedure was performed in triplicate.

Preparation of capsules by ionic gelation

Fourteen samples were prepared in duplicate. For this purpose, 2 mL of *L. acidophilus* suspension (LA) was mixed with 10 mL of 2% sodium alginate (SA) and blended with sterile solutions of Arabic gum (AG) and/or whey protein isolate (WPI) according to the experimental design. Samples were extruded using a syringe kit NUOBO™ X00206L1Q3 (Lüliang, China) into 50 mL of 0.2 M $CaCl_2$ solution. The syringe needle was held 10 cm above the calcium solution during extrusion. Samples were then stirred at 300 rpm at 25 °C ± 1 °C for 90 min to promote rigidity. The optimal interaction time between the biopolymers and the crosslinking agent was defined as 90 min, as it yielded completed, rounded, and well-structured capsules with adequate mechanical stability. The solidified beads were collected by filtration through Whatman #4 filter paper and washed with sterile water [34]. The capsules were refrigerated until further characterization.

Preparation of microcapsules by spray drying

Ten types of microcapsules were prepared in duplicate by spray drying. The LA solution (10 mL) was mixed with the biopolymers (AG, WPI or their blend), according to the experimental design. Each solution was sprayed into a mini spray dryer model B-290 (Büchi Labortechnik AG,

Flawil, Switzerland) with a flow rate of 6 mL/min, ensuring consistent droplet formation and particle size distribution. A co-current flow system was used. A double-fluid nozzle (0.7 mm diameter) was used to inlet the solution into the dryer. Drying conditions were set as follows: 140 °C ± 5 °C inlet temperature, 76 ± 5 °C outlet temperature, and 40–50 mBar atomizing pressure, pumped at 20%, and aspirator at 100% [12].

Particle size of capsules

To measure particle size, 20–25 capsules prepared by ionic gelation were placed on millimeter paper. Five sets of samples were taken from each experiment to complete at least 100 capsules. Subsequently, images were captured under 55x magnification using a digital single-lens reflex camera (Nikon D5500, 2015, Thailand) without flash, and under uniform laboratory lighting conditions to avoid interferences. For microcapsules obtained by spray drying, the images were taken by Scanning Electron Microscopy (SEM) as described in Sect. 2.7.

The captured images were saved in TIFF format and then processed with Image J software (Version 1.53k, USA), to adjust and convert to an 8-bit map (black and white), standardized to the same threshold (grayscale), and saved again in TIFF format [35–37]. The images were cleaned using MS Paint program to obtain binary (black and white) images, and then digital image analysis was performed using the Image J software (Version 1.53k, USA). The Feret diameter was determined from the processed images after calibrating the software using the millimeter paper.

Moisture content of the capsules

The moisture content was determined following the Association of Official Analytical Chemists method [38]. A sample of 1.5 g of each capsule was placed in an aluminum pan and dried at 100 °C ± 1 °C for 10 min to determine the moisture content based on weight loss. The test for each sample was performed in triplicate.

Morphological analysis of the capsules

Samples from capsules prepared by both ionic gelation and spray drying were observed in duplicate by Scanning Electron Microscopy (SEM). Briefly, samples were positioned on circular pans with double-sided conductive carbon tape. Unlike other SEM techniques, no gold coating was required for these samples. Morphology was observed with a Hitachi electron microscope model TM3030 Plus (Japan) at a 5 kV accelerating voltage and room temperature. The equipment was calibrated before each imaging session using a reference grid to verify magnification accuracy and contrast settings. Micrographs at different magnifications (40x and 3000x) were acquired to evaluate surface topology, porosity, and particle integrity. Samples showing the most homogeneous morphology and minimal structural defects were selected for further analysis based on smoothness, uniformity, and absence of visible fractures or deformations.

Encapsulation efficiency

Encapsulation efficiency (EE) was determined by quantifying viable bacteria using the plate count method. For capsules prepared by ionic gelation, 1 g of capsules was dissolved in 100 mM tribasic sodium citrate used as the calcium chelator, to release encapsulated microorganisms. For the microcapsules produced by spray drying, 1 g of capsule-containing powder was dissolved in water. Subsequently, the dissolved capsules were diluted in 9 mL of sterile 0.9% saline water and homogenized using a 3 Orbital Shakers Vortex (IKA, Germany), for 1 min. Finally, 1 mL of the appropriate dilution was spread on a Petri dish containing MRS agar and incubated at 37.2 ± 1 °C for 48 h. Colony forming units were counted and reported as CFU/g. This determination was performed in duplicate. The percentage of encapsulation efficiency was calculated according to Eq. 1.

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

Where N is the number of viable cells (CFU/g) in the capsules, and N_0 is the number of viable cells in the bacterial solution before the encapsulation process.

In vitro simulated Gastrointestinal digestion

Capsules showing the best encapsulation efficiency (by ionic gelation or spray drying process), the biopolymers used, and the free bacteria (*Lactobacillus acidophilus)*, were subjected to in vitro digestion according to the methodology proposed by Minekus et al. [23]. Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the mentioned reference.

Oral stage assay

The oral stage involved a 1:1 (w/v) dilution of the capsules with SSF. For this purpose, 5 g capsules were mixed with 3.5 mL of SSF electrolyte stock solution and the pH was adjusted to 7 with 1 M NaOH solution. 0.5 mL salivary α-amylase solution of 1500 U/mL prepared in SSF

electrolyte stock solution was added followed by 25 µL of 0.3 M $CaCl_2$ and 975 µL of pH 7 water. The solution was mixed and incubated for 2 min at 37 °C ± 1 °C. Independent samples were prepared in duplicate to determine the survival of *L. acidophilus* after this stage.

Gastric stage assay

For this assay, 0.375 g of capsules were weighed and rehydrated with 2.5 mL of water at pH 3.0 (the recommended average value for pepsin activity) and equilibrated at 37 ± 1 °C in 2 mL of SSF stock solution (without amylase) and 3.75 mL of SGF stock solution to obtain a bolus-oral: SGF 50:50 ratio, then the pH was adjusted to 3.0 with 1 M HCl solution and 2.5 µL $CaCl_2$ 0.3 M and 50 mg porcine pepsin (≥3,200 U/mg, EC 3.4.23.1) previously hydrated in 2.5 mL of water at pH 3.0, were added, reaching a final volume of 10 mL. Digestion was carried out for two hours, at 37 ± 1 °C in a water bath and stirring in an orbital shaking plate (at 330 rpm), because this is the approximate time of in vivo gastric digestion. Independent samples were prepared in duplicate for the digestion time of 120 min, and the reaction was stopped by freezing liquid nitrogen. The degree of protein hydrolysis in the gastric stage at acidic pH was calculated using Eq. 2.

$$\mathrm{DH}\ (\%) = 100 \times \frac{V \times N}{m \times h_{tot}} \times \frac{1}{1 - \alpha_{COOH}} \tag{2}$$

Where V is the spent volume of 0.05 N HCl (mL), N is the acid normality (meq/mL), m is the mass of protein (g), h_{tot} is the numer of peptide bonds per gram of protein (constant 8.8), and α_{COOH} is the average degree of dissociation calculated from Eq. 3.

$$\alpha_{COOH} = \frac{10^{pH-pK_A(COOH)}}{1 + 10^{pH-pK_A(COOH)}} \tag{3}$$

Where pH is the hydrogen ion concentration, and pK_A is the negative logarithm of the acid dissociation constant.

Intestinal stage assay

After the simulated gastric stage, the digest was transferred to simulate digestion in the small intestine. For this test, 5 mL of the gastric stage digest (gastric chyme) was mixed with 3.375 mL of SIF stock solution to obtain a final gastric chyme to SIF ratio of 50:50 (v/v) after adding the necessary amount of 1 M NaOH solution for neutralizing the chyme (pH 7.0) and the addition of 0.01 mL of 0.3 M $CaCl_2$, and 48 mg of pancreatin previously hydrated in 1.25 mL of SIF stock solution to achieve a final volume of 10 mL. Independent samples were prepared in duplicate for a 2 h digestion time, stopping immediately the reaction by freezing in liquid nitrogen. The degree of protein hydrolysis in the intestinal stage at basic pH was calculated using Eq. 4.

$$\mathrm{DH}\ (\%) = 100 \times \frac{V \times N}{m \times h_{tot}} \times \frac{1}{\alpha_{NH_2}} \tag{4}$$

Where V is the spent volume of 0.1 N NaOH (mL), N is the alkali normality (meq/mL), m is the mass of protein (g), h_{tot} is the number of peptide bonds per gram of protein (constant 8.8), and α_{NH2} the average degree of dissociation calculated from Eq. 5.

$$\alpha_{NH_2} = \frac{10^{pH-pK_A(NH_2)}}{1 + 10^{pH-pK_A(NH_2)}} \tag{5}$$

Where pH is the hydrogen ion concentration, and pK_A is the negative logarithm of the basic dissociation constant.

Reducing sugars test

The hydrolysis of sugars from encapsulant biopolymers and capsules that showed the best encapsulation efficiency (from both ionic gelation or spray drying processes), before and after in vitro digestion, was determined through the Fehling method. This volumetric titration method is based on the principle that all sugars having a free aldehyde or a ketonic group are converted to enediols, leading to the reduction of Cu^{2+} to Cu^{+} ions and the formation of a red precipitate of insoluble Cu_2O [39]. This test was used to measure the free monosaccharides after the in vitro digestion assay. Briefly, 0.5 g of each digested sample (from oral, gastric, and intestinal stages) was diluted to 25 mL with distilled water (solution X). Separately, 5 mL of distilled water was placed in an Erlenmeyer flask with Fehling A (7 g/dL copper (II) sulfate pentahydrate) reagent and Fehling B (potassium sodium tartrate tetrahydrate) reagent mixed in 1:1 ratio, to obtain the final Fehling solution. This solution was boiled and 20 µL of methylene blue (0.2% p/v) were added as a titration indicator (solution Y). Solution X was added dropwise solution Y until the red color corresponding to cuprous oxide (Cu_2O) was formed. The test for each sample was performed in duplicate. Reducing sugars were calculated using Eq. 6.

$$\mathrm{Reducing\ sugars}\ (\%) = \frac{(2500 \times T)}{(V \times P)} \tag{6}$$

Where T is the experimental factor of the Fehling A solution (0.059), V is the spent volume in the titration, and P is the sample weight (g).

Table 1 Characterization of the capsules obtained by ionic gelation using a simplex lattice mixture design

Run number	SA (%)	WPI (%)	AG (%)	pH	Feret diameter (mm)	Moisture (%)	EE (%)
1.5	100	0	0	8.16 ± 0.13^{g}	3.29 ± 0.20^{def}	64.29 ± 0.01^{de}	91
2.8	50	50	0	6.97 ± 0.21^{de}	3.16 ± 0.33^{cd}	55.43 ± 0.01^{a}	44
3.1	50	0	50	6.53 ± 0.15^{bcd}	3.42 ± 0.23^{fg}	55.45 ± 0.01^{a}	53
4.4	75	25	0	7.30 ± 0.10^{ef}	3.02 ± 0.22^{bc}	61.11 ± 0.01^{b}	31
5.14	75	0	25	6.97 ± 0.12^{de}	3.53 ± 0.24^{gh}	61.03 ± 0.01^{b}	76
6.12	50	25	25	6.10 ± 0.17^{ab}	2.86 ± 0.17^{a}	64.65 ± 0.01^{def}	94
7.13	66.7	16.7	16.7	6.49 ± 0.12^{bcd}	3.19 ± 0.16^{d}	63.68 ± 0.01^{cd}	63
8.11	83.3	8.3	8.3	6.85 ± 0.23^{cde}	3.30 ± 0.19^{def}	62.20 ± 0.01^{bc}	31
9.3	58.3	33.3	8.3	5.90 ± 0.20^{a}	2.85 ± 0.15^{a}	71.63 ± 0.01^{g}	63
10.7	58.3	8.3	33.3	5.86 ± 0.15^{a}	3.38 ± 0.17^{ef}	64.33 ± 0.01^{de}	88
11.6	50	0	50	6.50 ± 0.10^{bcd}	2.97 ± 0.31^{ab}	66.17 ± 0.01^{f}	86
12.2	50	25	25	5.90 ± 0.17^{a}	3.24 ± 0.14^{de}	64.71 ± 0.01^{def}	91
13.9	100	0	0	7.53 ± 0.15^{f}	3.62 ± 0.22^{h}	64.33 ± 0.01^{de}	82
14.10	50	50	0	6.47 ± 0.25^{bc}	2.84 ± 0.56^{a}	65.89 ± 0.01^{ef}	64

*SA: sodium alginate; WPI: whey protein isolate; AG: Arabic gum; EE: encapsulation efficiency

** To determinate the statistically significance difference between means, the Tukey's test ($p \leq 0.05$) was applied

***Values with different letters in the same column indicate a significant difference ($p \leq 0.05$)

Table 2 Characterization of the microcapsules obtained by spray drying using a simplex lattice mixture design

Run number	WPI (%)	AG (%)	pH	Feret diameter (μm)	Moisture (%)	EE (%)
1.4	100	0	5.27 ± 0.06^{bc}	14.18 ± 0.59^{b}	5.22 ± 0.01^{d}	9
2.8	50	100	5.06 ± 0.08^{a}	11.38 ± 0.23^{a}	6.83 ± 0.01^{a}	15
3.6	0	100	4.41 ± 0.06^{d}	36.27 ± 0.46^{f}	7.31 ± 0.01^{b}	2
4.3	75	25	5.35 ± 0.05^{bc}	18.48 ± 0.44^{d}	7.54 ± 0.01^{b}	21
5.2	25	75	5.06 ± 0.01^{a}	11.24 ± 0.19^{a}	8.28 ± 0.01^{e}	85
6.5	100	0	5.38 ± 0.09^{c}	14.82 ± 0.42^{bc}	4.59 ± 0.01^{c}	15
7.1	0	100	4.42 ± 0.05^{d}	33.28 ± 0.53^{e}	7.28 ± 0.01^{b}	4
8.7	50	50	5.19 ± 0.08^{abc}	10.52 ± 0.61^{a}	6.74 ± 0.01^{a}	17
9.9	20	80	5.23 ± 0.08^{abc}	15.59 ± 0.77^{bc}	4.43 ± 0.02^{c}	94
10.0	15	85	5.18 ± 0.06^{ab}	16.78 ± 0.36^{cd}	6.73 ± 0.02^{a}	88

*WPI: whey protein isolate; AG: Arabic gum; EE: encapsulation efficiency

** To determinate the statistically significance difference between means, the Tukey's test ($p \leq 0.05$) was applied

***Values with different letters in the same column indicate a significant difference ($p \leq 0.05$)

Statistical analysis

The proportions of wall materials used for encapsulation (via ionic gelation and spray drying) were determined using a Simplex Lattice Mixture Design implemented in Design-Expert software version 11 (Minneapolis, MN. USA). Each experimental run was subsequently performed in the laboratory, and the obtained responses are presented in Tables 1 and 2. The primary objective of these experiments was to maximize encapsulation efficiency (EE), which was therefore the sole response variable analyzed for both encapsulation methods. Analysis of variance (ANOVA) with a level of significance of 0.05 was carried out and the Tukey test was used to establish the differences between means by using the Statistica v12 software.

For the ionic gelation process, involving three mixture components, the software evaluated three possible regression models: linear, quadratic, and special cubic. In contrast, the spray drying process involved only two mixture components, allowing for the assessment of linear, quadratic, cubic, and quartic models. The software generated a fit summary to identify the most suitable model based on three criteria: the p-value for the regression (model significance), the p-value for lack of fit (model adequacy), and the adjusted coefficient of determination (R^2). The optimal model was selected based on a statistically significant regression ($p \leq 0.05$), a non-significant lack of fit ($p > 0.05$), and the highest R^2 value.

The analysis of variance (ANOVA) for the regression provides information on the variability explained by the model and serves as the foundation for statistical hypothesis

testing. For instance, in the case of a linear regression model, the hypotheses are:

Null hypothesis Ho: Y = a.

Alternative hypothesis H1: Y = a + bX.

If $p \leq 0.05$, H_0 is rejected, indicating the model is statistically significant. Conversely, if $p > 0.05$, H_0 is accepted, indicating the model is not significant.

Additionally, ANOVA includes p-values for each coefficient in the model to assess their significance:

Null hypothesis Ho: coefficient = 0.

Alternative hypothesis H1: coefficient ≠ 0.

A p-value ≤ 0.05 indicates the coefficient is statistically significant (≠ 0) and should be retained in the model. A p-value > 0.05 suggests that the coefficient is not significant and may be excluded from the model to improve parsimony.

A numerical optimization algorithm for the EE was used to find the best combination of encapsulating material that resulted in the maximal survival of *L. acidophilus*. The capsules obtained with the optimal mixtures of encapsulant material were subjected to a static in vitro digestion system to compare the survival of *L. acidophilus* at the end of the digestion for the different selected capsules, as well as to compare them with the survival of free bacterial cells subjected to the same digestion conditions.

Results and discussion

Particle size, moisture, and morphology of capsules

The results for capsules prepared by ionic gelation are shown in Table 1. The Feret diameter ranged between 2.84 and 3.62 mm, corresponding to mini capsules prepared with the 14 formulations. This range corresponds to the diameter of the NUOBO™ syringe needle (1.98 ± 0.005 mm) used for extrusion. For ionic gelation, factors influencing the particle size include nozzle diameter, flow rate, and the distance between solution outlet and the hardening bath [11]. In this study, higher alginate concentration resulted in larger Feret diameters of the capsules.

The particle size distribution for all formulations of capsules obtained by ionic gelation is illustrated in Fig. 1. As observed, the particle size distribution depended on the combination and the ratio of encapsulating materials. When only SA (Samples 1.5 and 13.9) was used, particle size ranged between 3 and 4 mm. A similar behavior was observed with mixtures of SA and AG (Samples 3.1, 5.14, 11.6), where the particle size distribution reached the highest values approaching 4 mm. Conversely, adding WPI to the wall materials (Samples 2.8, 4.4, 14.10), resulted in a smaller particle size distribution, ranging from 2 to 3 mm. Notably, sample 6.12 reached the highest encapsulation efficiency and showed the most normal particle size distribution.

While comparative data on particle size distribution in ionotropic gelation systems are scarce, our findings align with earlier reports. For example, Dehkordi et al. [14] demonstrated that varying alginate–whey protein ratios and cross-linking conditions significantly influenced microcapsule diameter and morphology for *Lactobacillus acidophilus* encapsulation. More recently, Rojas-Muñoz et al. [40] confirmed that wall-material composition in alginatesweet whey beads affects particle size (reported ~ 1.6 ± 0.2 mm) and supports viability under simulated gastrointestinal conditions.

Smaller particle size was reported by Dos Santos et al. [41] reporting the mean diameters of 40 microspheres prepared with sodium alginate containing *Lactobacillus helveticus* were 2.4 ± 0.1 mm. Conversely, other authors have reported smaller diameters of *Lactobacillus acidophilus* LA-5 microcapsules prepared under the emulsification/ionic gelation method in the range of 56.1 µm to 93.4 µm, confirming that both the method and the wall material influence the mean particle size [13].

As observed in Table 1, the moisture content of capsules obtained by ionic gelation ranged from 55.43 to 71.63% with a significant difference among all samples. This is mainly because encapsulation by ionic gelation with alginate results in hydrogel structures with a porous matrix, that allows the diffusion of small molecules into or out of them [15, 42].

Regarding morphology, capsules prepared with the highest concentration of SA showed the greatest homogeneity. As illustrated in Fig. 2, sample 1.5 (100% SA) presented a spherical shape and a smoother surface than capsules prepared with a mixture of SA and other biopolymers (WPI or AG). This result is consistent with the study carried out by [10], which found spherical morphologies in sweet whey–sodium alginate (SW-SA) beads observed by SEM. It is well known that ionic gelation typically produces spherical capsules. Several researchers suggest that low alginate concentrations result in capsules with sphericity [42–44]. However, a spherical surface does not seem to significantly contribute to the survival of *L. acidophilus* in this study, as survival rates are influenced by additional structural factors beyond morphology. This is confirmed by SEM micrographs, where formulation 1.5 (100% SA), reached 91% of EE whereas formulation 6.12 (50% SA, 25% WPI and 25% AG) yielded the highest encapsulation efficiency of 94%. This behavior suggests that adding WPI and AG enhances the protective matrix, likely due to their ability to interact with the encapsulated microorganisms used as probiotics and modulate the surface characteristics of the capsules.

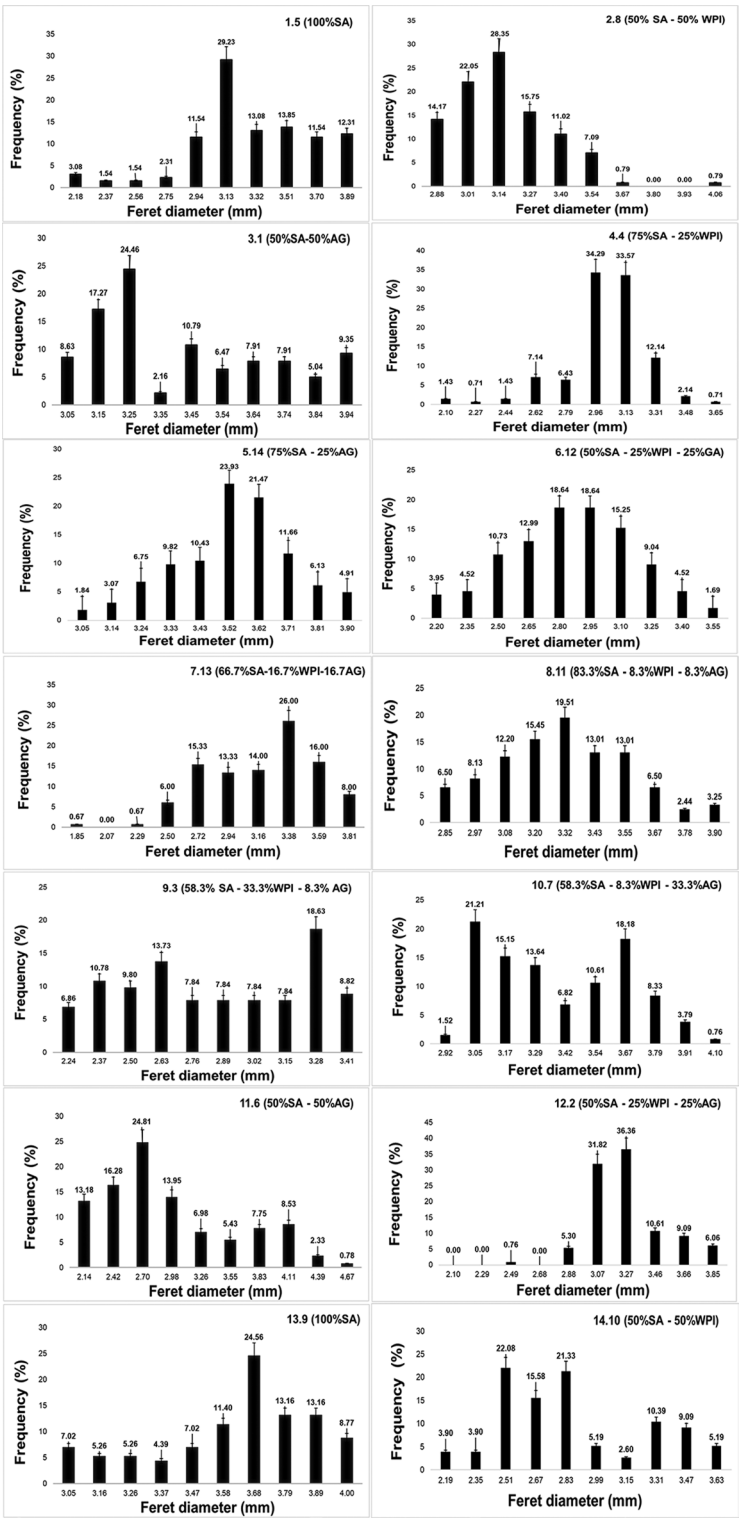

Fig. 1 Particle size distribution of capsules obtained by ionic gelation

The microstructure of the outer surface of capsules with and without *L. acidophilus* was captured for comparison (Fig. 2). The surface of capsules with *L. acidophilus* shows a rough texture and protrusions, suggesting that the bacterial strain was effectively entrapped within the biopolymer matrix. This observation aligns with previous findings on encapsulated microorganisms used as probiotics [13, 45]. Moreover, surface roughness and porosity are critical parameters that influence probiotic survival. Encapsulation studies using WPI and SA have demonstrated that denser, and less porous surfaces significantly enhance microcapsule stability [42]. Proteins such as WPI can contribute to

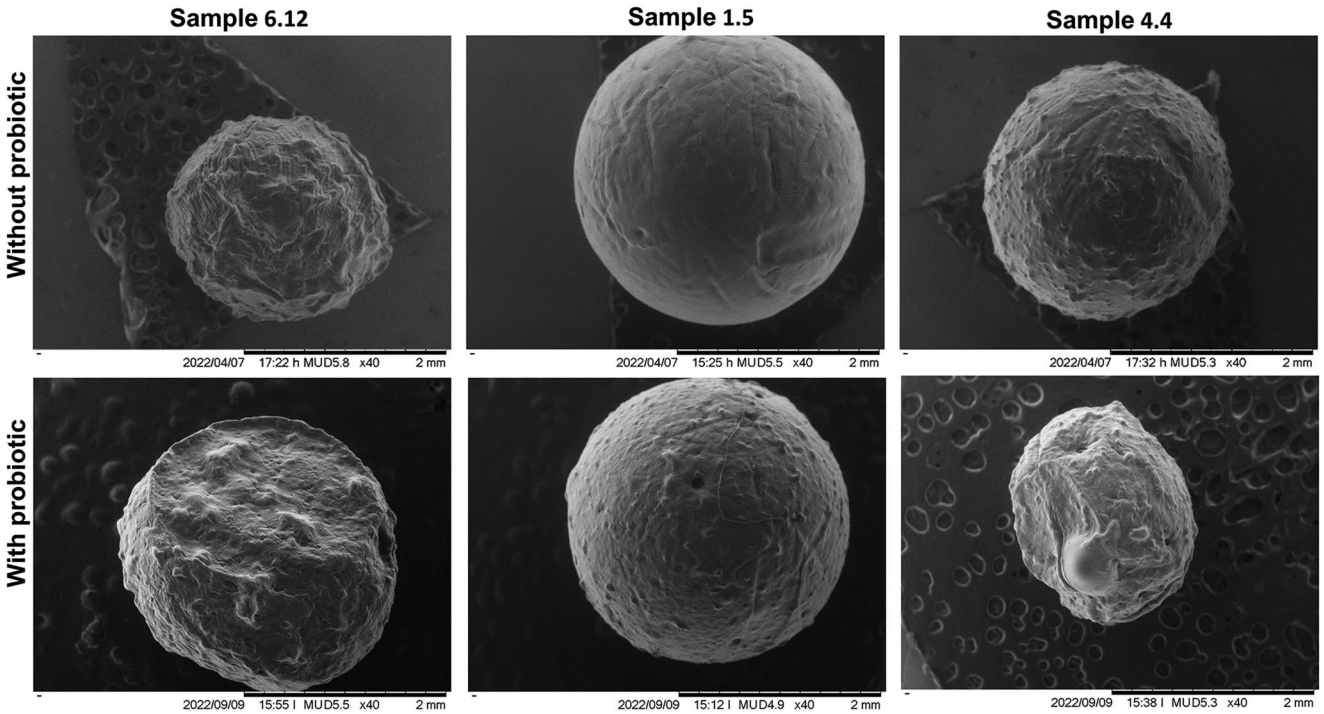

Fig. 2 Micrographs of capsules obtained by ionic gelation (Sample 6.12: 50% sodium alginate, 25% protein isolate, 25% Arabic gum; Sample 1.5: 100% sodium alginate, and Sample 4.4: 75% sodium alginate, 25% protein isolate) with and without *Lactobacillus acidophilus*

structural reinforcement by reducing surface porosity and limiting oxygen permeability, both essential factors for maintaining probiotic viability during storage. In contrast, more porous matrices may promote higher diffusion rates, potentially resulting in premature probiotic release and reduced viability [42].

These findings highlight the importance of evaluating external morphology and internal physicochemical interactions within the encapsulation matrix when assessing probiotic stability. Future research should focus on optimizing surface characteristics to improve encapsulation efficiency and ensure long-term probiotic viability under diverse storage and processing conditions.

The results for microcapsules obtained by spray drying are shown in Table 2. The Feret diameter from the ten formulations ranged between 10.52 and 36.27 µm across the ten formulations. These results indicate that spray drying encapsulation method produce fine particles with a diameter in the 10–150 µm range [3, 12]. Factors influencing the particle size of the spray-dried microcapsules include airflow, air temperature, flowrate, atomizer type, and the properties of the wall materials [5, 11, 46]. In this study, higher concentrations of AG resulted in larger Feret diameters. This could be explained because AG has a significantly greater molecular weight (350 KDa) than the proteins found in WPI (lactoglobulin, 18.37 KDa, and lactoalbumin, 14.18 KDa) [47, 48]. This is confirmed in the particle size distribution shown in Fig. 3, where the particle size distribution depends on the combination and ratio of encapsulating materials. When only WPI (formulations 1.4 and 6.5) is used, the particle size ranged between 13 and 14 µm. In contrast, when AG is used (formulations 3.6 and 7.1), the particle size distribution was higher, ranging from 30 to 40 µm.

These findings are supported by Behboudi-Jobbehdar et al. [49], who demonstrated that higher feed viscosity and reduced atomization airflow led to increased particle diameters in spray-dried *Lactobacillus acidophilus* microcapsules. Similarly, Leylak et al. [50] optimized the WPI-to-gum Arabic ratio. They reported a D_{50} of approximately 6.3 µm at specific spray drying settings, reinforcing the crucial role of wall material formulation on particle size control.

The moisture content of the spray-dried encapsulated *L. acidophilus* ranged from 4.43 to 8.28% with significant differences among all amples. Maintaining low moisture content is crucial for the stability and viability of encapsulated microorganisms used as probiotics during storage. Recent studies have emphasized that both moisture content and water activity (Aw) are critical parameters influencing probiotic stability. Moisture content below 4% and Aw below 0.3 are recommended to ensure optimal stability [51–53]. In this work, the sample with the best encapsulation efficiency was formulation 9.9, which contained 4.43% moisture (Table 2). Our findings align with those of Arepally et al. [3], who reported moisture contents ranging from 4.59 to 9.05% in probiotic-containing powders, which are within the recommended range for enhance stability during storage.

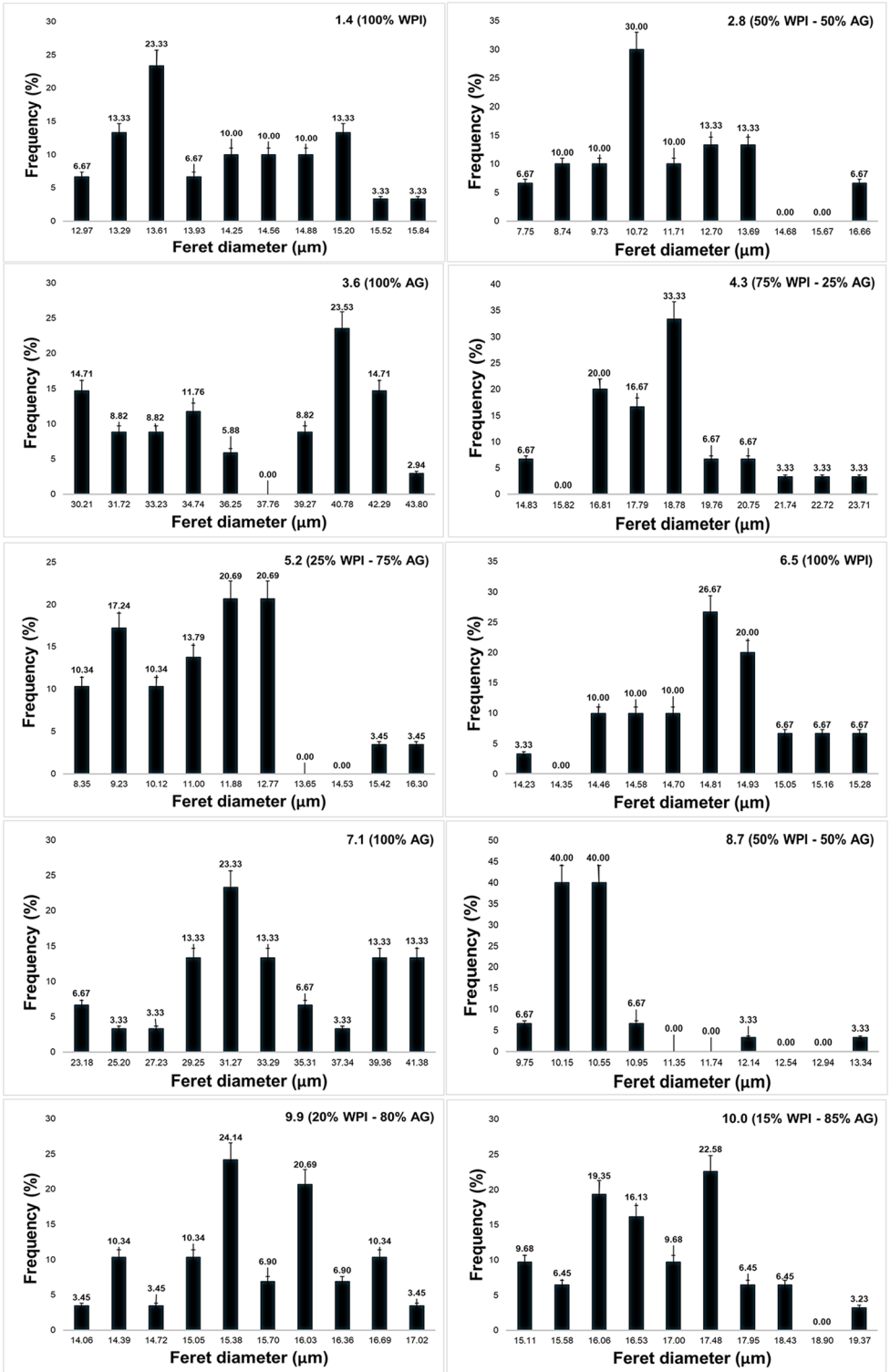

Fig. 3 Particle size distribution of microcapsules obtained by spray drying

Unlike moisture content, Aw indicates the availability of water for microbial growth and chemical reactions. Maintaining Aw below 0.3 ensure probiotic stability during storage. In this study, we evaluated the moisture content and survivability of encapsulated *L. acidophilus* under both low (0.11) and high (0.94) Aw conditions over a 12-week storage period at 15 °C.

Freshly prepared microcapsules via spray drying had a moisture content of 4.43% (Table 2) and a survivability of 3 log UFC/g. After 12 weeks of storage at Aw 0.11 (moisture content: 10.10%), survivability remained at 3 log UFC/g,

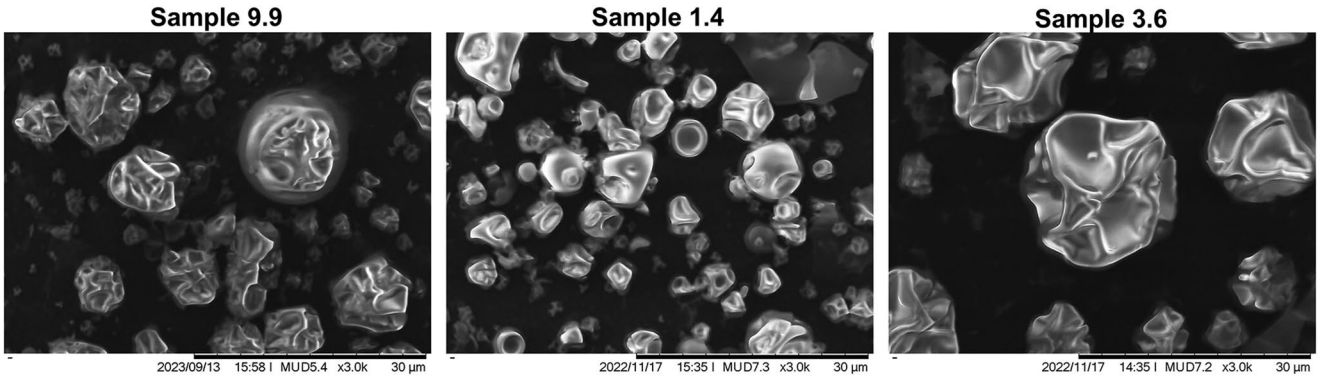

Fig. 4 Micrographs of microcapsules obtained by spray drying (Sample 9.9: 80% Arabic gum, and 20% whey protein isolate; Sample 1.4: 100% whey protein isolate and Sample 3.6: 100% Arabic gum)

whereas at Aw 0.94 (moisture content: 68.20%), no viable cells were detected. In contrast, freshly prepared microcapsules via ionic gelation had a moisture content of 64.65% (Table 1) and a survivability of 2 log CFU/g. After 12 weeks, survivability decreased to 1 log CFU/g at Aw 0.11 (moisture content: 27.03%) but unexpectedly increased to 4 log CFU/g at Aw 0.94 (moisture content: 62.32%). This increase may be attributed to high water availability promoting metabolic activity or reactivation of sub-lethally injured cells in the high-moisture environment.

Regarding morphology (Fig. 4), in general, the spray-dried microcapsules exhibited an oval, irregular, and collapsed shape, with variable sizes with dents but no evidence of cracks or fissures was observed, which is important to guarantee the protection of encapsulated *L. acidophilus* [12]. These findings are aligned with the results of [22], who concluded that spray drying is a complex process and together with the encapsulating materials plays a crucial role in the final morphology of the microcapsules.

A slight morphological difference was observed between sample 9.9 (80% AG and 20% WPI) and sample 1.4. (100% WPI) which seems to be smaller and showed less collapsed surface, probably due to the properties of proteins in the WPI wall material. These proteins can interact with the cell wall of the microorganisms used as probiotics and provide thermal protection during the spray drying process, minimizing collapsing [46]. Colín-Cruz et al. [12] reported that the incorporation of whey protein concentrate by spray drying produced smaller microcapsules due to the interaction of proteins present in the WPC.

As mentioned before, surface characteristics, such as smoothness and porosity, play a critical role in determining probiotic survival rates [42], since a smooth and regular surface tends to provide better protection by minimizing oxygen diffusion and moisture penetration, thereby reducing oxidative stress and maintaining cell viability over time [42]. These results emphasize the importance of optimizing spray-drying parameters to achieve a balance between structural integrity and functional properties for enhanced probiotic viability.

Table 3 Summary of the ANOVA for regression of the possible models describing the behavior of encapsulation efficiency using the ionic gelation method

Model Source	Regression *p*-value	Lack of fit *p*-value	Adjusted R^2
Linear	0.4399	0.1281	-0.0179
Quadratic	0.0220	0.4241	0.5536
Special Cubic	0.8472	0.3191	0.4927

Encapsulation efficiency after capsules formation

Ionic gelation

To compare the protective efficiency of capsules produced for *Lactobacillus acidophilus*, EE was measured, and an ANOVA for the regression was performed (Table 3). Only the quadratic model was statistically significant. Data were best fitted by the following reduced quadratic model (Adjusted $R^2 = 0.7173$):

$$EE\ (\%) = 0.809(SA) + 3.9497(WPI) + 0.5916(AG) - 0.0728(SA*WPI) + 0.05(WPI*AG).$$

All terms were significant ($p \leq 0.05$) except for term SA*AG ($p = 0.6422$) interaction term, which was excluded from the final model. After running the numerical optimization module of the software, the highest predicted encapsulation efficiency (94.165%) was obtained when the SA (50%) was blended with 25% AG and 25% WPI. Experimentally, this optimal combination was tested in runs 6.12 and 12.2 yielding an average encapsulation efficiency of 92.5 ± 1.5% (Table 1), thus, no cross-validation tests were necessary.

These results indicate that the selected biopolymer mixture was adequate for maintaining high survival rates of encapsulated *L. acidophilus*, suggesting an affinity among the three biopolymers, probably due to different kinds of

interactions taking place at the average pH value of 6.6 in this study. The interactions between Arabic gum and alginate have been studied by Sabet et al. [54] who demonstrated that these biopolymers (both having a net negative charge but containing positively charged amino acids) are able to interact at pH 4 and 7, suggesting similar behavior at pH 6.6. The authors proposed electrostatics interaction between the local positive charges of the arabinogalactan protein and the glycoprotein fractions of Arabic gum with the negatively charged carboxylate groups in alginate. Liu et al. [16] studied interactions between WPI and alginate using molecular docking demonstrating that the amino acid residues of β-lactoglobulin (the main protein responsible for interaction with polysaccharides) can form complexes with carboxyl and hydroxyl groups of SA. Electrostatic interactions (hydrogen bonding and van der Waals forces), play a key role in the formation of β-lactoglobulin-sodium alginate complexes mainly in the pH interval from 2 to 5. At pH 6.6, interactions between WPI and alginate may be relatively weak, indicating that although WPI contributes to the encapsulation matrix, its binding affinity with alginate is likely reduced under near-neutral conditions compared to more acidic environments. Additionally, temperature and polymer concentration are critical factors influencing encapsulation efficiency. The ionic gelation process is highly sensitive to temperature, which affects both the polymers' solubilities and the gel formation kinetics. Elevated temperatures can also lead to protein denaturation, thereby compromising the viability of encapsulated probiotics. Similarly, the concentration of encapsulating biopolymers plays a key role in determining the structural integrity, mechanical stability, and functional performance of the resulting microcapsules.

Erben et al. [47] investigated the interactions among whey protein, Arabic gum, and alginate in bio-based films, revealing that the polysaccharide mixture exhibited only weak associations with proteins. Such weak interactions may compromise encapsulation stability, leading to phase separation and structural heterogeneity. A higher concentration of WPI improves EE by promoting a more cohesive network with biopolymers, reducing permeability and enhancing mechanical resistance. In contrast, excessive AG content can result in irregular microcapsule structures due to its high solubility and weak protein interactions, potentially affecting the protective matrix surrounding the encapsulated compounds [16, 47, 54].

To the best of our knowledge, this study is the first research about the effects of different combinations of these three biopolymers on the encapsulation of probiotics. This is important because previous studies have demonstrated that the interactions taking place when mixing sodium alginate with other biopolymers enhance the structure of the wall material improving the survival of the encapsulated microorganism used as probiotic [30, 55, 56]. In contrast, the lowest encapsulation efficiencies were obtained in samples 4.4 (75% SA and 25% WPI), 8.11 (83.3% SA, 8.3% WPI and 8.3% AG), and 2.8 (50% SA and 50% WPI) with values of 31, 31, and 44%, respectively. In those samples, there was no Arabic gum, or it was in a low percentage. Sample 11.6 (50% SA and 50% AG) achieved an EE of 86%, indicating a better interaction between SA and AG compared to SA-WPI combinations [47, 54, 57].

Table 4 Summary of the ANOVA for regression of the possible models describing the behavior of encapsulation efficiency using the spray drying method

Model Source	Regression *p*-value	Lack of fit *p*-value	Adjusted R^2
Linear	0.3434	0.0003	0.0016
Quadratic	0.3156	0.0003	0.0221
Cubic	0.0112	0.0010	0.6404
Quartic	<0.0001	0.3728	0.9229

Spray drying

To compare the EE of capsules produced for *Lactobacillus acidophilus*, an ANOVA for the regression was performed for the spray drying method (Table 4). The quartic model was selected as the best fit as it had the highest R2 value and a non-significant lack of fit.

The data were best fitted by the following quartic model (Adjusted $R^2 = 0.9884$):

$$\begin{aligned}\text{EE } (\%) =& 11.7\,(WPI) + 3.195\,(AG) + 34.03\,(WPI * AG) \\ & - 363.107(WPI * AG * (WPI - AG) \\ & + 839.827(WPI * AG * (WPI - AG)2)\end{aligned}$$

The highest predicted EE (93.573%) was obtained with a biopolymer mixture of 80% AG and 20% WPI. Experimentally, this combination (sample 9.9) yielding an average EE of 94% (Table 2) so, no cross-validation was necessary.

The model indicates significant interactions between AG and WPI [44, 47], probably derived from local positively charged domains (pH 6.6) on the WPI that neutralize some negatively charged domains in AG due to their polarity, and electrostatic interactions between the carboxyl groups of AG, and the amino groups of WPI, so the polysaccharide-protein covalent conjugates could be generated [58]. These findings indicate that pH impacted EE by affecting the electrostatic interactions between AG and WPI. An optimal pH range (6.0–7.0) was necessary to maintain structural integrity and prevent phase separation during encapsulation [46, 47, 59].

Additionally, polymer concentration significantly influences EE. Arepally et al. [3] found that EE increased when Arabic gum concentration increased, attributed to

the presence of proteins and fibers in AG. Proteins in AG form a protective layer on probiotic microorganisms, while fiber provides partial substitution of water molecules in the microorganisms preventing cell membrane injury. Since AG is considered a prebiotic, it may be used as a source of carbon for probiotic metabolism, as adding prebiotics usually results in higher encapsulation efficiency [3, 55, 60]. Furthermore, the use of dairy proteins (like WPI) as wall materials offers thermal protection to probiotic microorganisms through interactions between WPI and the probiotics cell surface components, minimizing injury and consequently increasing probiotic survival [12, 46, 61]. Temperature plays a critical role in determining encapsulation success. Studies suggest that inlet temperatures above 150 °C may denature proteins and reduce probiotic viability, while temperatures below 100 °C may lead to incomplete drying and lower stability [46, 59].

The lowest encapsulation efficiencies were found in samples 3.6, 7.1 (100% AG), and 1.4 (100% WPI) with values of 2, 4, and 9%, respectively, indicating that the biopolymers alone did not enhance *L. acidophilus* survival after the spray drying process. This agreed with the conclusions of other researchers, who stated that combining at least two encapsulating materials enhances probiotic survivability and viability under simulated gastrointestinal conditions. They also found a higher viability of encapsulated probiotic cells compared to free cells under gastrointestinal conditions, which might be due to the modifications and interactions taking place within wall material structures depending on the encapsulation method parameters [3, 6, 12, 18]. These findings underscore the importance of optimizing pH and polymer concentration to maximize EE. Future research should investigate the effects of temperature variations and further refine encapsulation parameters to enhance stability and targeted delivery of microorganisms used as probiotics.

In vitro simulated Gastrointestinal digestion

Figures 5 and 6 show the degree of hydrolysis (DH) during the in vitro gastric and intestinal stage digestion of pure biopolymers, the capsules with the highest EE for each method, and free bacteria (*Lactobacillus acidophilus)*. The highest DH (3.4%) in the gastric stage was observed for WPI. This is expected, as pepsin, the primary enzyme active in this stage, to hydrolyze peptide bonds between aromatic amino acids such as tyrosine and phenylalanine [62]. Conversely, the lowest DH (0.6%) was recorded for AG, which contains approximately 2% protein in its composition [47].

For all samples, the DH in the gastric stage remained below 3.5%, which is a positive result, as an active ingredient release in the stomach should be limited to less than 10% [63]. Notably, the SA sample exhibited no hydrolysis after 100 min, consistent with findings by Samuels et al. [64], who reported that alginate can sequester pepsin and bile salts, thereby inhibiting pepsin activity. This property

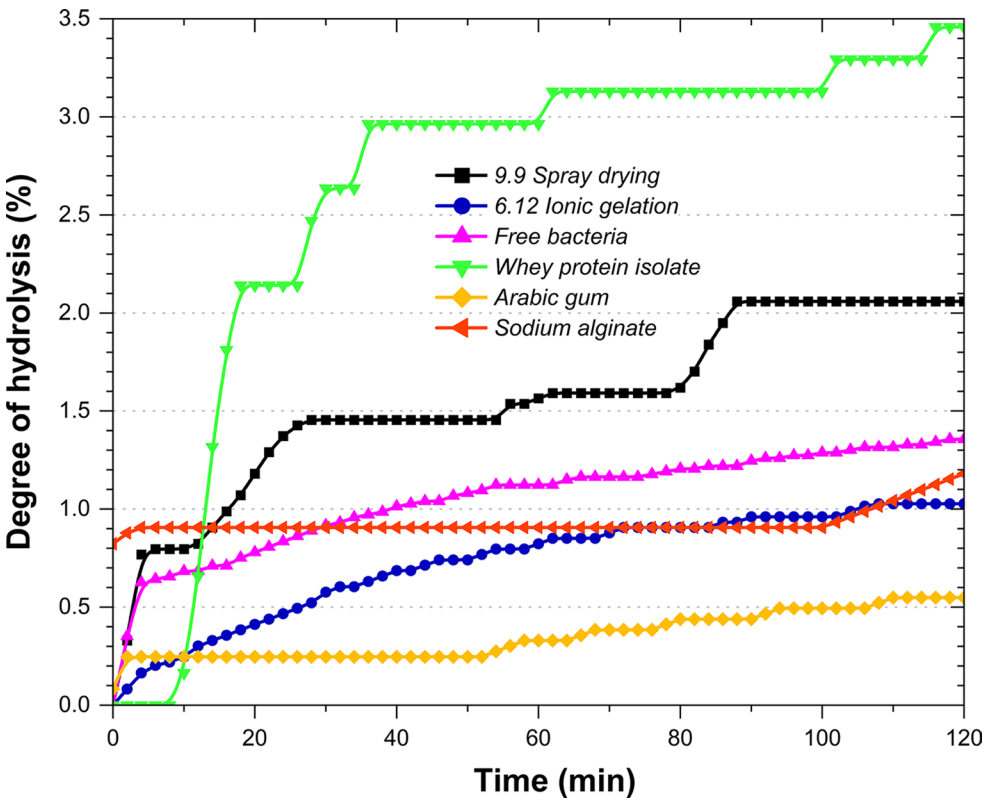

Fig. 5 Degree of hydrolysis during in vitro gastric stage digestion of capsules (9.9 Spray drying and 6.12 Ionic gelation), biopolymers used (Whey protein isolate, Arabic gum, and sodium alginate), and free bacteria (*Lactobacillus acidophilus)*

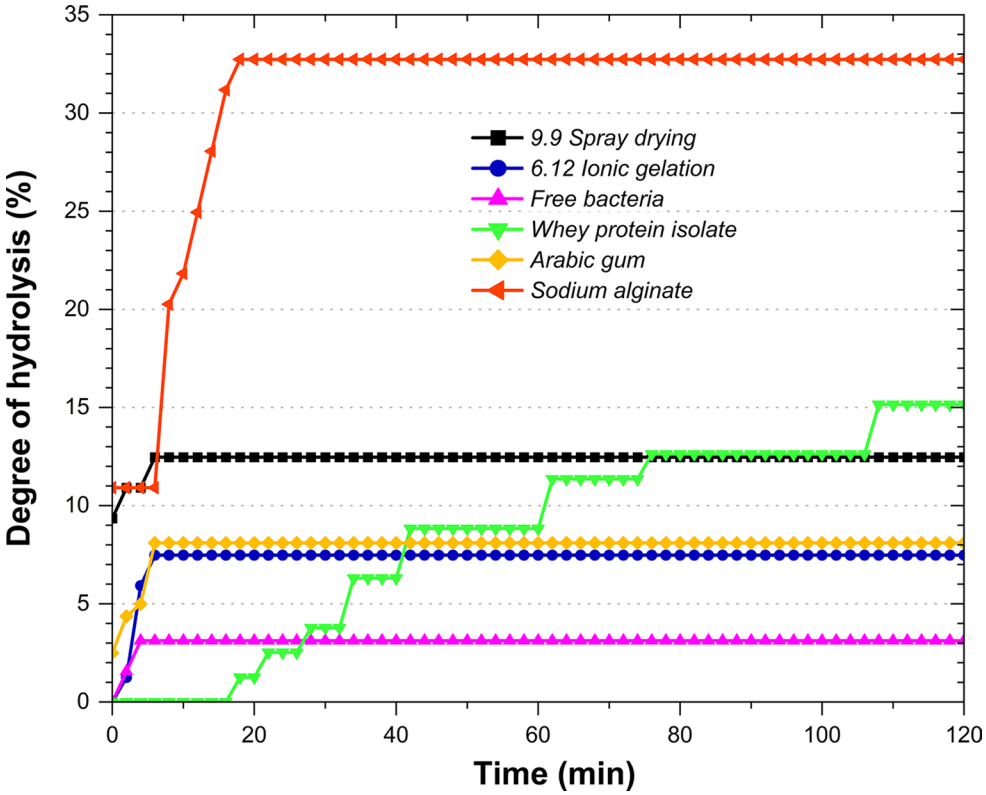

Fig. 6 Degree of hydrolysis during in vitro intestinal stage digestion of capsules (Samples 9.9 Spray drying and 6.12 Ionic gelation), biopolymers used (Whey protein isolate, Arabic gum, and sodium alginate) and free bacteria (*Lactobacillus acidophilus)*

is leveraged in gastroesophageal reflux treatment, where SA provides a protective effect.

During the intestinal stage, an overall increase in DH was observed, which is a desirable characteristic since probiotics exert their beneficial effects mainly in the intestine. The highest DH was recorded for SA (32.74%), aligning with literature reports that indicate progressive SA gel degradation due to pH shifts and pancreatin activity [65]. The lowest DH was observed in free *Lactobacillus acidophilus* (3.1%) suggesting a limited availability of hydrolyzable molecules in free bacterial cells.

A key aspect of this study is the correlation between biopolymer degradation and probiotic release and viability. The microcapsules produced by spray drying exhibited faster hydrolysis than those obtained through ionic gelation, likely due to their smaller particle size (10.52 to 36.27 µm) and consequently larger surface area compared to ionic gelation capsules (2.84 to 3.62 mm). While this increased degradation rate in spray-dried microcapsules may promote faster probiotic release, it also raises concerns about premature exposure to harsh gastric conditions. Conversely, ionic gelation capsules demonstrated more controlled degradation, indicating a potentially enhanced protective effect in the gastric stage, followed by a more gradual probiotic release in the intestine.

These findings are consistent with previous studies demonstrating that both encapsulation methods and biopolymer composition significantly influence the timing and efficiency of probiotic release. For example, a study investigating the stability of probiotics encapsulated in alginate, whey protein, and pectin microspheres reported that biopolymer composition plays a crucial role in maintaining probiotic viability during gastrointestinal transit. Specifically, smaller pore sizes and higher encapsulation efficiencies were associated with enhanced protection of probiotic cells [17]. Additionally, research works on biopolymer hydrogels have highlighted their capacity to improve probiotic viability and modulate release kinetics. Materials such as alginate and whey protein have been shown to regulate degradation rates and facilitate targeted delivery of microorganisms used as probiotics to the intestinal environment [66].

Reducing sugars test

Figure 7 shows the reducing sugars released before and after the in vitro digestion assays from free *L. acidophilus*, the biopolymers used to prepare capsules, and the capsules with the highest EE (from ionic gelation and spray drying methods). The highest value for reducing sugars was observed after the intestinal digestion assay. This is expected, since SIF contains pancreatin, which is composed of proteases, α-amylase, and lipases, responsible for the digestion of proteins, carbohydrates, and lipids, respectively. The degree of proteolysis from this enzymatic preparation was less than

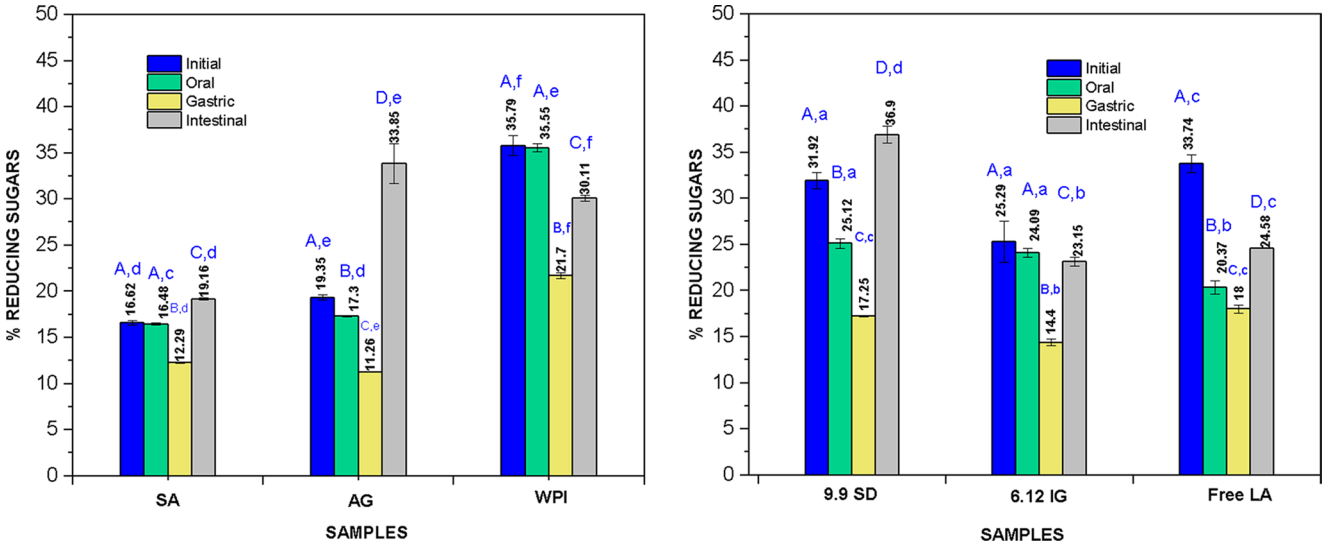

Fig. 7 Reducing sugars results before and after in vitro digestion assay of biopolymers used (SA: Sodium alginate, AG: Arabic gum, and WPI: Whey protein isolate), obtained capsules (9.9 SD: Spray drying and 6.12 IG: Ionic gelation), and free LA: Lactobacillus acidophilus

13% (Fig. 6), thus the pancreatin has predominantly a glycolytic effect on the capsules.

The spray drying method yielded a higher value for reducing sugars release compared to the ionic gelation method. This difference is due to the composition of the spray-dried microcapsules, which are constituted mainly of carbohydrates (80% AG). Additionally, the capsules prepared by ionic gelation are composed of 75% carbohydrates (SA 50% and 25% AG), and the SIF solution (pancreatin enzyme) resulted in a high value of reducing sugars (Fig. 7). This suggests that the SIF solution has a greater ability to hydrolyze AG than SA. This is why in biopolymers, a higher reducing sugars values was found in AG (19.35%) compared to SA (16.62%). Chemically, AG is composed of (1–3)-linked β-D-Galactose units and (1–6)-linked branches containing L-Arabinose in pyranose and furanose ring forms, L-rhamnose, β-D-glucuronate, and 4-O-methyl-β-D-glucuronate as single monomeric units or as oligosaccharide side chains. Also, a small amount (approximately 4%) of uronic acids (glucuronic acid) has been reported [48, 67]. In contrast, SA is a linear copolymer composed of (1→4)-linked α-L-guluronic (G) and β- D-mannuronic (M) residues [67]. WPI contains 3% lactose, which contributed to the high value of reducing sugars in the capsules. As observed in Table 2, the high content of AG and WPI in sample 9.9 (80%AG + 20%WPI) significantly contributed to the survival of *L. acidophilus*, because sugars are a carbon source for bacterial metabolism [68–71].

Free *Lactobacillus acidophilus* also showed a high value of reducing sugars (not significantly different from WPI). This could be attributed to the peptidoglycan layer that makes up the cell membrane [72], suggesting that sugars are being released as a result of degradation during the in vitro assay. The lowest values for reducing sugars were found in the gastric stage assay (Fig. 7). The SGF solution is composed of pepsin and has a pH of 3.0, primarily hydrolyzing peptide bonds, as discussed in Sect. 3.3. Any glycolysis in this stage is attributed to the acid pH [73], which promotes the hydrolysis of macromolecules or disaccharides into monosaccharides.

Correlation of the survival of free and encapsulated *Lactobacillus acidophilus* after exposure to simulated Gastrointestinal conditions

Table 5 summarizes the results of *Lactobacillus acidophilus* encapsulated by ionic gelation and spray drying, along with free bacteria as a control. Despite both encapsulation methods achieving similar EE after production (94%), there was a significant difference in survival between them after exposure to in vitro digestion. In the oral stage assay, both encapsulation methods showed a non-significant decrease in EE compared to their initial values after obtaining the capsules.

Lactobacillus acidophilus encapsulated by ionic gelation showed significantly lower survival rates (1% after the gastric stage and 3% after the intestinal stage) than microcapsules obtained by spray drying. These results indicate that the spray drying method offers better protection to *Lactobacillus acidophilus* against the harsh conditions in the gastrointestinal tract. The presence of proteins in the wall material might have contributed to the stability of the intracellular proteins of the bacterial strain cells during the drying process [3]. This is confirmed by SEM images in Fig. 4 where spray-dried microcapsules show no evidence of cracks or

Table 5 Encapsulation efficiency of the capsules obtained and free *Lactobacillus acidophilus* after in vitro Gastrointestinal digestion

Sample	Encapsulation method	Biopolymers	EE after production (%)	Survival after oral stage (%)	Survival after gastric stage (%)	Survival after intestinal stage (%)
6.12	Ionic gelation	50% SA 25% WPI 25% AG	94 ± 0.01^{Aa}	80 ± 0.01^{Ab}	1 ± 0.06^{Ac}	3 ± 0.02^{Ad}
9.9	Spray drying	80% AG 20% WPI	94 ± 0.01^{Aa}	74 ± 0.02^{Bb}	45 ± 0.06^{Bc}	36 ± 0.03^{Bd}
Free *LA*	NA	NA	NA	52 ± 0.02^{Ca}	4 ± 0.02^{Cb}	1 ± 0.08^{Cc}

**LA*: *Lactobacillus acidophilus*; SA: sodium alginate; WPI: whey protein isolate; AG: Arabic gum; EE: encapsulation efficiency; NA: Not applicable

**Results are presented as means ± SD ($n = 3$)

***Values with different capital letters in the same column indicate a significant difference ($p \leq 0.05$)

**** Values with different letters in the same row indicate a significant difference ($p \leq 0.05$)

fissures, an important factor to guarantee the protection of encapsulated microorganisms used as probiotics.

As evidenced in Table 5, the spray drying method, using a combination of 80% AG and 20% WPI as encapsulating materials, not only presented a high encapsulation efficiency immediately after the process but also reached the highest survival rate of *L. acidophilus* after in vitro digestion. Conversely, in ionic gelation method, despite the high initial EE (94%), survivability decreased dramatically during the gastric stage assay. As shown in Table 5, only 1% of free *Lactobacillus acidophilus* reached the intestine, which would reduce its bioaccessibility. Therefore, the encapsulating materials provide protection, delaying the diffusion of simulated gastrointestinal fluids into the capsules.

It has been hypothesized [74] that the protective mechanism of WPI for lactic acid bacteria is due to the hydrophobic domains of the unfolded ß-lactoglobulin fraction of WPI. During spray drying, these domains interact hydrophobically with the cell surface of the microorganism, followed by consolidation of the interface between the cells and WPI so, the higher the cell surface hydrophobicity (CSH), the higher the survival of the microorganism. In our study, an outlet temperature of 76ºC is known to induce the denaturation of 30% of the proteins in WPI, resulting in the unfolding of the ß-lactoglobulin fraction [75]. Bevilacqua et al. [76] also indicated that low pH values (the pH of fermented MRS broth is <4), as well as incubation temperatures of 37 °C, increased CSH in *Lactobacillus acidophilus* LA-5. Similarly, Shakirova et al. [77] found that the ability of LA-5 to survive under adverse conditions correlates with its CSH values.

The diffusion behavior of microcapsules can be attributed to the composition of the formulation [78], and their pore size. It has been well-established that hydrogel composition significantly influences pore size distribution [79]. Another critical factor affecting pore size is the encapsulation method. Hydrogels produced via ionic gelation using similar biopolymers typically exhibit pore diameters ranging from 5 to 50 nm, encompassing both micropores and mesopores. In contrast, microcapsules generated through spray drying tend to form structures with a predominant pore size of around 12.5 nm, primarily consisting of micropores [56, 79–82].

It is widely known that the release of encapsulated compounds from microcapsules may occur via two primary mechanisms: diffusion through the matrix or matrix erosion [79, 80]. In this study, the superior performance of spray-dried microcapsules under simulated gastrointestinal conditions, compared to those produced by ionic gelation, is likely attributable to differences in the dominant release mechanisms. Specifically, in the gastric stage (pH ~ 3), spray-dried microcapsules undergo structural breakdown and matrix erosion, facilitating the release of their contents. In contrast, the release from ionic gelation microcapsules is primarily driven by diffusion processes through their mesoporous structures [9].

The present work provides strong evidence that the encapsulating materials and processing conditions significantly influence the size and morphology of capsules, impacting the survival of *Lactobacillus acidophilus* both during the encapsulation process and under simulated gastrointestinal conditions.

Conclusion

This study evaluated the encapsulation of *Lactobacillus acidophilus* LA-5 using ionic gelation and spray drying. Both methods achieved comparable initial encapsulation efficiencies (~94%). However, microcapsules produced via spray drying, using a matrix composed of 80% Arabic gum and 20% whey protein isolate, exhibited superior probiotic survival after exposure to simulated gastrointestinal digestion. This enhanced protection was primarily attributed to the

presence of Arabic gum, which is recognized as a prebiotic that positively supports the metabolism of lactic acid bacteria. Our in vitro digestion studies revealed that carbohydrate hydrolysis played a more significant role in probiotic release than protein degradation. Key factors such as pH, moisture content, particle size, morphology, biopolymer composition, and matrix ratio significantly influenced probiotic viability and release dynamics. These findings highlight the critical role of both encapsulation method and biopolymer selection in ensuring probiotic protection and controlled release, ultimately impacting their ability to confer health benefits within the human gastrointestinal tract.

From an industrial perspective, spray drying offers a scalable and economically viable approach for probiotic encapsulation, although optimization of process parameters is necessary to minimize viability loss. In contrast, while ionic gelation is a gentler technique, it may be constrained by higher production costs and longer processing times. Further research should aim to enhance storage stability, improve release efficiency, and conduct comprehensive cost-benefit analyses to support the commercial application of these encapsulation technologies.

Future perspectives

Despite the promising results, certain limitations of this study must be acknowledged. Although in vitro digestion models offer valuable insights into the mechanisms of probiotic release, they do not fully replicate the complexity of in vivo gastrointestinal conditions, such as interactions with the gut microbiota, immune responses, and peristaltic activity. Furthermore, differences in storage stability between the encapsulation techniques were not thoroughly investigated, which could influence the long-term viability of probiotics in real-world applications, such as dairy products, functional beverages, and dietary supplements. Future research should incorporate comprehensive storage stability evaluations and in vivo studies to accurately assess the effectiveness of these encapsulation strategies in maintaining probiotic viability and functionality under physiological conditions.

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Declarations

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