Novel Strategies for enhancing the Stability and Efficiency of Probiotics for Gut Microbiota Support

In recent years, a major limitation of commercially available probiotics relates to maintaining cellular viability, the development of methods to overcome these challenges has become a topic of significant scientific interest. The aim of this study was to encapsulate Lacticaseibacillus casei (L. casei), Lacticaseibacillus rhamnosus (L. rhamnosus), Lactiplantibacillus plantarum (L. plantarum), and Bacillus clausii (B. clausii) strains, well -known probiotics for their beneficial health effect, within sodium alginate and starch matrices. Capsules developed using the extrusion method were evaluated in terms of microstructure, diameter, color, antioxidant activity, as well as encapsulation efficiency and probiotic survival in simulated gastric and intestinal fluids. The capsule diameter was relatively constant, ranging between 231.12 and 268.14 µm. Microstructural evaluation revealed a compact matrix. According to the results, the highest luminosity was observed in case of L. plantarum capsules. Encapsulation efficiency was over 91%. All formulations maintained their structural integrity throughout solubility testing. Results obtained in simulated gastrointestinal fluids demonstrated the resistance of biopolymeric coating under gastric conditions, allowing release in intestinal environments. Thus, the proposed encapsulation system is suitable for the development of targeted and sustained-release capsules.

Keywords: *Lacticaseibacillus casei; Lacticaseibacillus rhamnosus; Lactiplantibacillus plantarum; Bacillus clausii; biopolymers, dysbiosis.*

Introduction

Lately, due to their potential health-promoting benefits, probiotics have attracted scientific and technological interest. Defined by World Health Organization as live microorganisms that with beneficial effects when administered in sufficient amounts, they mainly consist of bacteria from gut microbiota (the largest microbiota from human organism). Scientific results presented that probiotics may contribute to the management of inflammatory bowel disease, metabolic, neurological and organs' disorders, and cancer. Unfortunately, the largest part of probiotics is sensitive to the harsh conditions encountered in the digestive tract (Shoukat, 2024).

Lacticaseibacillus casei (L. casei) is a gram-positive bacterium, commonly used as probiotics due to their health benefits in allergic disease, brain function, metabolic disorders (obesity and diabetes mellitus), and cancer (Hill, 2018). Due to their facultative heterofermentative metabolism, L. casei naturally tolerate acidic conditions. However, during gastrointestinal transit, the bacteria are exposed to strong gastric acidity, which can lower intracellular pH, damage proteins and DNA (Mills, 2011).

Lacticaseibacillus rhamnosus (L. rhamnosus) is a gram-positive bacterium, commonly found in human digestive and urogenital tracts. Due to its ability to survive in acidic conditions, it is widely used as probiotic to support gut microbiota. Furthermore, the studies indicated that it provides health benefits in different diseases: it may alleviate clinical symptoms of children's dermatitis, modulate gut microbiota to improve symptoms of patients with gastrointestinal pathologies, and improved diabetes mellitus or obesity (Yang, 2026).

Lactiplantibacillus plantarum (L. plantarum) is a gram-positive lactic acid bacterium, widely applied in the food industry, offering multiple health benefits. It is associated with antioxidant, antidiabetic, anti-obesity, anti-inflammatory, antiviral, and antidepressant effects, making it valuable for promoting overall well-being and supporting functional foods (Zare, 2024). In addition, this bacterium can inhibit the growth of food pathogens and extend the shelf life of bio-processed products due to its natural antimicrobial properties. *L. plantarum* also contributes to improving nutritional quality and taste, reducing undesirable compounds, increasing antioxidant and antimicrobial activity, and extending the shelf life of foods (Ge, 2021).

Bacillus clausii (B. clausii) is a gram-positive bacterium, widely used in medical in food industries as probiotic due to its capability to produce heat-resisting endospores, which allow it to survive in acidic conditions of the stomach. *In vitro* and *in vivo* studies highlighted the health benefits of *B. clausii*. Thus, its strains enhanced gut barrier function, contributed to gut homeostasis, presented antimicrobial and immunomodulatory activity, and relieve gastrointestinal distress (Ghelardi, 2022).

Many probiotics, including *Lactobacillus*, are very sensitive to environmental stressors, such as exposure to oxygen, temperature variations, which significantly reduce their viability during production and storage. In addition, most probiotics lose their ability to survive in the highly acidic environment of the stomach ($pH \leq 2.5$) (Xie, 2025). Therefore, different biopolymers have been tested as encapsulating substances to preserve cell viability and the beneficial effects of probiotic consumption.

Sodium alginate is an edible biopolymer, a biodegradable and biocompatible polysaccharide with low toxicity, non-immunogenic and non-allergenic properties. Due to its properties, it is widely used in the development of edible coatings, with good results in probiotics encapsulation (Wang, 2022).

Starch is a cost-effective biopolymer widely used for the encapsulation of bioactive compounds. Studies' results highlighted its ability to enhance the survival of probiotics in food biosystems or *in vitro* conditions. In this regard, starch molecules have been extensively used as encapsulation materials for probiotics or pharmaceutical formulations (Shoukat, 2024).

Our previous results evidenced the ability of sodium alginate and starch to develop a suitable matrix for encapsulation of bioactive compounds, respectively probiotics (Gheorghita, 2026). The present study aims to encapsulate four strains used for their probiotic potential, but susceptible to environmental conditions during manufacture, storage, and transit through the gastrointestinal tract.

Materials and Methods

Analytical grade sodium alginate (medium viscosity, ≥2000 cP at 2% at 25 °C), starch from wheat, simulated gastric fluids without enzymes, phosphate buffer saline (PBS, pH 7.2-7.6), and distilled water were purchased from Sigma Aldrich Romania. *L. casei* ATCC 393, *L. rhamnosus* ATCC 7469, *L. plantarum* ATCC 8014, and *B.*

clausii (Sun Wave Pharma, Romanian Distribution Branch) were reactivated twice on MRS and Vegitone agar at 37°C for 36 h. The culture obtained after the second reactivation was used for encapsulation in sodium alginate-starch matrices through extrusion method. Thus, in 110 ml distilled water volume, 2500 mg of sodium alginate and 500 mg starch were added. The mixture was homogenized under continuous stirring (500 rpm) and maintained for 10 minutes at 60 ± 3°C. Simultaneously, separately suspensions of probiotics were prepared and adjusted at 5 McFarland standard, corresponding to approximately 1.57 × 10^9 CFU/mL. 10 ml of each suspension was added in coating forming solution, after prior cooling at 30°C. The new solution thus developed was stirred for 10 more minutes at 500 rpm. The capsules were obtained using a Caviar-box system. Thus, the resulting mixture was extruded into a 5% (w/v) $CaCl_2$ solution and left for 10 minutes in order to allow coat formation. The capsules were washed with distilled water and kept under refrigerated conditions until further analysis.

For the morphological and topographic characterization of the probiotic-loaded biopolymer capsules, an Olympus SZX10 stereomicroscope was initially used, which allowed for detailed observation of the outer surface of the samples. Subsequently, the obtained microscopic images were processed and quantitatively analyzed using the specialized software MarSurf MFM 7.2. Through this three-dimensional micro-topographic analysis, statistical surface texture parameters were extracted, with a focus on the average roughness (Sa, Sq), the profile asymmetry (Ssk) and the degree of arching of the extremities (Sku), thus providing a rigorous assessment of the impact of each bacterial strain on the structure of the polymer matrix. The interactions among the constituents were investigated using Fourier Transform Infrared (FTIR) spectroscopy. Thus, capsules were crushed, mixed with KBr and compressed onto pellets. The infrared spectra were recorded between 1000-1400 cm^{-1} spectral range, allowing the identification of characteristic molecular vibration bands.

A digital Yato micrometer (Shanghai, China), with 0.001 m resolution was used to determine the capsules' diameter. Ten randomly capsules were selected and measured without applying compression. The results were expressed as mean ± standard deviation. A Konika Minolta CR-400 colorimeter was used to evaluate capsules' color. In this regard, a standard white reference plate (L^* = 95.12, a^* = -0.54, and b^*=-3,79) was used to calibrate the equipment. The CIELab color coordinates (L^*, a^*, b^*) were recorded for each formulation, and measurements were performed in ten replicates to ensure accuracy and reproducibility.

The antioxidant activity of the microcapsules was measured by the ABTS assay. This is based on the ability of bioactive compounds to reduce the radical cation ABTS•+, causing a color change directly proportional to the concentration of antioxidants. The protocol used was similar to that presented in other study (Gheorghita, 2024). For this purpose, 100 μg of microcapsules and 1400 μL of ABTS reagent prepared by mixing a solution of 2,2′-Azinobis (3-ethylbenothiazoline-6-sulfonic acid) (ABTS, 7 mM) with a solution of 2.45 mM potassium persulfate in a volumetric ratio of 1:1 (v/v) were incubated in the dark for 30 minutes at room temperature. The samples were centrifuged for 5 minutes at 5000 rpm before measuring the absorbance at 734 nm. Finally, the free radical scavenging activity was expressed as the percentage of free radical inhibition by the sample and was calculated with the following formula:

$$\text{Inhibition (\%)} = [(A_{734control} - A_{734sample}) / A_{734control}] \times 100$$

where $A_{734control}$ is the absorbance of the control sample and $A_{734sample}$ is the absorbance recorded in the presence of the sample extracts. The control sample consisted of 100 µL of distilled water mixed with 1400 µL of ABTS reagent.

The encapsulation efficiency (EE) was evaluated for formulations with probiotics. Thus, 0.2 g freshly prepared capsules were smashed in 9.8 mL sterile sodium citrate solution (1%, pH 6), followed by gentle homogenization at room temperature for 15 minutes. Serial dilutions (1:100) were prepared, and 10 mL of each dilution was inoculated onto Vegitone (*L. casei, L. rhamnosus*, and *L. plantarum* loaded capsules) and MRS agar (*B. clausii* loaded capsules). The plates were incubated at 37°C for 72 hours and EE was calculated according to the formula:

$$\text{EE (\%)} = N_1/N_0 \text{ x } 100$$

where N_0 is the viable cell count in the initial probiotic suspension (before encapsulation) and N_1 corresponds to the number of viable cells from capsules. The results were expressed as CFU/mL.

The survival of encapsulated probiotics under simulated gastrointestinal conditions was evaluated by determining the number of viable cells released form the capsules after subsequential exposure to gastric medium (SGF), intestinal medium (SIF), and cumulative gastric and intestinal medium (SGF + SIF). Thus, 0.2 g capsules were suspended in 9.8 mL simulated or intestinal simulated fluids. The mixture was maintained one hour in each experimental condition, with agitation at each 10-15 minute to promote the release of cells form matrix. Serial dilutions were prepared and form the final step; 1 mL aliquots were spread on culture medium and incubated at 37°C for 72 h. The results were expressed as percentage of survival rate (SR), according to the following formula:

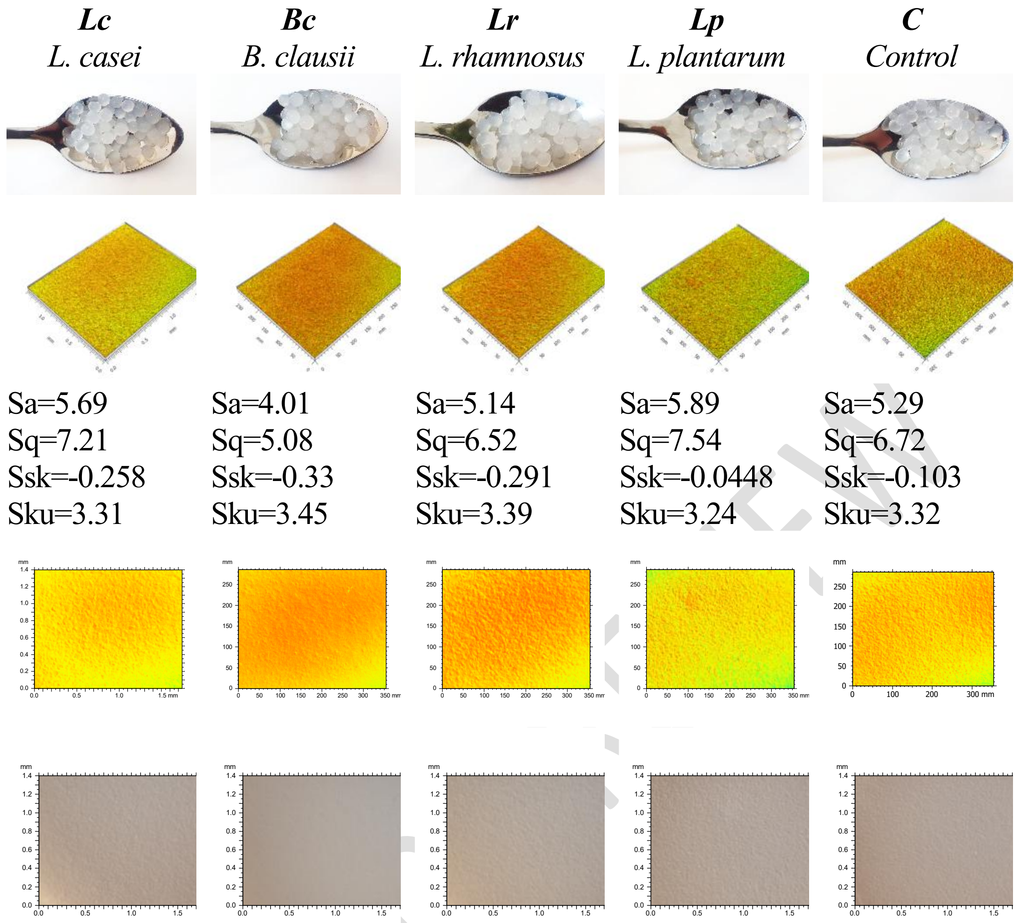
$$\text{SR (\%)} = N_t/N_0 \text{ x } 100$$

where N_t – number of viable cells after exposure to gastric, intestinal or cumulative gastrointestinal conditions, and N_0 represents the initial number of viable cells, before exposure.

Results

The image and microstructure of capsules are presented in Figure 1.

Figure 1. *Microstructure characteristics of developed capsules*



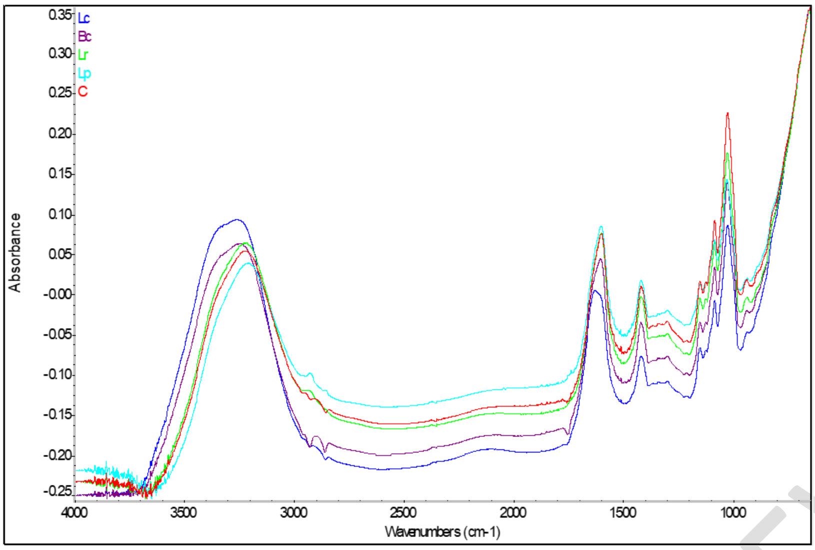
To ensure the statistical representativeness and reproducibility of the micro-topographic data, 4 randomly selected areas on the surface of each type of polymer capsule were analyzed. The results of the quantitative analysis, expressed as mean and standard deviation (Mean ± SD, n = 4) for the parameters of arithmetic mean roughness (Sa), profile asymmetry (Ssk) and height distribution excess (Sku), are summarized in Table 1.

Table 1. *Roughness parameters of capsules' micro topographies*

Sample	Probiotic strain	Sa (µm)	Ssk	Sku
Lc	*L. casei*	5.545 ± 0.274	-0.258 ± 0.027	3.547 ± 0.388
Bc	*B. clausii*	4.010 ± 0.041	-0.318 ± 0.055	3.400 ± 0.095
Lr	*L. rhamnosus*	5.275 ± 0.151	-0.277 ± 0.054	3.285 ± 0.099
Lp	*L. plantarum*	5.637 ± 0.334	-0.061 ± 0.034	3.267 ± 0.059
C	Control	5.295 ± 0.103	-0.086 ± 0.032	3.282 ± 0.033

The FTIR spectra recorded for the characterization of the capsules are presented in the following figure.

Figure 2. *FTIR spectra of capsules*



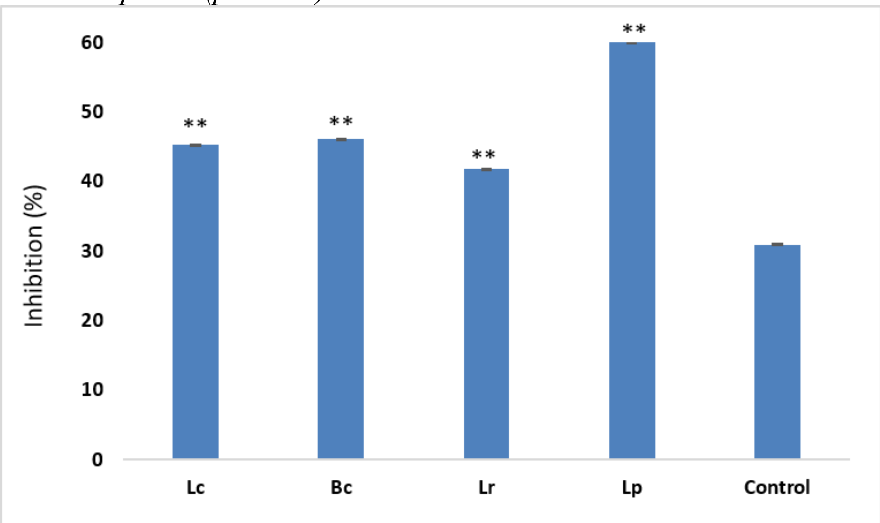
The color attributes and mean diameter of the capsules are presented as mean ± standard deviation in Table 2.

Table 2. *Physical and colorimetric features of capsules*

Sample	Diameter (µm)	Color		
		L*	a*	b*
Lc	231.12 ± 0.35	43.93 ± 0.25	-0.54 ± 0.02	-0.76 ± 0.08
Bc	247.98 ± 0.82	42.92 ± 0.33	-0.55 ± 0.05	-1.48 ± 0.06
Lr	250.27 ± 1.15	43.76 ± 0.12	-0.76 ± 0.02	-1.72 ± 0.06
Lp	242.32 ± 0.35	44.95 ± 0.21	-1.10 ± 0.12	-1.65 ± 0.02
C	268.14 ± 0.66	41.13 ± 0.38	-0.69 ± 0.07	-1.41 ± 0.40

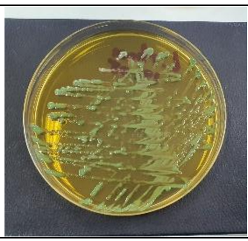
The antioxidant activity measured through ABTS method is presented in Figure 3.

Figure 3. *The antioxidant activity of capsules. Lc – capsules with L. casei; Bc – capsules with B. clausii; Lr – capsules with L. rhamnosus; Lp – capsules with L. plantarum; C – control capsules. The bars are expressed as mean value ± S.D. of triplicate experiments. The ** represent significant differences between probiotic and control capsules (p < 0.01)*



The encapsulation efficiency and survival rate of the probiotic strains after exposure to simulated gastrointestinal fluids are presented as follows:

Table 3. *Encapsulation efficiency and simulated gastrointestinal fluids survival rate*

Sample	EE (%)	SGF (%)	SIF (%)	SGF + SIF (%)
Lc	 91.67 ± 0.98	85.09 ± 0.49	88.01 ± 0.16	87.50 ± 1.06
Bc	 99.55 ± 0.12	90.24 ± 0.97	97.05 ± 0.41	96.08 ± 0.40
Lr		87.93 ± 0.65	90.82 ± 0.89	86.78 ± 0.73

	99.61 ± 0.08			
Lp	 99.28 ± 0.19	88.30 ± 0.93	84.12 ± 1.22	92.80 ± 0.28

Discussion

Comparative analysis of three-dimensional texture parameters (Table 1) reveals that the incorporation of different probiotic strains induces distinct structural changes at the biopolymer surface level, compared to the control sample (C). The parameter Sa (average roughness) indicates a clear variation depending on the bacterial strain used. It is observed that the samples encapsulated with *L. plantarum* (Sample 4: 5.64 ±0.33 µm) and *L. casei* (Sample 1: 5.55 ±0.27 µm) present a higher roughness than the control sample (5.30 ± 0.10 µm). This increase in roughness suggests that the bacterial cells modify the polymer network during the drying process, generating more pronounced local reliefs. In contrast, the sample containing *B. clausii* (Sample 2) recorded a significant decrease in roughness (4.01 ± 0.04 µm), demonstrating a much smoother surface and an extremely low standard deviation, indicating high structural homogeneity and possible denser packing of the biopolymer in the presence of this strain.

Regarding the profile asymmetry (Ssk), all the analyzed samples present strictly negative values, a clear indicator of a topography dominated by valleys, micro-pores or deep channels, on a background of flat plateaus. This feature is typical for hydrogel films or capsules subjected to dehydration. Sample 2 (*B. clausii*) presents the most negative value (Ssk = -0.32 ±0.05), suggesting a higher density of micro-pores on the surface. At the opposite pole, sample 4 (*L. plantarum*) has the value closest to zero (Ssk = -0.06 ±0.03), which indicates a much more symmetrical distribution of heights and depths on the capsule surface. The parameter Sku (kurtosis) presents super unit values compared to the normal distribution (Sku > 3) for all the samples, ranging between 3.27 and 3.55. This leptokurtic distribution demonstrates that the micro-valleys or pores identified by the Ssk parameter have relatively steep and sharp profiles, not rounded ones. Sample 1 (*L. casei*) presents the highest degree of vaulting (Sku = 3.55 ±0.39), suggesting a local presence of structural singularities (very sharp peaks or valleys), a fact also confirmed by the higher standard deviation recorded for this sample.

From a technological and biological point of view, these topographical changes can have a direct impact on the functional properties of the capsules. A rougher and more porous surface (as in the case of Samples 1 and 4) increases the specific contact area, a factor recognized in the literature as beneficial for improving mucoadhesive properties in the gastrointestinal tract and facilitating the transfer of nutrients to the encapsulated bacteria.

According to the FTIR spectra presented in Figure 2, it can be observed slight variations in band intensity and profile compared to the control formulation. These results may indicate possible interactions between the biopolymeric matrix and probiotics. The broad absorption band observed in the 3200-3500 cm^{-1} region is associated with O-H stretching vibrations characteristic to hydroxyl group from alginate and starch. Differences in the intensity of the band among formulations suggest that the probiotic strains can influence hydrogen bonding between polymer network.

The characteristic bands detected around 1600-1650 cm^{-1} vibrations and 1400-1450 cm^{-1} correspond to asymmetric and symmetric groups of carboxylate groups from sodium alginate. Slight intensity variations observed for probiotic capsules indicate electrostatic interactions between bacterial cell surface components and biopolymeric matrix. Variation in the 1000-1200 cm^{-1} region are associated with C-O and C-O-C stretching vibrations of polysaccharides, confirming that each probiotic strain differently affected the structural organization of alginate-starch network, confirming the results presented in Figure 1 and Table 1. Among the tested samples, capsules containing *L. plantarum* and *B. clausii* presented more pronounced modifications, that may indicate stronger interactions with the polymeric matrix compared to the other strains.

The FTIR results confirmed the incorporation of probiotics into biopolymers without major changes to the characteristic structures of alginate and starch.

The capsules' diameter ranged between 231.12 and 268.14 µm (Table 2). The results indicated that the incorporation of different probiotic strains influenced the capsule size. The control samples exhibited the largest diameter, while capsules containing *L. casei* showed the smallest one. These differences may be associated with specific interaction between probiotic cells and the alginate-starch matrix, that can affect gelation behavior and matrix compactness.

Regarding color parameters (Table 2), all formulations presented relatively similar L8, a*, and b* values, suggesting that probiotics' addition did not influence the overall appearance of the capsules. The highest luminosity was observed for *L. plantarum* capsules (44.95), whereas the control sample presented the lowest value (41.13). Negative a* values recorded for all samples highlighted a slight tendency to green hue, while the negative b* values indicated a blue coloration. Capsules with probiotic strains exhibited slightly higher lightness and more pronounced chromatic variations compared to the control formulation. These results may suggest that probiotic incorporation modify the optical properties of the polymer matrix.

Figure 3 presented the antioxidant activity of the capsule samples studied. Among the samples analyzed, *Lp* showed the highest antioxidant activity, reaching an inhibition of approximately 60%. On the other hand, samples *Bc* and *Lc* showed similar antioxidant activities, with inhibition values around 45-46%, while *Lr* showed a slightly lower activity, of approximately 42%. As expected, the control sample showed the lowest antioxidant activity, with an inhibition value of approximately 31%, this effect being attributed exclusively to the structure of the alginate used for encapsulation, known for its antioxidant capacity (Hentati, 2018).

The encapsulation efficiency and the survival rates of probiotic strains under simulated gastric and intestinal conditions are presented in Table 3. All formulations exhibited high encapsulation efficiencies, ranging from 91.67% to 99.61%. These values confirmed the suitability of the alginate-starch matrix for probiotic encapsulation. Among the tested strains, *L. rhamnosus* presented the highest encapsulation efficiency (99.61%), closely followed by *B. clausii* (99.55%) and *L. plantarum* (99.28%). *L. casei* presented a comparative lower value (91.67%).

Exposure to simulated gastric fluids resulted in a reduction in cell viability, regardless the formulation, even if the survival rates remained relatively high (85.09% and 90.24%). Capsules with B. clausii presented the greatest resistance under gastric conditions, that can be attributed to the spore-forming capacity of the microorganism.

Under stimulated intestinal fluid conditions, survival rates between 84.12% to 97.05%. The highest viability was observed for *B. clausii*, as well. These results indicated enhanced tolerance to intestinal stress conditions, while *L. plantarum* presented the lowest survival percentage. Following subsequential exposure to SGF and SIF, probiotic viability remained above 86% for all samples, highlighting the effectiveness of the encapsulation system in preserving probiotic viability.

Conclusions

The obtained results demonstrated that sodium alginate and starch capsules represent an effective system for probiotic encapsulation and protection under simulated gastrointestinal fluids. All tested formulations exhibited high encapsulation efficiency, confirming the synergism between biopolymeric matrix and probiotic strains tested. The addition of probiotics influenced the physical characteristics and the structural organization of the capsules, highlighted by variations in capsules' diameter, color parameters, and FTIR spectral profiles.

Among the tested results, B. clausii presented the highest resistance to simulated gastric and intestinal conditions, maintaining superior survival rates. Overall, the alginate-starch matrix provided sufficient protection to probiotic cells, ensuring high viability after exposure to stress conditions. These findings suggest that the developed biopolymeric capsules have strong potential for application as probiotic delivery systems in pharmaceutical formulation and innovative food.

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