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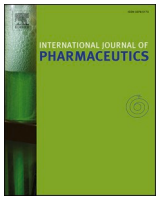

Multistimuli responsive microcapsules produced by the prilling/vibration technique for targeted colonic delivery of probiotics

Vita D'Amico [a], Antonio Lopalco [a], Rosa Maria Iacobazzi [a], Mirco Vacca [b], Sonya Siragusa [b], Maria De Angelis [b], Angela Assunta Lopedota [a,*], Nunzio Denora [a]

[a] Department of Pharmacy-Pharmaceutical Sciences, University of Bari "Aldo Moro", 4, E. Orabona Street, 70125 Bari, Italy
[b] Department of Soil, Plant and Food Sciences, University of Bari "Aldo Moro", 165/A, G. Amendola Street, 70126 Bari, Italy

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ABSTRACT

This study aimed to microencapsulate the probiotic strain *Lactiplantibacillus plantarum* 4S6R (basonym *Lactobacillus plantarum*) in both microcapsules and microspheres by prilling/vibration technique. A specific polymeric mixture, selected for its responsiveness to parallel colonic stimuli, was individuated as a carrier of microparticles. Although the microspheres were consistent with some critical quality parameters, they showed a low encapsulation efficiency and were discarded. The microcapsules produced demonstrated high yields (97.52%) and encapsulation efficiencies (90.06%), with dimensional analysis and SEM studies confirming the desired size morphology and structure. The results of thermal stress tests indicate the ability of the microcapsules to protect the probiotic. Stability studies showed a significant advantage of the microcapsules over non-encapsulated probiotics, with greater stability over time. The release study under simulated gastrointestinal conditions demonstrated the ability of the microcapsules to protect the probiotics from gastric acid and bile salts, ensuring their viability. Examination in a simulated faecal medium revealed the ability of the microcapsules to release the bacteria into the colon, enhancing their beneficial impact on gut health. This research suggests that the selected mixture of reactive polymers holds promise for improving the survival and efficacy of probiotics in the gastrointestinal tract, paving the way for the development of advanced probiotic products.

1. Introduction

The human gut microbiota has a significant role in maintaining human health and wellness, and oral probiotics have been shown to promote a healthier gut microbiota. As such, there has been a significant increase in interest in recent years from companies who have responded to this growing demand by launching a wide range of probiotic and prebiotic products. This interest has been raised due to a heightened awareness of the significance of the gut microbiome for human health.

Probiotics are live microorganisms which, when consumed in sufficient densities, can provide health advantages, and maintain or improve the balance of the gut microbiota (Khalighi et al., 2016). These microorganisms can compete with pathogens, regulate the inflammatory response, produce beneficial metabolites, and promote the microbiota's balance (Wang et al., 2021). Dysbiosis, or the disruption of this balance, leads to negative health effects and the onset of various pathological conditions (Appanna, 2018). Therefore, probiotic supplementation is essential to maintain proper homeostasis, support a healthy gut microbiome, maintain health and prevent disease. Nevertheless, several hurdles persist. Probiotics must be safe for human consumption. Additionally, to effectively facilitate their beneficial actions, it is essential to maintain a minimum viable count of approximately 6 log CFU/mL until they reach their optimal site of colonization (Terpou et al., 2019).

The results of several studies (Dodoo et al., 2017; Naissinger da Silva et al., 2021) investigating the viability of commercial probiotic products have surprisingly shown significantly lower than reported concentrations and deficiencies in the viability of probiotic strains during passage through the gastrointestinal tract (GIT). This raises critical concerns about the feasibility of probiotics in commercial products and suggests that many of the products on the current market may not be as effective as believed, due to their inability to survive the processes of processing and storage (Caillard and Lapointe, 2017; Huff, 2004).

Probiotics are sensitive to external factors such as temperature and

* Corresponding author at: Department of Pharmacy – Pharmaceutical Sciences, University of Bari "Aldo Moro", Orabona St. 4, I-70125 Bari, Italy.
E-mail address: angelaassunta.lopedota@uniba.it (A.A. Lopedota).

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humidity, rendering them unsuitable for long-term storage (Kiepś and Dembczyński, 2022; Wendel, 2022). Furthermore, they must endure the harsh conditions imposed by protease-rich gastric acid and bile acids (Centurion et al., 2021). Although they successfully reach the colon, they may face challenges in integrating into the gut microbiome and could potentially be excreted with faeces. Therefore, it is necessary for the formulation containing the probiotics to be designed and effectively developed for colonic delivery (Awad et al., 2022; McCoubrey et al., 2023) to maintain their viability during GIT, allow sufficient delivery of the strains to the target site, and subsequently enable them to have positive effects in the colonic environment.

Microencapsulation of probiotic cells is an approach of growing interest in recent years, as it aims to improve their stability and survival, particularly during production, storage, and passage through the GIT (Cook et al., 2012). This process involves the incorporation of probiotic cells within biopolymer matrices that have a physical barrier action, protecting the cells from stressors and increasing their viability (Rajam and Subramanian, 2022). In addition, the selection of biopolymers is crucial, as they must be biocompatible and non-toxic to cells (Sharma et al., 2023; Sun et al., 2023). They could allow a targeted delivery of the probiotics to the colon, where they will provide their beneficial effects, thus improving the efficacy of the final product.

Among the different physical methods of preparation of microparticles, such as the well-known spray drying, fluid bed coating, lyophilization, extrusion etc… (Lopedota et al., 2016; Tolve et al., 2023), prilling/vibration is an interesting technique capable of producing homogeneous-sized microparticles or microbeads of homogeneous size. This encapsulation process is efficient, reproducible, industrially scalable and yields high-quality results to produce scalable modified-release formulations (D'Amico et al., 2024; Lopalco et al., 2020). The process is based on the breaking of the laminar flow of a polymer solution pumped through a syringe into a nozzle into one-dimensional droplets by applying a vibration frequency. To prevent the droplets from coalescing, an electrostatic charge is induced on the surface of the droplets, which then fall into a consolidation bath where they solidify into microspheres (Racaniello et al., 2024).

In this study, a bacterial strain previously isolated and belonging to the culture collection of the Department of Soil, Plant and Food Science and taxonomically belonging to basonym *Lactobacillus plantarum* (strain 4S6R) was microencapsulated.

The microcapsule envelope comprises a polymer, such as swellable, pH- and time-responsive calcium alginate, enzyme-responsive inulin, and Eudraguard® control. This combination offers gastroprotection and ensures controlled, sustained release in the GIT. In detail, alginates are subject to proton-catalysed hydrolysis, which relies on time, pH, and temperature (Tønnesen and Karlsen, 2002). Indeed, it is common knowledge that the release process from alginate microcapsules can be categorised into two distinct pathways: drug release through the degradation of the alginate network or via diffusive processes (Uyen et al., 2020). Alginate is a polymer that responds to changes in pH (Han et al., 2007), therefore, its application in the advancement of oral drug delivery systems specific to the colon is widely recognised (Agüero et al., 2017). Furthermore, alginate is metabolised via fermentative processes by the microbiota, resulting in the production of a significant amount of short-chain fatty acids (SCFAs) that have an important role in maintaining gut health and preventing the development of colon diseases (Shang et al., 2018). Its function as a superior probiotic carrier enhances the survival rate of probiotics; however, it does have some disadvantages on its own, e.g., it does not protect probiotics well in low pH conditions, and the high porosity of alginate microspheres results in rapid release of the loaded molecules, low encapsulation efficiency of probiotics, easy degradation in an acidic environment, and poor transport of probiotics in the gut (Wang et al., 2022). To overcome this limitation, Eudraguard® control and inulin were additionally employed to enhance the shell structure and bolster its strength. Inulin, a prebiotic food component that cannot be metabolised by the human body and remains unhydrolysed and unabsorbed in the upper GIT, was chosen due to its potential for bacterial fermentation in the colon. Such fermentation stimulates the growth and activity of bacterial species that are already present in the colon (You et al., 2022). In addition is effectively digested by trillions of bacteria present in the colon, ensuring efficient delivery.

This work aimed to develop a simple and potentially scalable method of encapsulating probiotics for improved viability and targeted delivery to the colon. This was achieved by utilising trigger-dependent polymer blends to produce microparticles capable of the targeted release of probiotics. Trigger-dependent polymer mixtures incorporate independent release mechanisms: pH-, time-, and microbiota-dependent activation. The microcapsules were characterised based on their encapsulation efficiency, size, and morphology. Additionally, were conducted release studies in simulated gastric, intestinal, and faecal media to demonstrate the effectiveness of our formation of protective microencapsulated probiotics.

2. Materials and methods

2.1. Materials

Lactiplantibacillus (L.) plantarum (basonym *Lactobacillus plantarum*) 4S6R was isolated from pig faces of Large White sows from farms in the Montepetriolo hamlet, Perugia, Italy (De Angelis et al., 2006) and belonged to the Culture Collection of the Department of Soil, Plant and Food Science of the University of Bari Aldo Moro.

Inulin 90 % from *Cichorium intybus* (MW≈ 5000 Da), sodium alginate with a ratio of mannuronic and guluronic acids (M/G) of 1.8–2.2, viscosity = 500–600 cps and D-mannitol were gifted by Farmalabor Srl (Canosa di Puglia, Italy). Alginic acid sodium salt (MW = 120,000–190,000 g/mol, with M/G of 1.56, viscosity = 15–25 cps) was purchased from Sigma-Aldrich (Milan, Italy). Eudraguard® control (E 1206) was kindly donated by Evonik Nutrition & Care GmbH (Rofarma Italia Srl, Gaggiano, Italy). All solvents and other salts used were of analytical/technical grade and purchased from Sigma Aldrich.

2.2. Preparation of cell culture and growth conditions

The lactic acid bacterium used in this study was taxonomically identified as *L. plantarum 4S6R* and it belongs to the Culture Collection of the Department of Soil, Plant and Food Science, University of Bari Aldo Moro, Italy. Frozen stock cultures were propagated in de Man, Rogosa and Sharpe (MRS) medium broth (Oxoid Ltd., Basingstoke, Hampshire, England) at 37 °C for 16–24 h under aerobic conditions. Subsequently reaching the exponential phase of growth, assessed by optical density (OD) at 620 nm, cells were harvested by centrifugation at 10000 rpm for 10 min at 4 °C, washed twice with 50 mM sterile potassium phosphate buffer (pH 7.4) and the bacterial suspension was finally centrifuged under the same conditions mentioned above. The pellets were resuspended in saline (0.9 % NaCl) solution.

2.3. Preparation of polymeric feeds for microcapsules

To produce microspheres (F_{sphere}), different polymer ratios were prepared and tested by prilling/vibration technique until the best-performing one was chosen. Initially, a 2 % w/v aqueous solution of alginic acid sodium salt was prepared and combined with the aqueous suspension of Eudraguard® control 30 % w/w in different ratios until a 1:2 w/w ratio was chosen, to which 40 % w/w inulin and 3 % w/v of mannitol were added.

To produce the microcapsules ($F_{capsule}$), initially, a 0.5 % w/v aqueous solution of sodium alginate was prepared with 3 % w/v mannitol added as a cryoprotectant to form the core (S_{core}). For the shell (S_{shell}), different concentrations of sodium alginate (from 4 to 2 % w/v) and different polymer ratios were prepared and tested until the best

performing was chosen. 2 % w/v aqueous solution of alginic acid sodium salt was initially prepared and combined with the aqueous suspension of Eudraguard® control in a 1:2 w/w ratio to which 40 % w/w inulin was added.

Both resulting polymer feeds were autoclaved at 121 °C for 15 min. Once cooled to room temperature, the pellet of the selected probiotic strain (12 log CFU/mL) was added to obtain a total volume of 20 mL for the microcapsules or 50 mL for the microspheres and gently agitated for 5 min to obtain a homogenous suspension. This was processed using the prilling/vibration technique to obtain the microcapsule formulation.

2.4. Preparation of microparticles by prilling/vibration technique

Microbead formulations were obtained by aseptically processing the different polymeric feeds with the prilling/vibration technique using the B395 Pro Encapsulator (Büchi Labortechnik AG, Switzerland) equipped with a concentric nozzle to produce microcapsules or single nozzle to produce microspheres, and a reaction vessel to work under sterile conditions. Preliminary tests were carried out to choose the best parameters to process the polymer feeds and produce microparticles with the required properties in terms of yield and size range. The parameters evaluated were both those of the formulation (Eudraguard® control w/w ratio: alginate) and those of the instrument (nozzle diameters, vibration frequency, electrode potential, consolidation bath, etc.). The feed prepared for the microcapsules was pressed using a concentric nozzle (450 μm for the inner nozzle or core and 700 μm for the outer nozzle or shell) or a single nozzle (750 μm) for the microspheres, the vibration frequency set to break up the laminar jet of liquid was 1000 Hz, and the electrode potential was 1500 V. After several tests to optimise the operating parameters, the best core flow rate was chosen to be 3.15 mL/min, while the shell was pumped at an optimal rate of 22.5 mL/min. The most suitable flow rate for microsphere production was 21 mL/min. The distance between the vibrating nozzle and the gelling bath was 20 cm. The aqueous gelling solution consisted of 500 mL of 0.3 M $CaCl_2$ in which the droplets were consolidated and kept, under mechanical agitation to avoid aggregation of the microcapsules or microspheres, for 10 min. Subsequently, the obtained microparticles were separated from the gelling solution by filtration through paper filters, washed twice with sterile water to remove the unencapsulated probiotics present on the surface, and frozen at − 20 °C and then dried by the sublimation technique for 24 h using an Alpha 1–4 LSCbasic (Christ, Germany) freeze-dryer under reduced pressure (0.018 mbar) at − 50 °C.

2.5. Characterization of microparticles

2.5.1. Viable cells enumeration and calculation of yield and encapsulation efficiency

The viability of the bacteria cells in microparticles (microcapsules or microspheres) was evaluated using the plate count method. In detail, 1 g was added in 9 mL of sterile PBS with Ultraturrax T 25 basic for 5 min at 11000 rpm to facilitate a complete disintegration of microparticles. After that, the samples were serially diluted to appreciate the bacterial cell density and poured plated in MRS agar. The plates were incubated at 37 °C for 48 h. The viable cell number was expressed as logarithm colony forming units (CFU/mL) by using the following Eq. (1):

$$CFU/mL = (Numberofcolonies \times dilutionfactor \times volumeofcultureplate) \quad (1)$$

The production yield percentage (Y%) of the process was determined using Eq. (2):

$$Y\% = \frac{Weightobtainedofmicrobeads}{Totalweightoffeedcomponents} \times 100 \quad (2)$$

The encapsulation efficiency percentage (EE%) of the probiotic cells in the microparticles was determined after their disintegration as described above. EE%, which is a combined measurement of the efficacy of entrapment and survival of viable cells during the microencapsulation procedure, was determined by using the following Eq. (3):

$$EE\% = \frac{N}{N_0} \times 100 \quad (3)$$

where N is the number of viable bacteria (log CFU/mL), after the disintegration of the microparticles and N_0 is the initial number of the bacteria cells (log CFU/mL) in the feed before the process.

In addition, using the plate count method, the gelled solution was analysed to determine the number of viable free cells present in the consolidation bath and thus not encapsulated in the microparticles. The analyses were conducted for each formulation on three different batches and the results were reported as mean ± standard deviation (SD).

2.5.2. Size and morphology

The size of microparticles before and after drying was measured by optical microscopy (Inverted Laboratory Microscope Optech IB 4) equipped and interfaced with an image analysis program (Capture 2.1 software) and the relative reduction in size was expressed by the shrinkage factor (SrF) calculated according to Eq. (4):

$$SrF = \frac{dhydrated - ddry}{dhydrated} \quad (4)$$

where d hydrated and d dry are the diameters of microspheres before and after freeze-drying respectively.

Furthermore, to assess the size homogeneity of microparticles in each production batch, the width of the particle distribution (Span), as defined in Eq. (5), was evaluated.

$$Spanvalue = \frac{d_{90} - d_{10}}{d_{50}} \quad (5)$$

where d_{10}, d_{50}, and d_{90} represent the diameters of 10, 50 and 90 % of the sample microparticles, respectively.

Scanning electron microscopy (SEM) studies were conducted to analyse the morphology of the lyophilised microcapsules. For this purpose, the Hitachi Tabletop Microscope TM300 equipped with a secondary electron detector operating in high vacuum mode, an acceleration voltage of 20 kV, and an effective magnification ranging from 60 × to 1000 × was utilized and interfaced with an analysis program (AZtecOne software).

The sample was dispersed onto an electrically conductive adhesive pad and coated with a gold/palladium layer (sputter coating 15–20 nm). To gain insight into the internal structure of each sample, cross-sections were created and analysed uniformly.

2.6. Swelling behaviour

The swelling properties of the dried microcapsules alone ($F_{capsule}$), having discarded the microspheres for further study, were evaluated using the gravimetric method (Lopedota et al., 2021). Precisely weighed samples were placed in a cylinder with a square mesh base and a known tare. Swelling tests were performed at a temperature of 37 ± 0.5 °C in fluids that simulated the gastric-enteric environment: gastric fluid (SGF pH 1.2) for 2 h and subsequently intestinal fluids at pH 6.8 (SIF) for 3 h and pH 7.4 (SCF) for the remaining time of the analysis. The sample in the cylinder was lightly dried with paper and reweighed at predetermined intervals. The degree of swelling (Sw) was calculated by determining the weight ratio of the final weight of the rehydrated microcapsules (Wf) and their initial dried weight (Wi), using the following equation (6):

$$Sw = \frac{Wf}{Wi} \quad (6)$$

2.7. Viability of bacteria to stress conditions

2.7.1. Survival of free and encapsulated probiotics during simulated GIT.

The survival rate of encapsulated cells was assessed in vitro under GIT conditions and compared to the survival rate of free cell suspensions used as a control.

The VanKel VK 7000 with a rotating paddle at 50 rpm and a temperature bath of 37 ± 0.5 °C was used. The simulated gastric and intestinal fluids were obtained as described by Fernández (Fernández et al., 2003). The simulated gastric fluid (SGF pH 1.2) contained NaCl (125 mM), KCl (7 mM), $NaHCO_3$ (45 mM), and pepsin (3 g/L). The simulated intestinal fluid (SIF pH 6.8) contained 0.1 % w/v of pancreatin and 0.15 % w/v of bile salt (Zárate et al., 2000), while the simulated colonic fluid (SCF) was PBS 0.1 M at pH 7.4. The simulated fluids were sterilised (121 °C for 15 min), stored at 4 °C and used within 24 h.

As assessed by OD at 620 nm, cells grown to the exponential (LOG) phase have been harvested by centrifugation at 8000 rpm for 10 min. The bacterial cell pellet was washed with physiological solution (0.9 % NaCl) and suspended in 100 mL of SGF, to achieve a theoretical concentration of 8.5 log CFU/mL, to evaluate gastric digestion. At the end of gastric digestion (2 h), bacterial suspension was centrifuged (10000 rpm, 4 °C, 10 min). The pellet was moved in 100 mL of SIF (pH 6.8) for 3 h for intestinal digestion. At the end of this study, bacterial suspension was centrifuged and the pellet was harvested and suspended in 100 mL of SCF for the remaining time of the analysis up to 24 h. At each sampled time point aliquots of 2 mL were withdrawn maintaining a sink condition and the bacterial viability was monitored using the plate count method.

The same procedure was used for the microcapsules. 1 g of microcapsules was poured into 100 mL, to achieve a theoretical concentration of 8.5 log CFU/mL, of SGF for 2 h followed by 3 h in SIF and then in SCF for the remaining time of the analysis up to 24 h. Samples with a volume of 2 mL were withdrawn from the medium at specific times, maintaining a sink condition, the bacteria cell survival (log CFU/mL) was determined, and results were expressed as mean ± SD of three independent experiments.

2.7.2. Simulated faecal microbiota fermentation

The release study of probiotics from microcapsules was also carried out in a simulated colonic medium containing exclusively enzymes from faecal microbiota.. This research activity aimed to evaluate how the enzymatic activity of faecal microbes enhances the release of probiotics from the microcapsules. Simulated human faecal conditions were based on previously established protocols (Vacca et al., 2021) with slight modifications. In details, the faecal medium was prepared by adding K_2HPO_4•2 g/L, $C_2H_3NaO_2$•5 g/L, $C_6H_{17}N_3O_7$•2 g/L, $MgSO_4$•0.2 g/L, $MnSO_4$•0.05 g/L, glucose•2 g/L, Tween 80 polysorbate•1 mL/L to faecal supernatants recovered from a faecal suspension with faeces to distilled water ratio of 20 % w/v. The pH was adjusted to 7.2 ± 0.2 using a 6 M NaOH solution. Before proceeding further, the faecal medium was sterilised at 121 °C for 15 min.

In parallel, the faecal slurry inoculum was prepared by homogenising fresh faeces (32 g) provided by a healthy volunteer. The sample was collected according to the guidelines of the Declaration of Helsinki and after the approval by the Ethics Committee of *Azienda Ospedaliero-Universitaria Consorziale Policlinico di Bari* (28th February 2020) and registered with the code NCT04689074. Informed consent was obtained from the subject. The fresh faecal sample, collected within 1 h before further processing, was in 100 mL of sterile saline solution (NaCl 9.0 g/L) within filtered bags (250 μm). This mixture was homogenized (3 min) in a lab stomacher (Bag Mixer, Interscience International; Roubaix, France) and the pellet was recovered after centrifugation (8000 × rpm for 5 min).

Hence, faecal batches accounted for the combination of the faecal medium with the faecal inoculum, which was subsequently sonicated to maintain the enzymatic activity of faecal microbiota while avoiding any bias during probiotics plate counting. The microcapsules were added to vials containing sonicated faecal batches and placed into jars under simulated colonic conditions (anaerobically, at 37 ± 0.5 °C, with slight stirring at 150 rpm in the Thermo Scientific MaxQ 4000 Orbital Incubator Shaker). The pH of the medium was continuously monitored throughout the analysis to assess variations during the experiment.

During the fermentation process, the dissolution of the microcapsules was visually monitored, and at predetermined time intervals, each jar was opened to stop bacterial fermentation. The containers with the microcapsules were then centrifuged, and the supernatant was collected and analysed by performing cell counts. The negative control sample accounted for the same incubation step of the faecal batch to which were not added microcapsules.

2.7.3. Heat tolerance of free and microencapsulated cells

Free cells of *L. plantarum* 46SR were cultured in MRS broth at the optimum growth temperature of 37 °C until they reached the exponential phase of growth measured by OD at 620 nm. The cells were then harvested through centrifugation at 10000 rpm for 10 min at 4 °C and washed twice with PBS solution. The concentrated cell culture suspension was placed in sterile distilled water in glass capillary tubes and heated in an oil bath at either 65 °C for 30 min or 90 °C for 1 min. After this stress, the samples were allowed to cool on ice for 2 min. Following this, a cell count was conducted on MRS agar and subsequent incubation was carried out at 37 °C for 48 h. Similarly, 1 g of microcapsules, encapsulating 10.5 log CFU/mL of *L. plantarum* 46SR underwent treatment. After cooling on ice, the microcapsules were homogenized to facilitate the release of cells, and counts were performed on MRS agar. The data expressed in mean ± SD of three independent experiments.

2.7.4. Long-term stability test

The free cells and microencapsulated bacteria were stored for up to 7 months in a hermetically sealed jar in the fridge (4 ± 0.5 °C), at room temperature (25 ± 0.5 °C), and in a climatic chamber (40 ± 0.5 °C and 75 % of relative humidity). The viability of the probiotics was then determined by the plate count method by taking 1 g of microcapsules (with an initial concentration of 10.5 log CFU/mL) at specific time points and placing them in 9 mL of a PBS solution to disintegrate the microcapsules, and similarly, counts were performed on free cells. The number of viable bacteria in the solution was determined by plate counts on MRS agar through serial dilutions of the solution at 37 °C for 48 h. The physical stability of the samples was assessed by visual inspection and the staining test was performed to identify the bacteria present in the sample. Results were expressed as mean ± SD of three independent experiments.

2.8. Statistical analysis

The experimental data were obtained in triple replicates and are reported as the mean ± SD (standard deviation). Student *t*-test or one-way ANOVA analysis, followed by Bonferroni's post hoc tests were performed to compare statistical significance by using Graph Prism version 8.0.1 (GraphPad Software Inc., La Jolla, CA, USA) adhering to a p-value of < 0.0021. Statistically differences are reported as follow: ns = p-value > 0.05; *= $p < 0.0332$; **= p value < 0.0021; ***= p value < 0.0002; ****= p value < 0.0001.

3. Results and discussion

This study examined the feasibility of developing an oral formulation to deliver probiotics to the colon. The study aimed to evaluate the influence of microencapsulation on the viability and stability of probiotic bacteria under simulated GIT conditions and thermal stress. *L. plantarum* (strain 4S6R) was encapsulated by the prilling/vibration technique in microparticles. This proof of concept was validated through experiments wherein Multiparticulates (MPs) effectively shielded probiotics from

stress, outperforming the performance of free bacteria and facilitating their release in the faecal medium.

3.1. Preparation of cell culture, growth conditions and bacterial characterisation

The selected probiotic *L. plantarum* strain 4S6R, used as a model bacterial strain, was previously isolated from pig gut microbiota (Siragusa et al., 2014). It is a Gram-positive, facultatively anaerobic, non-spore-forming lactic bacterium, that grows between 15 °C and 45 °C and has a large industrial application for fermented foods, such as dairy products, cheese, sourdough, pickles, fermented sausages (Todorov and Franco, 2010). *L. plantarum* is generally regarded as safe (GRAS) according to the U.S. Food and Drug Administration (Todorov and Franco, 2010). Of particular interest are the lactic acid bacteria due to its large number and purported benefits for gut function and health. In line with this, *L. plantarum* is increasingly recognised as a probiotic due to its diverse benefits for gut health, including IBD, metabolic syndromes, dyslipidaemia, hypercholesterolaemia, obesity and diabetes. Additionally, it has potential benefits for metabolic and brain health (Liu et al., 2018).

3.2. Production of microparticles, yield, and EE%

The initial phase of the investigation focused on selecting the most suitable polymers and determining the optimal feed composition to obtain a mixture that could be easily processed by the prilling/vibration technique. The polymer mixture had to ensure the viability of the microencapsulated probiotics until their release in the colon.

Firstly, the most suitable polymer blend was identified based on our previous work (D'Amico et al., 2023). For this case, the polymer ratios were adjusted to select the best alginate/Eudraguard® control 1:2 w/w ratio, as high-viscosity sodium alginate was used. Higher values of Eudraguard® control were excluded during the mix production due to the formation of gelatinous clogging. Similarly, higher values of alginate were avoided due to the high viscosity of the alginate, which made the feed unprocessable. The selection of nozzles and optimization of operating parameters were critical in achieving both F_{sphere} and $F_{capsule}$, ensuring easy processing of the feed and achieving homogeneous particle sizes. Optimisation of the production process was crucial for the realisation of the $F_{capsule}$. It was necessary to fine-tune the total flow rate to ensure that the core carrying the probiotic strain was adequately enveloped by the polymeric casing. This would allow consolidation once the drop fell into the underlying $CaCl_2$ bath.

Preliminary studies were conducted to assess the viability of probiotics in the polymeric core food alone. The purpose was to demonstrate that the food did not have a detrimental effect on their viability.

F_{sphere} demonstrated yield values of 85.16 % and EE values of 53.62 %, as shown in Table 1. Although the process was efficient, some bacteria were lost during the consolidation bath. This resulted in the bath appearing light yellow at the end of the process, due to the easy diffusion of probiotic strains from the polymer matrix, still present in a semi-solid transition state (Jyothi et al., 2010) towards the aqueous phase of the consolidation bath. EE values are mainly related to the loss of viability of probiotics during the freeze-drying process as it damages the cell membrane and reduces their viability (Wang et al., 2020).

$F_{capsule}$ production yield is 97.52 % which confirms the importance of microcapsule production and confirms the success of the process used. There were no losses either in the consolidation bath or during the subsequent lyophilisation process. The EE% (90.06 %) values are also very high showing that almost all bacteria were microencapsulated without damaging their viability. This was further confirmed by performing a cell count on the consolidation bath where no traces of bacteria were found, highlighting that the bacteria had been successfully encapsulated in the core of the microcapsules. The addition of 3 % mannitol significantly (p value < 0.0021) influenced the EE% after freeze-drying, ensuring the viability of 90.06 % compared to 77.48 ± 3.59 of the same microcapsules but without mannitol in the core. The addition of mannitol was performed for its cryoprotective actions to reduce osmotic stress during freezing to which bacteria may be exposed before the freeze-drying process. However, it also has prebiotic properties due to its ability to help enhance the growth of probiotics (Patel and Goyal, 2012). In addition, the interaction, probably via hydrogen bond formation during the microencapsulation process, between the prebiotic and polymer constituting the core of the microcapsule (mannitol and calcium alginate), protects the probiotics (Dianawati et al., 2012).

Table 1
Y%, EE%, and size of microcapsules. Data are reported as mean ± SD.

Formulation Code	Yield %	EE%	Size hydrated microparticles (μm)	Size dried microparticles (μm)	Shrinkage Factor (SrF)
F_{sphere}	85.16 ± 2.11	53.62 ± 6.84	1749.54 ± 35.38	1242.62 ± 78.06	0.290
$F_{capsule}$	97.52 ± 1.07	90.06 ± 2.84	1220.39 ± 60.39	792.75 ± 39.44	0.350

3.3. Micromeritic studies of microcapsules

The size and encapsulation yield of the prepared microparticles can be influenced by several factors, such as nozzle size, concentration, and composition of the polymeric feel. The mean diameters of the lyophilised and hydrated microparticles are shown in Table 1. The hydrated microcapsules ($F_{capsule}$) had a mean diameter of 1220.39 μm, i.e., about twice the diameter of the shell nozzle used (Nemethova, 2015; Whelehan and Marison, 2011).

There was a slight reduction in the diameter of both the dried $F_{capsule}$ and F_{sphere} compared to the diameter of the hydrated $F_{capsule}$, as shown by the calculated SrF values. SrF is a parameter that measures the degree to which microparticles shrink or contract after lyophilization and depends on several factors, including the nature of the microparticles, their composition, initial size, and the specific conditions of the lyophilization process (Chan et al., 2011). This is a consequence of the freeze-drying process; the matrix of the hydrated microparticles only slightly collapsed during water sublimation, as there was a high amount of polymers constituting the shell, and the microcapsules retained similar dimensions to the hydrated ones.

As shown in Table 2, the span value for $F_{capsule}$ was calculated. The larger the value, the wider the particle size range; the lower the span value, the narrower the particle size range. An ideal span value of 0 would indicate a monodisperse particle size distribution (Varela-Fernández et al., 2022).

$F_{capsule}$ showed a spherical shape, indicating the adequacy of the feeds and process parameters used. Indeed, to maintain the shape of the droplets, a good balance of forces such as viscosity, surface tension, and the impact of the droplets on the surface of the consolidation bath is required. The microparticles produced, having a spherical shape, have a low surface-to-volume ratio. In addition, hydrated spherical microparticles are more prone to make dried spherical microparticles that may have better fluidity influencing the breakdown into dosage forms such as

Table 2
Diameter d50, d10, d90 and SPAN of dried microparticles.

Formulation Code	Size microparticles (μm) d50	Size microparticles (μm) d10	Size microparticles (μm) d90	SPAN
F_{sphere}	1242.62	1035.09	1537.03	0.40
$F_{capsule}$	792.75	690.32	896.17	0.26

capsules, sachets, tablets, etc (Reque and Brandelli, 2021).

The SEM technique was employed to assess the morphology of microcapsules. The high resolution of the images enables the size, shape, surface structure, roughness, and presence of porosity of the microcapsules to be determined in detail. This detailed characterisation is essential for understanding the structure–property relationships of the microcapsules. From the SEM images, the lyophilised microcapsules (Fig. 1A) appear quite spherical, with regular, if in some areas collapsed edges probably caused by the lyophilisation process. The detail of Fig. 1B shows the cross-section of the microcapsules and their details (Fig. 1C), with a core–shell structure that is not clearly visible but in which a denser, more compact shell can be observed, compared to the core with a more inhomogeneous microstructure due to the lower concentration of polymeric material in the core.

These microcapsules are MP delivery systems, i.e., oral dosage forms consisting of many small units, each of which has the desired properties. The individual microcapsules have a patient-friendly diameter (<1.8 mm), flexibility in dosing and administration, and could potentially overcome swallowing difficulties. Hence, they might be suitable for specific populations such as geriatric patients with dysphagia and paediatric patients (Martinez Teran et al., 2017).

3.4. Swelling study

Since the microcapsules produced had a good amount of alginate, swelling studies were conducted with the main objective of analysing their behaviour during gastrointestinal transit. In fact, the release of any drug or biological encapsulated material in a delivery system, alginate-based is usually modulated by a swelling-dissolution-erosion process (Tønnesen and Karlsen, 2002).

The rehydration and swelling behaviour of obtained $F_{capsule}$ makes it possible to assess the evolution of this system during gastrointestinal transit and predictively the release of probiotics from them. (Colombo, 1993). In swelling-controlled release systems, several physicochemical processes, including polymer water absorption, gel layer generation, and polymeric chain relaxation, regulate the transport of microencapsulated material through the polymer network (Argin et al., 2014). We believe that the hydration of the hydrophilic groups of alginates is the main reason for the swelling of the dry microcapsules (Hoffman, 2012). In this case, free water permeates the microcapsules to fill the inert spaces between the polymer chains, which results in further swelling.

To explore the behaviour and rehydration of $F_{capsule}$, swelling tests were conducted in simulated gastric and intestinal fluids (buffers at pH 1.2, pH 6.8 and pH 7.4). The findings of the gravimetric method-assisted swelling investigation are shown in Fig. 2.

The swelling behaviour of lyophilised microcapsules indicates the velocity and simplicity of liquid infiltration into the polymeric network of the matrix, which is imperative for the liberation of bacterial cells. In detail, swelling is a compromise between two actions: at low pH, alginate becomes protonated, resulting in an uncharged polymer with limited solubility. This leads to rapid rearrangement of the polymer, which expels water from the gel network and causes it to contract back to its original volume. However, despite the pH, alginate manages to swell due to its internal porous structure (Huq et al., 2017).

At pH 1.2, the addition of Eudraguard® control into the

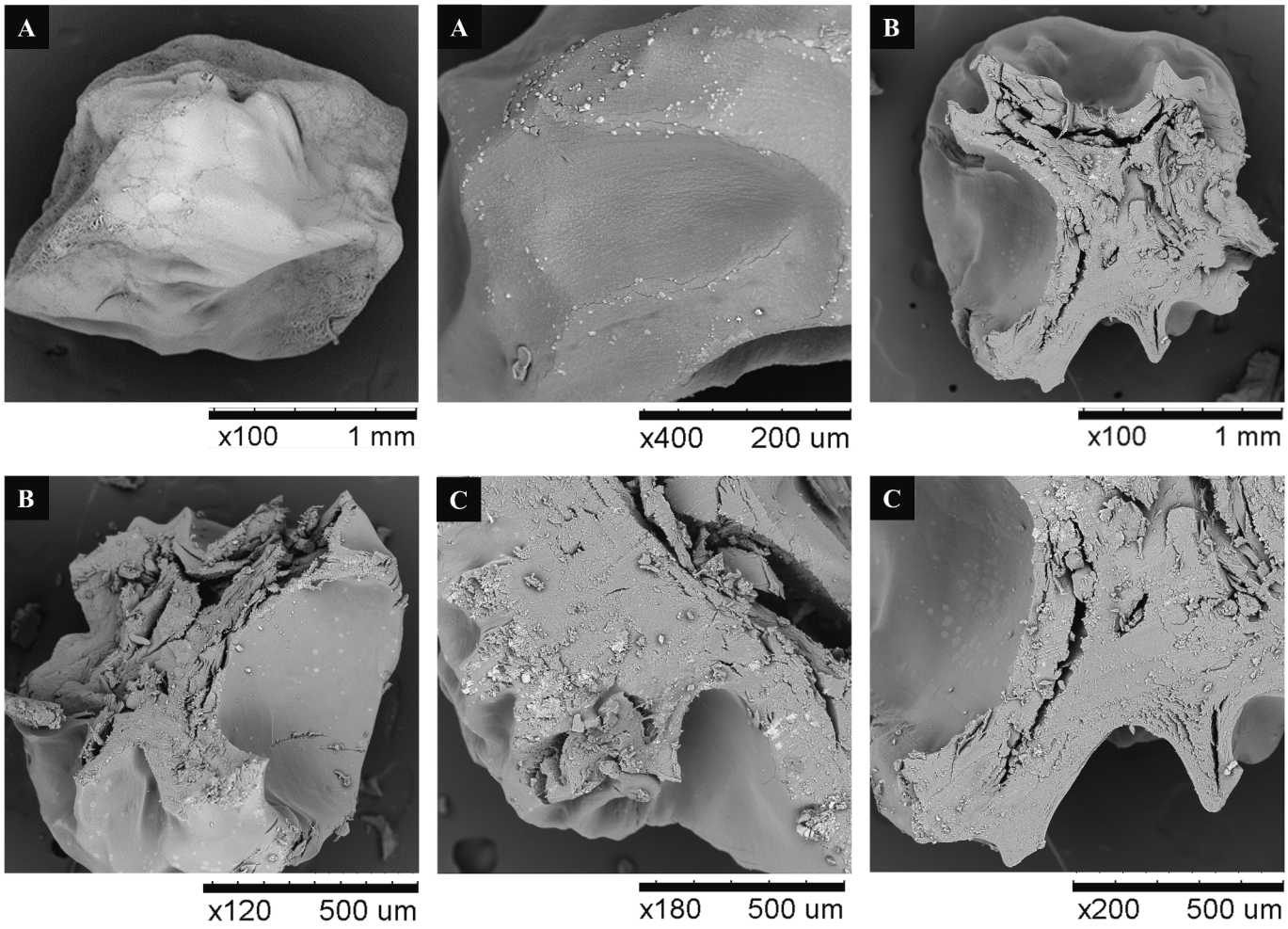

Fig. 1. SEM images of microcapsules ($F_{capsule}$) with probiotics, their surface (A) and cross-sections at different magnifications (B, C).

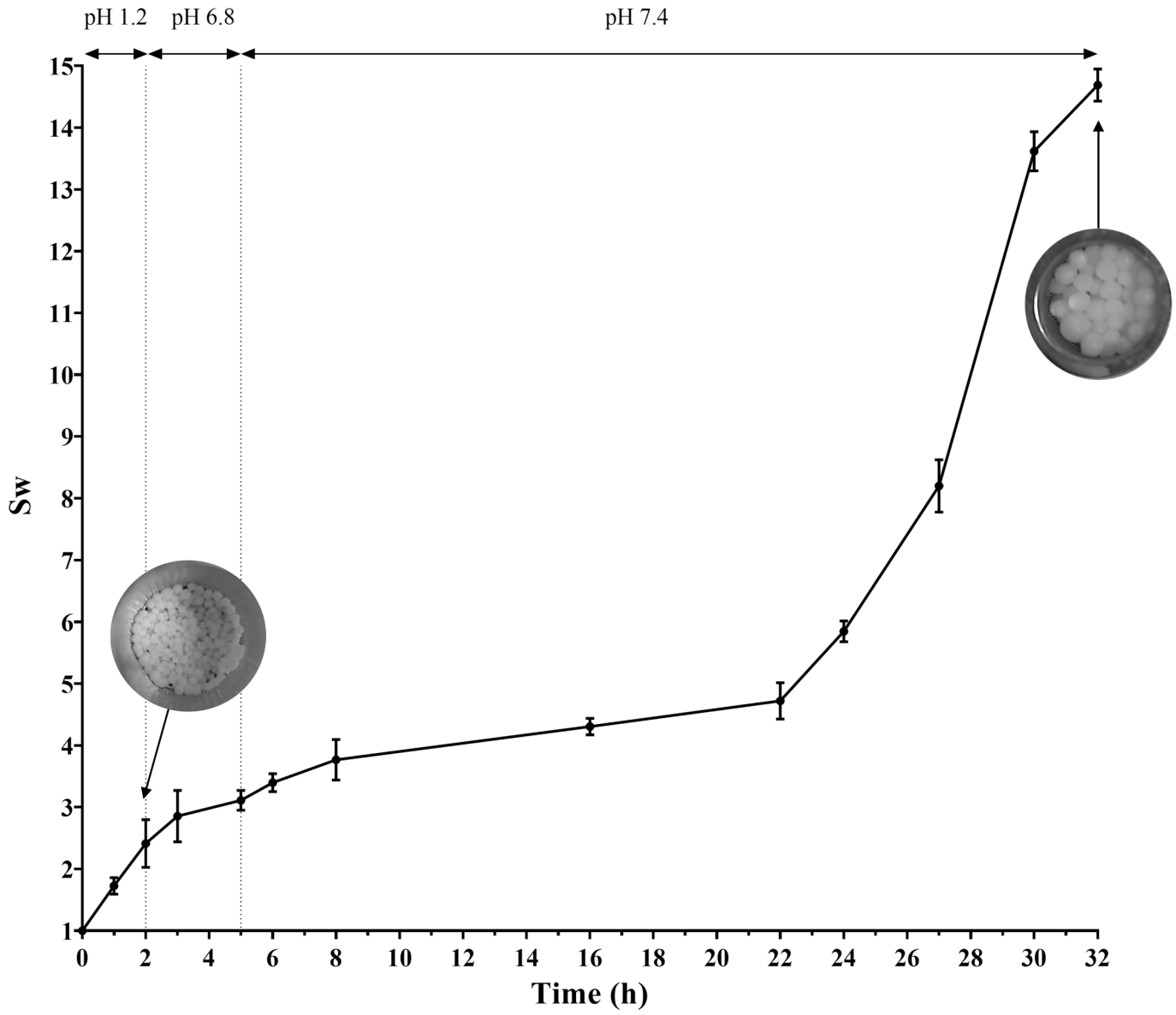

Fig. 2. Swelling profile and details of microcapsules carried out in simulated gastric fluid (SGF pH 1.2) and intestinal fluids (SIF pH 6.8 and SCF pH 7.4).

microcapsule matrix reduces permeability, forms a structure with reduced swelling capacity and prolongs the disintegration time of the microcapsules. Furthermore, the inclusion of inulin is thought to enhance increased cross-linking within the alginate microcapsule matrix, leading to the development of a denser, more compact gel. This, in turn, results in slower diffusion processes in aqueous media resulting in slower aqueous media diffusion processes (Zhang et al., 2020).

This justifies the outcomes of the gravimetric swelling study, where $F_{capsule}$ exhibited a 2.5-fold increase in weight, preventing the establishment of a disintegration release system. Consequently, this system was evaluated in intestinal media. The known pH-responsive characteristics of alginate would have led it to dissolve in pH conditions of 6.8 and 7.4 with complete dissolution of the shell. However, the presence of Eudraguard® control in the shell influenced the swelling, delaying the pH-responsive disintegration process of the alginate and giving the shell characteristics that allow for prolonged release.

Eudraguard® control not only delays swelling but also brings out a controlled time-responsive behaviour, evidenced by constant weight for 22 h of analysis followed by rapid and intense swelling (from 4.51 at 22 h to 7.90 after only 5 h). This is due to the two forces of alginate dissolution and Eudraguard® control, which are synergistic and promote rapid water uptake in the shell. However, the absence of enzymes in the media that can digest inulin avoided increased swelling, which is the cause of the disintegration of the $F_{capsule}$. In fact, after 32 h of study, complete microcapsules, even when swollen, were recovered.

3.5. *Survival of free and encapsulated probiotic bacteria in simulated GIT*

For effective oral supplementation, probiotics must be able to survive the harsh conditions of the GIT, such as highly acidic stomach juices, bile salts in the small intestine and different digestive enzymes, and then be released at the colon level in sufficient cell density to allow colonisation and proliferation (Nezamdoost-Sani et al., 2024). Maintaining the viability of probiotics during administration through the digestive tract is a challenge. Therefore, free cells of *L. plantarum* and $F_{capsule}$ were tested during simulated GIT to understand whether the microencapsulation process had a beneficial effect on bacterial survival as microencapsulation is known to improve survival rates of probiotics during gastrointestinal transit (Shori, 2017).

Fig. 3A compares the viability of the free cells of *L. plantarum* with

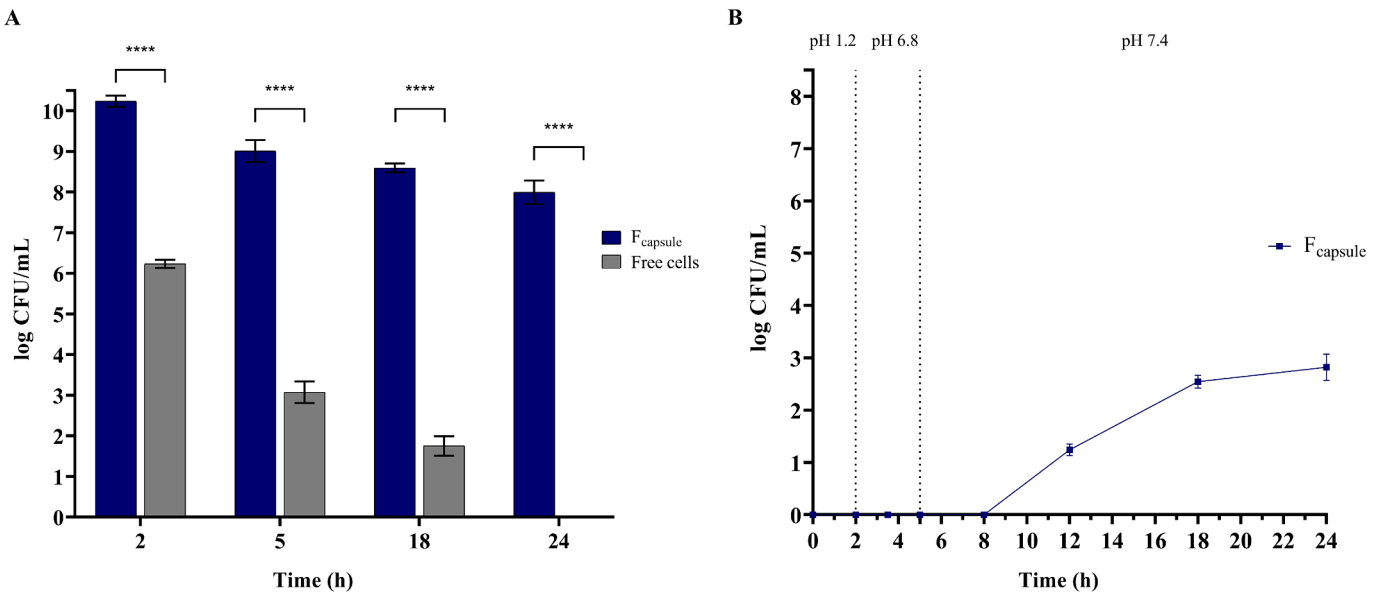

Fig. 3. Release of probiotic cells profile for $F_{capsule}$ (A), and bar graphs (at 2, 5, 18 and 24 h) comparing the viability of free bacteria and the viability of bacteria in the microcapsules at different time points (B).

$F_{capsule}$ formulation by a bar graph, while Fig. 3B shows the release profile of the probiotics from the $F_{capsule}$.

For the first study, it was evaluated the released bacterial cell density at specific time points, as well as the residual viability within the $F_{capsule}$ (Fig. 3A). The results indicate that there is a reduction in the viability of the free cells after two hours in the SGF at pH 1.2 of 40.63 % due to the highly acidic nature of the gastric fluids and the presence of pepsin maintaining the viability of 6.23 log CFU/mL. Unlike free cells, when the probiotics are microencapsulated, they maintain a significantly ($p < 0.0001$) higher viability of 97.52 % (10.24 CFU/mL). This effect can be attributed to the protective capacity of the microcapsule shell, which forms a physical barrier, making it difficult for the acidic medium to penetrate the core where the probiotic bacteria are encapsulated. The improved survival of the microencapsulated bacteria compared to the free bacteria demonstrates the efficiency and effectiveness of the polymer mixture chosen for the preparation of $F_{capsule}$ to protect the bacteria from harsh gastric conditions. In addition, at acidic pH, alginate sequesters protons and converts them into insoluble alginic acid, effectively buffering the gastric pH at higher values and thereby promoting the survival of encapsulated probiotics (Lee and Mooney, 2012). However, the porosity of the alginate could be responsible for some limitations of the microcapsules made, such as the rapid release of loaded probiotics, low encapsulation efficiency, easy degradation in an acidic environment and limited transport of probiotics in the gut. However, choosing an alginate with a high mannuronic acid content allows the formation of softer, less porous gels that easily disintegrate over time. Also, the addition of Eudraguard® control and inulin makes the shell more compact, reducing the limitations associated with the use of sodium alginate alone. Inulin is not hydrolysed by digestive enzymes in the upper GIT, demonstrating a protective effect on the survival of bacteria in the acidic gastric environment (García-Gamboa et al., 2020). The robustness of the protection provided by the microcapsules was further confirmed by the residual presence of bacteria within the microcapsules at the end of the test, which retained their viability despite the gastrointestinal challenges to which they were subjected.

The free bacterial strain showed a further reduction of 30.09 % after 3 h in SIF (pH 6.8) with a halving of their viability compared to the residual value recorded in the gastric environment (Fig. 3A). This apparent loss of viability is due to the bile salts that can alter the integrity of the cell membrane (Badgeley et al., 2021). Indeed, bile salts self-assemble into micelles and can hurt bacterial membrane proteins, can be incorporated into cell membranes, increasing their permeability, resulting in altered cell membrane integrity, leakage of cell contents and bacterial cell death (Li, 2012). The viability of microencapsulated bacteria, on the other hand, is significantly higher; in fact, a viability of 9.01 log CFU/mL is maintained in the microcapsules due to their protective capacity. The ability to tolerate digestive stress is one of the most important properties for the successful supplementation of probiotics in functional foods and food supplements (Ross et al., 2005).

In addition, to the comparative study of the survival of free and microencapsulated probiotic bacteria, their release from the microcapsules was also analysed. Fig. 3B shows that no bacteria were detected in the external medium at pH 1.2 and pH 6.8. It should be noted that although a small quantity of probiotics may have been released in both cases, as soon as release occurred, the bacteria in the external medium were completely killed due to the stressful conditions to which they were exposed. A slow release of the microencapsulated bacterial cells begins only when the pH changes to 7.4. Indeed, the pH-responsive nature of the alginate (Agüero et al., 2017) is counterbalanced by the swellable/time and pH-dependent nature of the Eudraguard® control ("Eudraguard® Portfolio," n.d.). At the end of the 24-hour test, there was less than 35 % release of bacterial cells from the microcapsules. This is due to the presence of inulin in the shell which was not degraded due to the absence of the stimulus to which the microcapsules would be responsive, i.e., the enzymes produced by the colonic microbiota.

3.6. Release study of the encapsulated probiotic bacteria in the faecal medium

The same $F_{capsule}$ previously studied were also tested in a faecal medium simulating the colonic environment in vitro, which was featured by viable bacterial cells of the gut microbiota to evaluate their enzymatic contribution to inulin metabolism. The Fig. 4 shows the results of this study that was carried out to evaluate the contribution of the resident microbiota on the release profile of the microcapsules. The disintegration of the particles was monitored by visual observation, while the amount of released *L. plantarum* in the faecal medium was quantified by plated bacterial counts.

As previously demonstrated for the first hours of digestion (at pH 1.2 and 6.8), no bacterial cells were found in the external media. Subsequently, when the $F_{capsule}$ were transferred to faecal medium (pH 7.2 ± 0.2), they exhibited a release of 3.28 log CFU/mL (36.44 %) after 4 h of

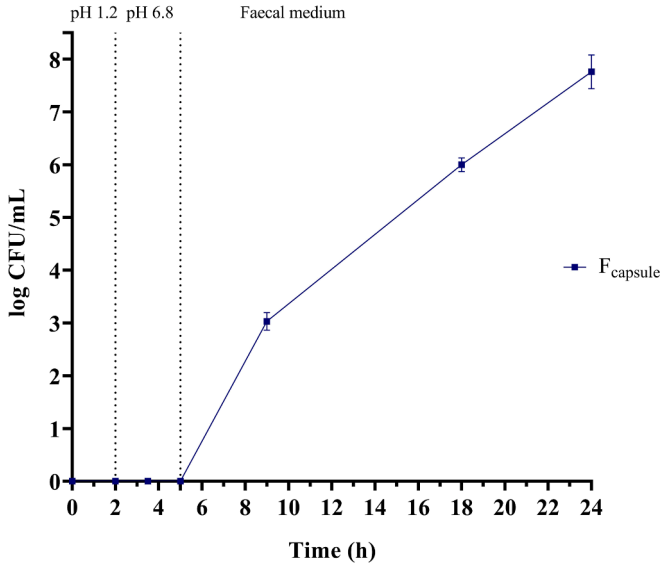

Fig. 4. Release of probiotic cells from the microcapsules ($F_{capsule}$) at pH 1.2, 6.8 and faecal medium.

study in faecal medium and 7.75 log CFU/mL (86.11 %) after 24 h. These values were significantly different from the release values obtained from the same microcapsules studied in the medium without enzymes at pH 7.4. It is hypothesized that the 24-hour release may be complete, as the remaining 13.89 % may have been released in the initial media at pH 1.2 and 6.8 but could not be counted due to the stresses that caused their death.

Indeed, microcapsules can respond to parallel triggers present in the colon ensuring a specific and targeted release in the colon. Alginate can swell, allowing time-dependent drug release. It is also metabolised by colon enzymes, allowing faster degradation of the envelope and a targeted release profile at the specific site in the colon (Strich et al., 2023). Eudraguard® allows pH-responsive release, whereas inulin is a dietary fibre, which is not absorbed after oral intake but is degraded when it reaches the colon, fermented, and utilised by the gut microbiota (Shang et al., 2018).

Fermentation and utilisation of inulin by specific gut microbes could change the structure and composition of the gut microbiome and benefit the host by producing short-chain fatty acids (Fu et al., 2021). However, this statement requires additional experiments because the inulin-based microbiota modulation is dependent on its quantity. Nonetheless, inulin has been documented also to prevent the development and progression of ulcerative colitis by modulating the intestinal microbiota (Fu et al., 2023). These encouraging results indicate that the developed systems are a promising formulation for the delivery of probiotics to the colon.

3.7. Heat tolerance of free and microencapsulated cells

The industrial use of probiotic foods presents significant challenges when they are exposed to high temperatures, such as pasteurisation (Rezaei et al., 2020), an important thermal process used to deactivate pathogenic microorganisms, and ensure food safety and product preservation. However, it involves the inevitable killing of non-pathogenic organisms. In fact, probiotics are generally sensitive to heat, and it is very important that they maintain their viability during heat treatment in a significant percentage to perform their beneficial actions (Cook et al., 2012). It is therefore essential to selectively protect these microorganisms during heat processing, e.g. through microencapsulation (Teoh et al., 2011) Indeed, microcapsules can provide a physical barrier against harsh environmental conditions, including heat (Kailasapathy, 2002), preventing the loss of viability of bacteria during thermal processing. However, it is important to note that the ability of microcapsules to protect bacteria depends on their resistance to heat and their ability to keep cells intact during heating.

Starting from this premise, the viability of free *L. plantarum* and $F_{capsule}$ was studied before and after heating at 65 °C and 90 °C for 30 and 1 min respectively, and the results are shown in Fig. 5.

The viability of the bacteria alone is drastically reduced when exposed to temperatures of 65° C for 30 min starting from values of 10.5 log CFU/mL and reaching values of 2.42 log CFU/mL with a 71.42 % reduction in viability indicative of the bacteria's inability to withstand this thermal stress. In contrast, the same $F_{capsule}$ maintain a viability of 7.64 log CFU/mL. Similarly, there is a significant difference ($p < 0.0001$) when free and microencapsulated bacteria are subjected to heat stress at 90 °C for 1 min, no free bacterial cells survive after exposure to the heat treatment while the same microencapsulated bacterial cells maintain a viability of 6.96 log CFU/mL. In both thermal stress conditions to which the bacteria are exposed when microencapsulated, they demonstrate the ability to maintain values above 6 log CFU/mL. This corresponds to the 'minimum therapeutic' level of viable probiotic microorganisms that must be sustained throughout the product's shelf life (Terpou et al., 2019). This confirms that microencapsulation with the chosen polymer mixture, and the inclusion of these microcapsules in probiotic-enriched foods, can improve the thermal resistance of the bacteria by providing a physical barrier against harsh environmental conditions.

3.8. Long-term stability test

To ensure the benefits of probiotic products, their viability must be maintained not only after the microencapsulation process but also, more importantly, during storage. Hence, stabilising probiotic cultures during preparation, storage, and transport is one of the major challenges for researchers.

Generally, the long-term stability of microencapsulated probiotics by the prilling/vibration technique depends on several factors, including the choice of encapsulation materials, storage conditions and the nature of the probiotics themselves. Nonetheless, prilling microencapsulation can substantially enhance the long-term stability of probiotics compared to non-encapsulated probiotics.

For this research, it was evaluated the stability of both the encapsulated and the non-encapsulated probiotic strain. We visually examined $F_{capsule}$ stored at different temperatures and at different times. The microcapsules exhibited no changes in dimension, colour, stickiness, or deterioration during the 7-month physical stability assessment when stored at 4 °C and 25 °C. However, when stored at 40 °C and 75 % of

relative humidity, they experienced browning after 2 months, a phenomenon that could be attributed to different types of reactions such as oxidation and protein denaturation that are related to both bacterial death and browning processes (Kurtmann et al., 2009).

In addition, the stability of the probiotic strain alone, which was simply stored hydrated for one month at 4 °C, was analysed but suffered an 89.32 % reduction in viability. The literature already reports low storage stability of aqueous phase or encapsulated probiotics if the water is not removed from the delivery system, leading to the hypothesis that the combined approach of microencapsulation and lyophilization is ideal for increasing the viability of probiotics during storage (Albertini et al., 2010).

As depicted in Fig. 6, there is a decline in viability in the samples as storage time increases, and this reduction is accentuated with higher temperatures. Indeed, viability demonstrates an inverse relationship with storage temperature (Barbosa et al., 2015). This phenomenon is primarily attributed to the oxidation of membrane lipids and denaturation of proteins which results in the denaturation of macromolecules in bacterial cells (Fu and Chen, 2011).

In detail, storage at 40 °C had a considerable negative impact on the viability of free probiotics, although microcapsules exhibited higher viability than free cells. Specifically, storage of free cells at 40 °C for one month resulted in an 84.58 % reduction in viability, while microencapsulated cells only decreased by 38.86 % (data not shown). At 25 °C, microencapsulation provides increased stability for the bacteria over 4 months. On the contrary, free cells experience a loss of 63.55 % of their viability in just two months. However, concerning refrigeration, studies have reported that lower temperatures improve cell viability by reducing potential chemical reactions that are damaging to microorganisms (Corcoran et al., 2004). Microcapsules stored at 4 °C showed greater stability than both the same microcapsules stored at room temperature and free cells. The non-encapsulated cells reported values of 6 log CFU/mL only for 2 months, unlike the microencapsulated cells, which preserved this density for more than 4 months without losing viability during the 7-month studied time.

These findings endorse the selection of both the polymer mix and the microcapsules, which protected and stabilised the bacteria during storage by prolonging their viability compared to non-microencapsulated bacteria. Specifically, the inclusion of inulin in the chosen polymeric mixture could serve as an additional nutrient source for *L. plantarum* during storage, enhancing its survival (Parhi et al., 2023). In addition, mannitol protected probiotic bacteria during storage, likely due to its role as a hydroxyl radical scavenger (Efiuvwevwere et al., 1999).

4. Conclusion

This study aimed to microencapsulate *Lactiplantibacillus plantarum 4S6R* (formerly known as *Lactobacillus plantarum*) as a probiotic, employing the prilling/vibration technique with a specifically chosen polymeric blend for constructing the microcapsule shell. Initially, the focus was on selecting the optimal combination of polymeric materials to encapsulate the probiotic strain, investigating the different variables influencing processability and viability, and refining the microencapsulation technology. The microparticles containing probiotics are

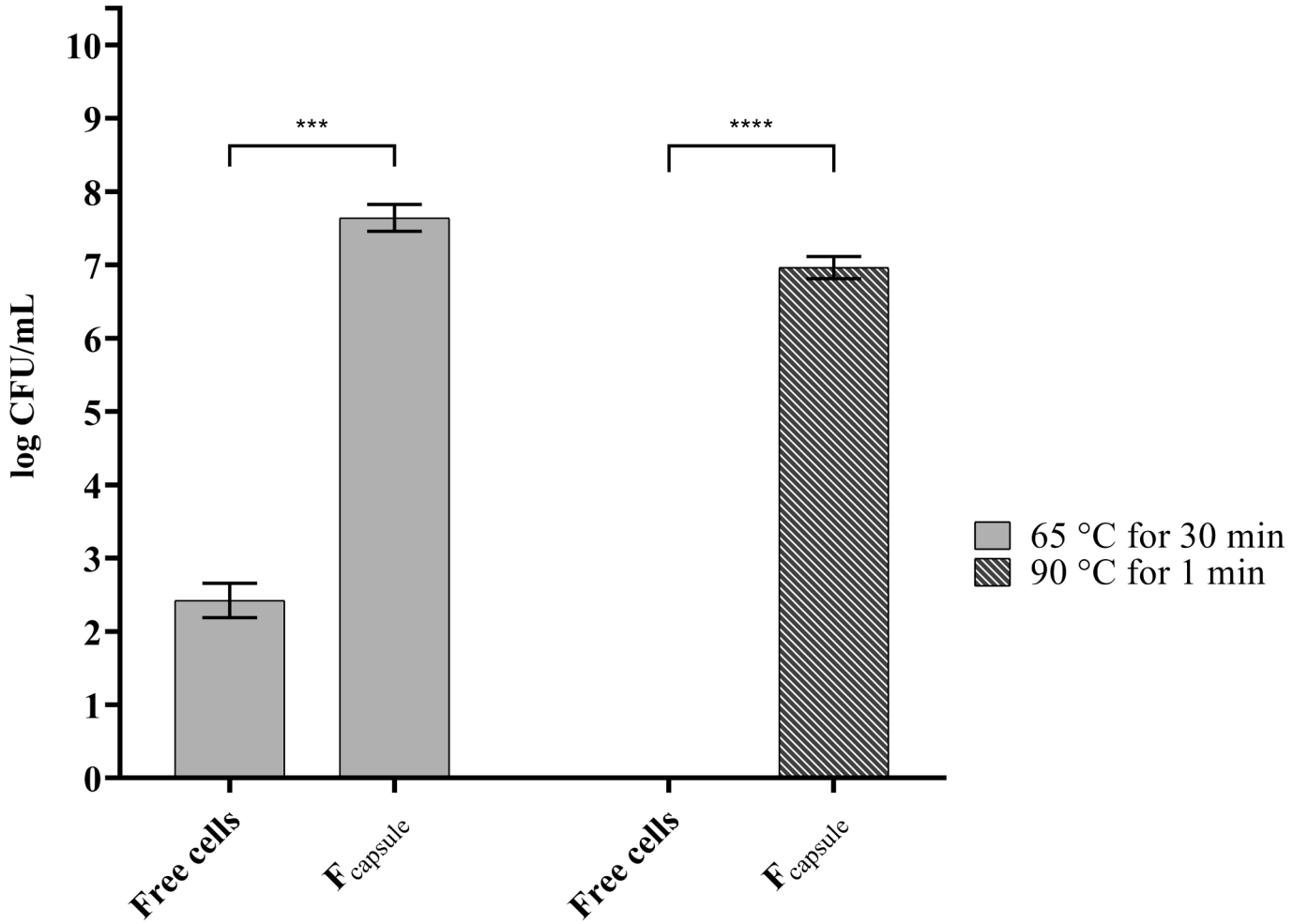

Fig. 5. Survival of free cells and $F_{capsule}$ after exposure of 30 min at 65 °C (left side) and after 1 min at 90 °C (right side).

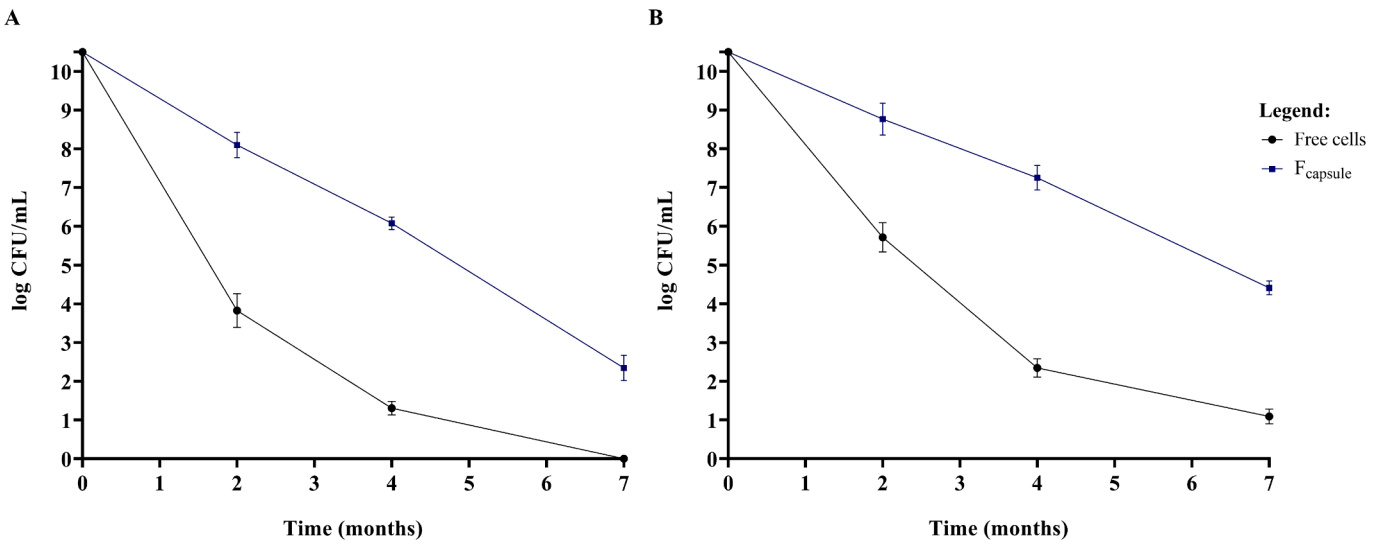

Fig. 6. Viability of free and microencapsulated cells at 25 °C (A) and 4 °C (B).

designed to respond to multiple parallel trigger mechanisms, including time, pH, and microbiota, ensuring a targeted release of probiotics into the colon. This guarantees that the probiotics can exert their beneficial effects at the intended site of action. Microspheres with uniform distribution of the probiotic in the polymer matrix exhibited low encapsulation efficiency (EE%) values, prompting the rejection of this formulation in favour of more efficient microcapsules. The realised core–shell formulation underwent thorough characterization, including viability tests of the probiotics post-production, in the GIT, release tests in the faecal environment, and stability assessments at different temperatures and over extended periods. In conclusion, this study endeavoured to enhance probiotic delivery through the production of microcapsules with targeted colon release, capable of stabilising the encapsulated probiotics. This approach holds promise for providing potential health benefits and addressing limitations observed in several currently commercialised formulations.

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CRediT authorship contribution statement

Vita D'Amico: Writing – original draft, Methodology, Data curation. **Antonio Lopalco:** Validation. **Rosa Maria Iacobazzi:** Validation. **Mirco Vacca:** Writing – review & editing, Methodology. **Sonya Siragusa:** Investigation. **Maria De Angelis:** Resources, Funding acquisition. **Angela Assunta Lopedota:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Nunzio Denora:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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