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Effect of microencapsulation on the bio-preservative and probiotic properties of *Enterococcus durans* F21

Ghita Benkirane^{a,b}, Samir Ananou^a, Géraldine Agusti^b, Laila Manni^a, Nour-Eddine Chihib^c, Emilie Dumas^b, Adem Gharsallaoui^{b,*}

^a Laboratoire de Biotechnologie Microbienne et Molécules Bioactives, Faculté des Sciences et Techniques, Université Sidi Mohamed Ben Abdellah, Route Immouzer, BP 2202, Fez, Morocco

^b Laboratoire d'Automatique, de Génie des Procédés et de Génie Pharmaceutique, Université Claude Bernard Lyon 1, CNRS 5007, 69622, Villeurbanne, France

^c CNRS, INRA, UMR 8207-UMET-PIHM, Université de Lille, 369 Rue Jules Guesde, 59651, Villeneuve D'Ascq, France

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ABSTRACT

This study aimed to assess the probiotic potential of *Enterococcus durans* F21 and its microencapsulation. Two microencapsulation methods, spray-drying (SD) and freeze-drying (FD), were employed using sodium caseinate (Cas) as a cell protectant at concentrations of 0.035% and 1% and at two pHs, 3 and 7. Maltodextrins (MD) served as wall material (10%). Microcapsules were analysed for cell viability and membrane damage after drying, survival under simulated gastro-intestinal conditions, antimicrobial activity, stability during storage, and physicochemical characterization. Results showed that *E. durans* F21 exhibited promising probiotic properties, including moderate auto-aggregation, high co-aggregation with pathogens, moderate biofilm formation, and resistance to simulated gastrointestinal conditions. The encapsulation pH showed to be a crucial factor affecting the viability of microencapsulated cells. Microencapsulation at pH 3 adversely affected cell viability during drying. However, microencapsulation at pH 7 using Cas (at 0.035 and 1%) was found to be most effective in maintaining higher cell viability under similar conditions. No significant difference was detected between both Cas formulations, suggesting that 0.035% Cas concentration might be sufficient for the microencapsulation process at pH 7. Moreover, FD proved to be the most effective method to produce *E. durans* F21 microcapsules with high viability (cell viability of 93%) and stability during storage (cell viability of 99%). Conclusively, microencapsulation of *E. durans* F21 using Cas, at pH 7, and employing FD method could be a promising strategy for producing highly viable microcapsules containing *E. durans* F21 cells with high probiotic and bio-preservative potentials in foods.

1. Introduction

The genus *Enterococcus* belongs to the large group of lactic acid bacteria (LAB) that produce lactic acid by fermentation (Estilarte et al., 2021). They can be found in several ecological niches including water surfaces, foods, dairy products and human and animals gastrointestinal tract (Li et al., 2018). In the last years, many of *Enterococcus* strains have been extensively studied as bio-preservative cultures to ensure food safety while maintaining the sensorial and nutritional properties, which meets the consumers demand for naturally and minimally processed foods (Benkirane et al., 2023). In addition to their protective role, some bacteria of the genus *Enterococcus* have been reported for probiotic features, or even used as starter cultures in food industries due to their

contribution to the organoleptic properties of fermented products (ripening, taste and flavour improvement, etc) (Daba et al., 2021; Hanchi et al., 2018). The protective effects of most common *Enterococcus* strains are related to their ability to produce antimicrobial compounds commonly called “bacteriocins” (Wu et al., 2022).

In a previous study, we have isolated a bacteriocin-producing *Enterococcus* strain identified as *E. durans* F21 (Benkirane et al., 2023). The strain was isolated from a Moroccan traditional fermented milk called « *Lben* », which has shown interesting bioprotective and biotechnological properties. In particular, this strain was active against various pathogens and food spoilages (*Listeria monocytogenes*, *L. innocua*, *E. faecalis*, *Mycobacterium smegmatis*, and *Brochothrix thermosphacta*) and revealed absence of undesirable properties, including virulence factors

* Corresponding author.

E-mail address: adem.gharsallaoui@univ-lyon1.fr (A. Gharsallaoui).

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and the main antibiotic (ATB) resistance. Indeed, *E. durans* F21 was not hemolytic, sensible to ATB commonly used for the treatment of enterococcal infections, and did not produce biogenic amines or other virulence enzymes. In addition, this strain showed satisfactory biotechnological features (high acidification power and antioxidant activity). These properties reinforced its potential application as a bio-preservative agent for functional foods.

In recent years, considerable interest has been given to the use of probiotics in functional food products due to their potential in promoting human health (Romyasamit et al., 2022). Probiotics can provide many beneficial effects to the host, such as prevention of inflammation, stimulation of gut immunomodulation, and production of some substances such as organic acids and antimicrobial agents (Maldonado Galdeano et al., 2019). Some of the criteria that LAB must meet to be considered as probiotics, starter cultures, or bio-preservative cultures in foods include: (i) their ability to survive and remain active during their passage through the gastrointestinal tract and during food processing; (ii) their capability to grow during food storage; and (iii) their effectiveness to control undesirable microorganisms, including pathogenic and spoilage bacteria (Ribeiro et al., 2021). However, ensuring the stability and viability of these cultures can be a major challenge to food and biotechnological industries. Microencapsulation is a promising technology used as a possible way to protect LAB cultures from harsh environmental conditions (Ribeiro et al., 2021).

Several drying techniques (used as microencapsulation methods) can be applied to facilitate the industrial chain supply of protective LAB cultures, reduce storage associated costs, and increase the product shelf life (Romano et al., 2021; Terpou et al., 2019). Among them, spray-drying (SD) has been the most commonly used technique to produce dry powders (Ilha et al., 2015). It is generally useful for microencapsulation of heat sensitive materials because of its rapid processing. Moreover, SD provides high productivity, reproducibility and adaptability to most industrial equipment (Gul & Atalar, 2019). However, freeze-drying (FD) method has also become more widespread in the food industry (Assegehegn et al., 2019). It has been used as an effective alternative to SD and the mechanism behind is based on sublimation, where the water in the feed solution undergoes freezing at an extremely low temperature, then removed into water vapour through the sublimation process (Yudhiastuti et al., 2019). In spite of their well demonstrated efficiency, the dehydration stress involved in these drying methods can lead to various structural damages to the membrane lipids and proteins of bacterial cells (Gul & Atalar, 2019; Romano et al., 2021). These structural changes, in turn, lead to a decrease in bacterial viability and can adversely impact their technological performance (e.g. decrease in the shelf life and alteration in the production of antimicrobial compounds, among others) (Romano et al., 2021).

Various biopolymers have been used to reduce the damaging effect of SD and FD methods. The most commonly used are proteins due to their functional properties, good amphiphilicity, biodegradability, and biocompatibility (Dianawati et al., 2017). Their ability to interact and bind with a large range of active compounds makes them ideal agents for microencapsulation. Milk-based proteins are among the favourable wall materials used singly or in combination with other biomaterials for microencapsulation (Abd El-Salam & El-Shibiny, 2015). Sodium caseinate (Cas) is a promising encapsulation material due to its structural and physicochemical properties (Koh et al., 2022). It has the advantage to offer ideal physical and functional properties because of its amphiphilic character and emulsifying characteristics, which provide suitable conditions for microencapsulation of probiotics without causing any marked effect on their viabilities (Koh et al., 2022). Starch derivatives such as maltodextrins (MD) can also be used as the primary wall materials. They are mainly used in drying process due to their good solubility at high concentration and their relatively low cost (Koh et al., 2022).

The present study focuses on the probiotic characterization of *E. durans* F21 strain and its microencapsulation. Several operational

parameters were assessed during the microencapsulation process, including Cas concentration, encapsulation pH, and drying method. Specifically, Cas was evaluated at two different concentrations, as a bacterial cell protectant during drying, at different pHs (acidic and neutral). MD were used as wall material in all formulations. The aim of this research was to identify the most appropriate microencapsulation formulation and drying method to produce *E. durans* F21 microcapsules with high viability, prolonged stability during storage and high potential for application as probiotics and bio-preservatives in foods.

2. Materials and methods

2.1. Materials

The bacterial strains used in this study were *E. durans* F21 (GenBank accession no. OQ572352) isolated from a Moroccan traditional fermented milk called "Lben" (Benkirane et al., 2023). *E. faecalis* S5, *M. smegmatis* MC₂-155, were obtained from BM2B laboratory collection (Faculty of science and technology of Fez, University of Sidi Mohamed Ben Abdellah, Morocco); *Salmonella enterica* B801, *Staphylococcus aureus* B804 and *L. monocytogenes* B806 were obtained from CNRST (Rabat, Morocco). The encapsulating agents used were Cas containing 92% of protein obtained from Acros Organics, (Belgium) and MD (Glucidex DE 28) obtained from Roquette (France). All other reagents were of analytical grade.

2.2. Probiotic profiling of *E. durans* F21

2.2.1. Auto-aggregation

The auto-aggregation ability of *E. durans* F21 was carried out as described by Collado et al. (2008) with some modifications. *E. durans* F21 cells were grown in Brain Heart Infusion (BHI, Biokar diagnostics, France) for 24 h at 37 °C. Cells were collected by centrifugation (5000 rpm, 10 min, 4 °C) and washed twice with phosphate-buffered saline PBS (pH 7.4) and suspended in the same buffer. Suspension of approximately 8 log CFU/mL (4 mL) was incubated at 37 °C for 4 h and 24 h and monitored by optical density (OD) measurements at 600 nm without vortexing. The auto-aggregation percentage (Ap) was calculated as following:

$$A_p = (1 - A_t/A_0) \times 100$$

Where: A₀: Absorbance measured at 600 nm before incubation at 37 °C; A_t: Absorbance measured at 600 nm after 4 h (or 24 h) of incubation at 37 °C.

2.2.2. Co-aggregation with pathogens

The co-aggregation capacity of the strain was assessed according to the method described by Tuo et al. (2013) by mixing suspensions of pathogenic bacteria with a suspension of *E. durans* F21. The pathogenic bacteria tested were *L. monocytogenes* B806, *S. enterica* B801, *St. aureus* B804, *M. smegmatis* MC₂-155 and *E. faecalis* S5. A cell suspension (8 log CFU/mL) was prepared for each bacteria using the same procedure described above (Section 2.2.1.). Equal volumes of each pathogen suspension (2 mL) and *E. durans* F21 (8 log CFU/mL) (2 mL) were mixed and incubated at 37 °C. The absorbance at 600 nm of the mixture was monitored at different durations: 4 h and 24 h. The co-aggregation percentage (Cp) was calculated as following:

$$C_p = [1 - A_{mix}/(A_{pathogen} + A_{F21})/2] \times 100$$

Where, A_{mix}: Absorbance measured at 600 nm of the mixed bacterial suspension after 4 h (or 24 h) of incubation at 37 °C; A_{pathogen}: Absorbance measured at 600 nm of pathogenic suspension after 4 h (or 24 h) of incubation at 37 °C; and A_{F21}: Absorbance measured at 600 nm of *E. durans* F21 suspension after 4 h (or 24 h) of incubation at 37 °C.

2.2.3. Biofilm formation

Biofilm-forming ability of *E. durans* F21 was assessed using the method described by Khalil et al. (2023) with some modifications. Aliquots of 100 μL (from suspension of 6 log CFU/mL) were distributed into 96 well-microplate and incubated at 37 °C for 24 h. Next, each well-microplate was washed with PBS (pH 7.2), fixed with 150 μL of ethanol (96 %, v/v) (Prodisphar, Morocco) for 5 min, then stained with 150 μL of crystal violet (1 %) for 15 min. For quantification, 150 μL of glacial acetic acid solution (33% v/v, Sigma Aldrich, France) were added to the wells after washing with sterile distilled water. Finally, the OD was measured at 590 nm using a MultiScan sky microplate reader (Thermo-Fisher, France). A negative control was prepared using only the culture medium without the bacterial strain and was subjected to the same steps previously mentioned. The strain was classified as (a): non/low biofilm producer (when $\text{OD} \leq 0.12$), (b) moderate biofilm producer (when $0.12 < \text{OD} \leq 0.24$), or (c) strong biofilm producer (when $\text{OD} > 0.24$) (Khalil et al., 2023).

2.2.4. Cell surface hydrophobicity

Cell surface hydrophobicity of *E. durans* F21 was determined as described by Jardak et al. (2017). Briefly, 0.2 mL of *n*-hexane (Sigma Aldrich, France) was added to 1.2 mL of bacterial suspension ($\text{OD}_{400\text{nm}}$ of 0.4) (A_0). The solution was then vortexed and maintained at room temperature for 15 min. OD of aqueous phase (A_1) was measured at 400 nm and the cell surface hydrophobicity (CSH) percentage was calculated using the following formula:

$$\text{CSH (\%)} = (1 - A_1/A_0) \times 100$$

The bacterial strain is considered hydrophobic, moderately hydrophobic or hydrophilic, when the bacterial adhesion to hexane exceeds 50 %, comprised between 20 % and 50 %, or below 20 %, respectively (Krepesky et al., 2003).

2.2.5. Resistance to *in vitro* simulation of gastrointestinal conditions

The resistance to *in vitro* simulation of gastrointestinal conditions was tested, using 7 log CFU/mL of *E. durans* F21, as following (the viability was checked by plate count and data were expressed as logarithmic value of survival cells):

- The ability to survive under acidic conditions was tested on sterile aqueous solutions of NaCl 0.2 % (w/v) previously adjusted to pH 2.5, 3.0 and 4.0 for 4 h at 37 °C (Gul & Atalar, 2019).
- The resistance to bile was realized on sterile bile salts solutions (0.3 and 1 %, w/v, pH 7.0) (Sigma Aldrich, France), for 3 h at 37 °C (Bhagwat et al., 2020).
- The tolerance to pancreatin was tested on sterile PBS solution (pH 8.0) containing pancreatin at 1 mg/mL (Oxford, India) for 4 h at 37 °C (Akmal et al., 2022).

2.3. Characterization of the surface charge of *E. durans* F21 and Cas: Zeta potential measurements

The measurement of the zeta potential was conducted on imidazole/acetic acid buffer solutions at 5 mmol/L (a low ionic strength buffer) adjusted to different pHs (2, 3, 4, 5, 6, 7 and 8). The pH adjustment was carried out with HCl and NaOH solutions prepared at 0.1 M. The zeta potential of *E. durans* F21 was measured from a bacterial suspension of the strain on MRS broth, recovered after 24 h of incubation at 37 °C and washed two times with a saline solution (0.85 % w/v). The cells were resuspended in imidazole/acetic acid buffer solutions to obtain a final concentration of 8 log CFU/mL. For Cas, 1 g/L stock solution was suspended in the imidazole/acetic acid buffer solutions to obtain a final concentration of 0.01 g/L.

Zeta potential measurements were performed using a Zeta equipment (NanoZS90, Malvern Instrument, Malvern, UK). Mean zeta

potential values as well as standard deviations were obtained from the instrument.

2.4. Formation and characterization of Cas/*E. durans* F21 complexes

The formation of Cas/F21 complexes was studied at two different pHs: 3 and 7. Solutions of Cas (10 g/L) and *E. durans* F21 (8 log CFU/mL) were prepared separately in imidazole/acetic acid buffer at pH 3 (or pH 7). Cas solution was then added to the bacterial suspension to obtain a Cas/F21 ratio ranging from 0 to 800 $\mu\text{g}/8$ log CFU. The number of F21 cells was kept constant at 8 log CFU. The resulting suspensions were vortexed for 1 min and pH was readjusted prior to each test. The zeta potential of Cas/F21 complexes formed at pH 3 (or pH 7) and at different Cas/F21 ratios was determined using a zeta equipment NanoZS90 (Malvern Instrument, Malvern, UK). Particle size measurements of Cas/F21 complexes formed with different Cas/F21 ratios and at two different pHs (3 and 7) were carried out using a laser diffraction instrument Mastersizer 3000 (Malvern instruments, Malvern, UK). Prior to measurement, the complexes were diluted with imidazole/acetic acid buffer (adjusted to pH 3 or 7). The complexes were stirred continuously throughout the measurement to ensure sample homogeneity.

2.5. Microencapsulation process

E. durans F21 was grown in MRS broth at 37 °C for 24 h and then collected by centrifugation at 5000 rpm, for 15 min at 4 °C using a refrigerated centrifuge (Ohaus, France). The cell pellets were washed three times with saline solution (0.85%) and resuspended in the same solution to obtain a cell suspension. Feed solutions for drying experiments were prepared at a final pH of 3 or 7 as given in Table 1. Cas were first dispersed in imidazole/acetic acid buffer (5 mmol/L) at room temperature and mixed until complete dissolution. The pH was adjusted to 3 or 7 with 0.1 N NaOH or 0.1 N HCl. MD (10%, w/v) were subsequently added to all Cas solutions, and the ingredients were left to fully hydrate for 1 h under continuous agitation. Finally, the solutions were mixed with 20 % (v/v) of *E. durans* F21 suspension (final concentration of 8 log CFU/mL). The final pH was readjusted to pH 3 or 7. Other formulations were also prepared using only MD and served as controls.

Microencapsulation of *E. durans* F21 by SD process was performed with a laboratory scale spray-dryer (Buchi B-290 Mini Spray Dryer, France). Experiments were carried out under the following conditions: inlet air temperature of 160 °C, outlet air temperature of 85 °C, airflow of 1L/h and air pressure of 3.2 bar. The microcapsules containing the bacteria were collected from the base of the cyclone and placed into sterile bottles with screw-on lids and stored at 4 °C. For FD experiments, encapsulating material solutions containing the bacterial suspension were frozen at -20 °C for 24 h in a freezer. Then, frozen samples were introduced into a laboratory scale freeze-dryer (Lyovapor™ L-200, France) operating at vacuum pressure of 0.08 mbar and condensing temperature of -54 °C for 72 h. The dried samples were placed into sterile bottles with screw-on lids and stored at 4 °C.

Table 1
Composition of the solutions used for *E. durans* F21 encapsulation.

Encapsulation pH	Encapsulant formulation	Concentration (% w/v)	
		MD	Cas
3	MD (Control)	10	–
	MD/Cas a	10	0.035
	MD/Cas b	10	1
7	MD (Control)	10	–
	MD/Cas a	10	0.035
	MD/Cas b	10	1

2.6. Survival of microencapsulated *E. durans* F21 after drying

The viability of *E. durans* F21 was assessed immediately after drying by plate count method. SD and FD powders were dispersed in Tryptone Salt (TS, Biokar diagnostics, France) in order to make the initial solid content. Aliquots of 100 μ L were plated on MRS agar plates after appropriate 10-fold serial dilutions of released samples using TS broth. Colony-forming units (CFU) were enumerated after the incubation at 37 °C for 72 h. The survival (S%) of encapsulated cells after drying, with respect to initial counts before drying, was determined and calculated as follows:

$$S (\%) = (\log \text{CFU/mL viable after drying} / \log \text{CFU/mL viable before drying}) \times 100$$

2.7. Membrane damage analysis

The determination of membrane damage in the obtained microcapsules was carried out according to the method described by Romano et al. (2021) with some modifications. The microcapsules were rehydrated and then diluted in a sterile saline solution (0.85%). Their OD at 600 nm was adjusted to 0.1. Next, 50 μ L of a 15 μ M stock propidium iodide (PI) solution were added to 100 μ L of bacterial suspension, giving a final PI concentration of 5 μ M. All samples were incubated for 60 min at 25 °C in the dark. Sample fluorescence was measured using a Varioskan-Lux microplate reader (Thermo-Scientific, France). The emission wavelength was 530 nm, while the excitation wavelength was 618 nm. For each sample, thermally-dead cells (85 °C for 30 min) obtained under the same tested conditions (OD_{600 nm} of 0.1) were used as controls in the experiment. These cells were also labelled with PI to provide fluorescence references corresponding to dead cells. To subtract background noise, a blank containing 50 μ L of PI stock solution and 100 μ L of sterile saline solution (0.85%) was used. Finally, the rate of damaged cells in the 12 obtained microcapsules was estimated in relation to control samples (corresponding to 100% damaged cells) and was calculated as follows: **Damaged cells (%) = (Fluorescence signal detected in sample/Fluorescence signal detected in control) x 100%**

2.8. Survival of microencapsulated *E. durans* F21 under acidic and bile salts conditions

The resistance of microencapsulated *E. durans* F21 to acidic conditions (pH 2.5 and 3) or bile salts (0.3 and 1.0%) was evaluated as described previously (Section 2.2.5.). One gram of the microencapsulated cells (or 1 mL of free cells) was transferred to 10 mL of sterile aqueous solutions with the specified conditions, then vortexed for 20 s for complete dissolution. The samples were incubated at 37 °C for 4 h and 3 h under acidic and bile salts conditions, respectively. The survival of cells (%) after the incubation period was determined and calculated using the formula:

$$S (\%) = (N/N_0) \times 100$$

Where, N is the CFU/g (or CFU/mL) of the powders (or free cells) quantified after incubation and N₀ is the CFU/g (or CFU/mL) of the initial suspensions before incubation.

2.9. Antimicrobial activity of microencapsulated *E. durans* F21

Antimicrobial activity analysis was conducted according to Ananou et al. (2020) with some modifications. In brief, 1 mL of *L. monocytogenes* B806 used as indicator strain (viability 6 log CFU/mL) was plated into 19 mL of buffered Tryptone Soy Agar (TSA, Biokar diagnostics, France) and waited until the medium solidified. The solid medium was then

plugged for making well (5 wells in each Petri). One gram of each powder (or 1 mL of an overnight culture of free cells) was incubated at 37 °C for 24 h in tubes containing 9 mL MRS broth. Cell free supernatant from each powder culture was used for antimicrobial analysis. Approximately 100 μ L of supernatant were dropped into the wells. Plates were incubated at 37 °C for 18 h. Antibacterial activity was determined by clear zone measurement around the wells (mm).

2.10. Stability of powders under storage conditions

Viability of cells in the microcapsules was determined during storage at room temperature (28 °C–33 °C) and refrigeration temperature (4 °C) for 8 weeks. At selected times (0, 1, 2, 4 and 8 weeks), viable cell counts (in log CFU/g) were carried out on MRS agar for 72 h at 37 °C.

2.11. Physicochemical characterization of powders

2.11.1. Yield

The powder yield is a representation of the ratio between the weight of the product and the weight of the suspension. The yield (%) was expressed as the amounts of dried powders (in g) per 100 g of dry solid in feed solution before drying and was calculated using the following formula:

$$Y (\%) = [(g \text{ of powder produced} - g \text{ of residual water}) / g \text{ of solid in feed solution}] \times 100$$

2.11.2. Residual water content and water activity (*a_w*)

The residual water content was calculated according to the method described by Bhagwat et al. (2020). Two grams of each powder were placed in an aluminium pan and dried at 105 °C for 24 h. Moisture content (%) of each sample was calculated using the following formula:

$$MC (\%) = [(w_0 - w_1) / w_0] \times 100$$

Where w₀ and w₁ are the weights of powders before and after drying at 105 °C, respectively.

Moreover, the a_w of the obtained powders was measured using an a_w-meter (Novasina, France).

2.11.3. Hygroscopicity

The hygroscopicity of powders was determined according to the method described by Soukoulis et al. (2014). One gram of each powder was placed in a desiccator equilibrated at 75% RH containing a saturated solution of NaCl. Powders were kept for 7 days and hygroscopicity (%) was calculated according to the formula:

$$H (\%) = [(w_1 - w_0) / w_0] \times 100$$

Where w₀ and w₁ are the weights of powders before and after storage at 75% RH, respectively.

2.11.4. Particle size analysis

The particle mean size analysis was measured using a Malvern Mastersizer 3000 instrument (Malvern Instrument Ltd, Worcestershire, UK). The powders were dispersed in pure ethanol and the mean size was expressed as the mean diameter D_[4,3] of the particle size distribution.

2.11.5. Scanning electron microscopy

The Scanning Electron Microscopy (SEM) was performed with an FEI Quanta 250 FEG microscope at the "Centre Technologique des Microstructures" at the university of Lyon 1 (Villeurbanne, France). Powders were deposited on a flat steel holder. The samples were coated under vacuum by a sputter before doing the microscopy analysis.

2.12. Statistical analysis

The average data from triplicate trials \pm standard deviations were determined with Excel program (Microsoft Corp., USA). The statistical analysis was performed using a trial version of SPSS-PC software 17.0 (SPSS, Chicago, USA). The results were submitted to ANOVA analysis. Value of $P \leq 0.05$ considered significant difference.

3. Results and discussion

3.1. Probiotic profiling of *E. durans* F21

Cell surface properties such as auto-aggregation, biofilm formation and hydrophobicity are fundamental properties for probiotic bacteria, as they are closely linked to their adhesion to host epithelial cells (Sharma et al., 2019). This adhesion is considered a prerequisite for the colonization and persistence of probiotics in the gastrointestinal tract, enabling them to withstand the various conditions of the digestive system, colonize the intestinal mucosa and exert their beneficial effects on health (Campana et al., 2017). The auto-aggregation ability of *E. durans* F21 increased significantly ($P \leq 0.05$) from 25.1 % after 4 h to reach 47.7 % after 24 h of incubation at 37 °C (Fig. 1 A). These results suggest that this strain has a moderate auto-aggregation capacity (30%–60%) (Li & So, 2021), which could give it the ability to survive in sufficiently high numbers and effectively colonize the gastrointestinal tract.

Moreover, *E. durans* F21 had important co-aggregation ability with all tested pathogens, including *L. monocytogenes*, *S. enterica*, *E. faecalis*, *St. aureus* and *M. smegmatis* with 81.9, 77.6, 80.4, 76.1 and 79.1 % after 24 h of incubation, respectively (Fig. 1B). No significant differences were observed in co-aggregation ability between all the tested pathogens ($P > 0.05$). This high co-aggregation ability between bacteriocin producing strains and pathogens -permitting the production of antimicrobial substances in their proximity-could be an effective barrier in preventing pathogens in foods and in the host gastrointestinal tract (Reuben et al., 2020).

Regarding the strain's ability to form a biofilm, *E. durans* F21 revealed a measured OD_{590nm} of 0.162. This result suggests that this strain has a moderate ability to form biofilm ($0.1 < OD \leq 0.24$) (Khalil et al., 2023). In fact, biofilm formation plays an important role in probiotic's ability to firmly adhere to the host intestinal mucosa, enabling the establishment of stable microbial communities and contributing to their prolonged beneficial effects on host health (Shridhar & Dhanashree, 2019). Other *Enterococcus* strains, including *E. durans* KLD56.0930, *E. faecalis* and *E. faecium*, have revealed a moderate capacity for biofilm production (Khalil et al., 2023; Li et al., 2018).

Hydrophobicity values provide insights into the potential hydrophobic affinity between intestinal environment and bacterial cell membrane. The hydrophobicity of *E. durans* F21 was determined using hexane (a non-polar solvent). The adhesion to this solvent enables the assessment of hydrophobic/hydrophilic characteristics of this strain.

E. durans F21 showed 65.4% of microbial adhesion to hexane. So, this strain can be considered hydrophobic (hexane affinity exceeds 50%). Similar hydrophobicity results were observed for other *E. durans* strains isolated from Turkish dairy products and Brazilian soft cheese (61.2% and 66.8%, respectively) (Pieniz et al., 2015; Yerlikaya & Akbulut, 2020).

In order to establish the probiotic potential of bacteria, it is essential to consider additional important criteria, including their ability to survive under *in vitro* simulated gastrointestinal conditions including acidic pH and the presence of bile salts and pancreatin. Probiotics resistance under these conditions is of great importance, as it enables them to survive in the gastrointestinal tract and reach the intestine, where they can exert their beneficial effects on the host. According to our results, *E. durans* F21 was resistant to all *in vitro* gastric conditions (acidic pH, bile, and pancreatin) (Fig. 2). In fact, this strain was resistant to acidic conditions with reductions of 0.24 (3.2%), 1.57 (20.7%), and 3.1 (42.4%) log units at pH 4, 3 and 2.5, respectively, and reductions of 2.7 (40.4%) and 4.2 (61.7%) log units, respectively, under 0.3 and 1 % of bile with respect to controls (before incubation) ($P \leq 0.05$). Moreover, this strain displayed higher resistance to pancreatin (1 mg/mL) with only reduction of 0.1 (1.37%) log unit ($P > 0.05$). Thus, the probiotic profiling of *E. durans* F21 suggests the possibility of its use as a probiotic strain.

3.2. Zeta potential measurements of *E. durans* F21 and Cas

Prior to microencapsulation, it was important to assess the surface properties of the various components involved in this process. In our case, characterization of the surface charge of *E. durans* F21 and Cas through zeta potential measurements was conducted. However, MD

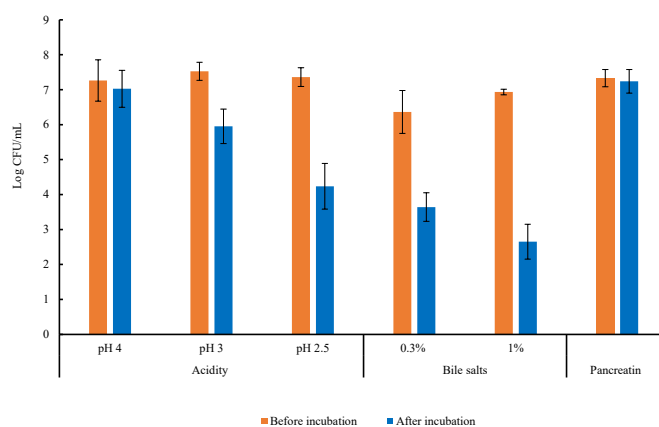


Fig. 2. Viability (log CFU/mL) of *E. durans* F21 after exposure to acidic conditions, bile salts and pancreatin.

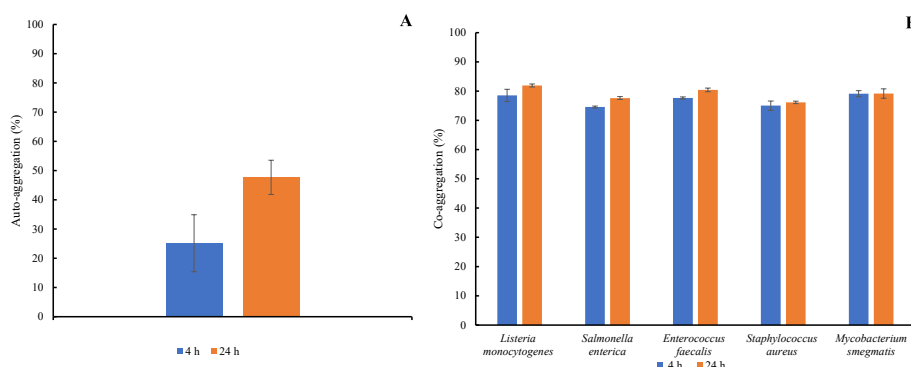


Fig. 1. Auto-aggregation (A) and co-aggregation (B) abilities of *E. durans* F21.

were not subjected to zeta potential characterization, as they do not exhibit any electrical charge. The zeta potential measurements of *E. durans* F21 cells and Cas were carried out at different pH values (from 2 to 8) (Fig. 3). The zeta potential of the cells was negative for the whole pH range (from -21.2 mV to -9.9 mV) and similar to the zeta potential profiles that were previously reported for other *Enterococcus* strains (Tariq et al., 2012; Van Merode et al., 2006). Such profiles indicate that the surface of the cells was largely dominated by anionic compounds. These compounds include weak acids, such as the carboxylate combining polysaccharides and proteins, and strong acids, such as the phosphate based (lipo) teichoic acids (Deepika et al., 2009). It is also interesting to point out that between pH 2 and pH 8, the overall charge of Cas was successively positive, neutral, and negative varying from 18 mV to -13 mV. The isoelectric point (*pI*) associated with no zeta potential was around 4.5. This value aligns with other authors reporting a *pI* of Cas between 4.4 and 5.2 (Asaduzzaman et al., 2022; Yesiltas et al., 2018). The positive and negative net charges of sodium caseinate are related to the solution pH, which promotes the protonation of the amino groups or deprotonation of the carboxyl groups, respectively (Navarrete et al., 2022).

3.3. Formation of Cas/*E. durans* F21 complexes at pH 3 and pH 7

pH is a key factor in the behaviour of mixed molecule solutions due to its direct impact on the charge distribution of ionizable groups in these molecules. The formation of Cas/*E. durans* F21 complexes was studied at different Cas/F21 ratios (0–800 $\mu\text{g}/8$ log CFU). Complex formation was assessed at pH 3 (where Cas carries a net positive charge) and pH 7 (where Cas carries a net negative charge).

3.3.1. Zeta potential of complexes

The zeta potential of complexes formed at pH 3 and 7 is shown in Fig. 4 (A, B). The negative zeta potential of Cas/F21 particles formed at pH 3 decreased with the addition of Cas until electro-neutrality was reached (Zeta potential = 0) (Fig. 4 A). Subsequent addition of Cas lead to progressively higher positive zeta potential values. This result indicates that electrostatic interactions have indeed occurred between Cas and F21 cells. According to these results, the electro-neutrality was achieved at a Cas/F21 ratio of 350 $\mu\text{g}/8$ log CFU of bacteria. Above or below this ratio, the Cas/F21 complexes exhibited positive and negative charges respectively. At this stage of electro-neutrality (or charge neutralization), Cas/F21 particles exhibited high stability and the most intense interactions were established. Furthermore, all the negative binding sites on F21 cells become saturated by the positive Cas sites at a ratio of 350 $\mu\text{g}/8$ log CFU, meaning that the excessive amount of protein in the medium resulted in an increase in zeta potential, which reached its highest value (16.36 mV) at a Cas/F21 ratio of 800 $\mu\text{g}/8$ log CFU (the

highest ratio tested).

Concerning the complexes formed at pH 7 (Fig. 4 B), for all tested Cas/F21 ratios, the zeta potential remained consistently negative. This seems evident, because within this pH range, both Cas and *E. durans* F21 cells carry net negative charges.

3.3.2. Particle size of complexes

The particle size of complexes formed at pH 3 and 7 is shown in Fig. 4 (C, D). The average particle size of Cas/F21 formed at pH 3 increased after the addition of small amounts of Cas due to the formation of Cas/F21 complexes followed by aggregation (Fig. 4 C). This result indicates that electrostatic interactions between Cas molecules and *E. durans* F21 cells took place. The highest average particle size of these complexes, with a maximum diameter of 5.6 μm , was obtained at a Cas/F21 ratio of 400 $\mu\text{g}/8$ log CFU. The formation of these large complexes signifies a high degree of aggregation of individual soluble complexes. Above this ratio (400 $\mu\text{g}/8$ log CFU), the average size of complexes decreased, indicating the dissociation of the large aggregates into smaller and insoluble complexes. This may suggest that large aggregates were formed through a mechanism of rapprochement between complexes. Indeed, in the presence of small amounts of Cas, at least one positively charged Cas molecule could interact with more than one formed complex having free anionic sites, resulting in the association of individual complexes. However, as the concentration of Cas increased, the size of complexes decreased through dissociation of aggregates due to repulsive forces (Eghbal et al., 2016).

Concerning the complexes formed at pH 7 (Fig. 4 D), the average size of these complexes remained relatively constant for all tested Cas/F21 ratios ($P > 0.05$). The stability of particle size of these complexes at pH 7 can be attributed to the similarity in negative charges of Cas and F21 cells. Indeed, as previously reported (Section 3.2.), both Cas and F21 cells carry net negative charges at pH 7, indicating that electrostatic interactions could not be involved.

3.4. Microencapsulation of *E. durans* F21

Based on the previously reported zeta potential measurements (Section 3.3.1.), the ratio of Cas/F21 required to achieve electrical neutrality at pH 3 corresponds to 350 $\mu\text{g}/8$ log CFU of bacteria. At this ratio, all negative charges of *E. durans* F21 cells were neutralized by the positive charges carried out by Cas molecules. After appropriate calculations, this ratio was equivalent to 0.035% (w/v) of Cas (w/v) for a suspension of *E. durans* F21 of 8 log CFU/mL. This Cas concentration (0.035%) was assessed in the microencapsulation process of the strain (at 8 log CFU/mL), at pH 3 and pH 7, in the presence of 10% (w/v) MD serving as wall material, and using SD and FD methods. However, to assess whether this concentration will be sufficient for the microencapsulation process, another Cas concentration at 1% (w/v) (where they are in excess) was also evaluated. Furthermore, in order to examine the effect of Cas in this process, other identical formulations were prepared in the presence of only MD, and used as controls.

The different formulations were referred: “MD” (control), “MD/Cas a” (with 0.035% Cas), and “MD/Cas b” (with 1% Cas). These formulations were prepared at both pH 3 and pH 7 and were all subjected to SD and FD methods.

3.4.1. Survival of microencapsulated *E. durans* F21 after drying

The survival of *E. durans* F21 after drying is presented in Fig. 5 A. According to our results, the viability of the strain was significantly affected by the encapsulation pH ($P \leq 0.05$). Specifically, higher cell viability was detected when the cells were encapsulated at pH 7 compared to pH 3 ($P \leq 0.05$). The survival rates in microcapsules produced at pH 7 ranged between 89 % and 96 %, while those obtained at pH 3 ranged between 59 % and 78 %. However, no significant difference was detected between both drying methods ($P > 0.05$). For microencapsulated cells at pH 7, the addition of Cas at both concentrations,

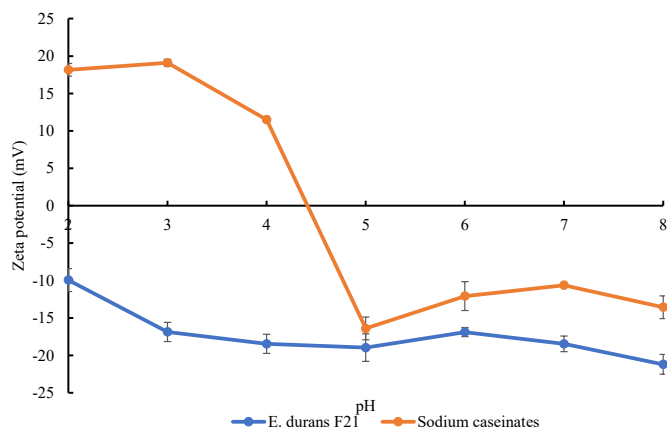


Fig. 3. Zeta potential of *E. durans* F21 and Cas.

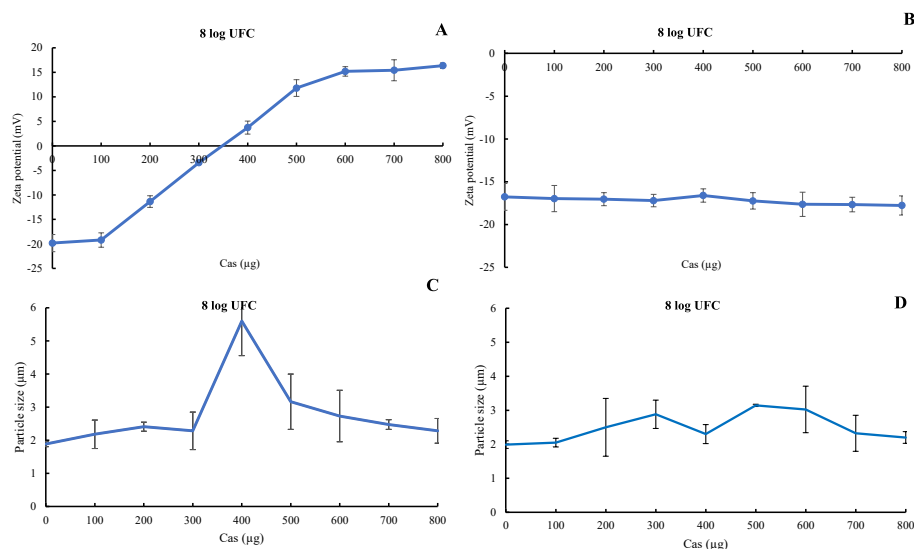


Fig. 4. Zeta potential and particle size of Cas/F21 complexes as a function of Cas/F21 ratios. Zeta potential of Cas/F21 complexes formed at pH 3 (A) and pH 7 (B), and particle size of complexes formed at pH 3 (C) and pH 7 (D).

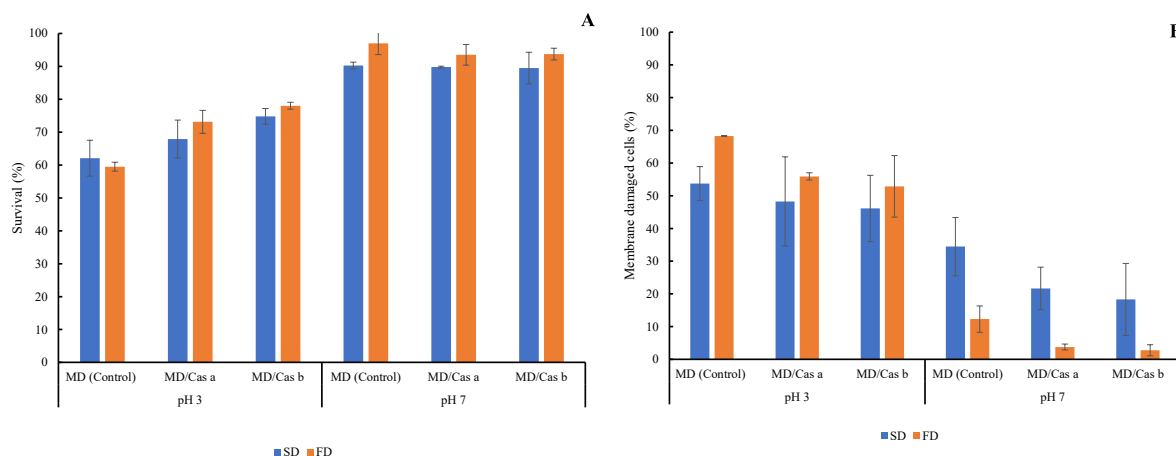


Fig. 5. Survival of microencapsulated *E. durans* F21 (A) and rate of damaged cells (with membrane damage) (B) in the different microcapsules obtained after drying.

0.035 and 1% (“MD/Cas a” and “MD/Cas b”), did not significantly affect cell viability after drying when compared to the control (MD) ($P > 0.05$). However, during microencapsulation at pH 3, a significant difference was detected between the cell viability in Cas-based microcapsules (“MD/Cas a” and “MD/Cas b”) and the control (MD) ($P \leq 0.05$). In fact, the survival rates in Cas-based microcapsules produced at pH 3 ranged from 67.85 to 78.02%, while those of MD (control) ranged from 59.47 to 62.06%.

According to the obtained results, the presence of Cas during microencapsulation at pH 3 significantly improved the survival of *E. durans* F21 cells during drying compared to the control (but not to microencapsulated cells at pH 7). Furthermore, no significant difference was detected between both Cas concentrations (0.035% and 1%), indicating that the concentration of 0.035% of Cas might be sufficient for the microencapsulation at pH 3. These findings emphasize the substantial impact of pH on *E. durans* F21 cell viability during microencapsulation, and the use of an acidic pH seemed to negatively affect survival of cells after drying. Other studies have also reported the negative impact of acidic pH on bacterial cell viability (Liu et al., 2023; Ryan et al., 2008; Sanhueza et al., 2015). According to Liu et al. (2023), microbial exposure to highly acidic environments can lead to intracellular acidification at levels capable of damaging or disrupting essential biochemical

processes, such as decreasing enzymatic activity, cellular process disruption, protein denaturation, and alteration or damage to RNA and DNA.

Moreover, these results indicate that the use of “Cas as a” bacterial cell protectant during drying can be pH-dependent. Indeed, pH is a crucial parameter in the interactions between molecules and the improved cell viability in Cas-based microcapsules (“MD/Cas a” and “MD/Cas b”) produced at pH 3 compared to the control (MD) supports the contribution of electrostatic interactions between Cas molecules and *E. durans* F21 cells (Section 3.3.2.). However, despite the positive effect of Cas during drying at pH 3, its presence (at 0.035% or 1%) proved ineffective to effectively protect *E. durans* F21 cells (from acidity and dehydration steps), resulting in significantly lower viability compared to microencapsulated cells at pH 7. Therefore, these results emphasize the importance of pH selection in the encapsulation process of *E. durans* F21, and the application of a neutral pH seems more advantageous for maintaining high cell viability after drying.

3.4.2. Membrane damage analysis

The membrane integrity of microencapsulated cells at pH 3 and 7 was determined using the PI uptake experiment (Fig. 5 B). PI is a fluorescent dye used to assess the integrity of cell membranes (Romano

et al., 2021). Intact cell membranes are impermeable to PI, but this probe can penetrate damaged membranes and bind to DNA. According to our results, the microcapsules produced at pH 3 exhibited significantly higher rates of damaged cells (with membrane damage) than those obtained at pH 7 ($P \leq 0.05$). These findings align with the results obtained in the previous section (Section 3.4.1.), where higher cell viability was observed in microcapsules obtained at pH 7 compared to those obtained at pH 3. Specifically, the rate of damaged cells (with membrane damage) in microcapsules obtained at pH 3 ranged from 46.11 to 77.26% ($P \leq 0.05$), while that of microcapsules produced at pH 7 varied from 2.77 to 34.48%.

In the case of microencapsulation at pH 3, the FD method resulted in higher membrane damage compared to SD ($P \leq 0.05$). Specifically, damaged cells detected after SD method were 53.72, 48.23 and 46.11% ($P > 0.05$) in “MD” (control), “MD/Cas a” and “MD/Cas b” microcapsules, respectively. In contrast, damaged cells reported after FD method were 77.26, 55.9 and 52.87% in “MD”, “MD/Cas a” and “MD/Cas b” ($P \leq 0.05$) microcapsules, respectively. Several factors may have contributed to the increased membrane damage in *E. durans* F21 microcapsules produced at pH 3 through FD process, including their prolonged exposure to acidic pH (at least 24 h in a freezer), as well as aggressive temperature changes, particularly during the freezing phase. Indeed, according to Wang et al. (2020), the FD process can lead to various cellular alterations, including disruptions in membrane fluidity and integrity, as well as modifications in sensitive protein structures. These cellular damages can be mainly attributed to the formation of ice crystals during the freezing steps, prolonged exposure to extreme environments, as well as high osmolarity due to water removal.

Furthermore, Cas-based microcapsules (“MD/Cas a” and “MD/Cas b”) produced at pH 3 were associated with significantly lower rates of damaged cells compared to the control (“MD”) ($P \leq 0.05$), indicating the protective effect of Cas during the microencapsulation at pH 3. However, despite its positive effect during drying at an acidic pH, the presence of Cas (at both tested concentrations) could not effectively prevent membrane damages when compared to microencapsulated cells at pH 7. In the case of microencapsulation at pH 7, microcapsules produced using SD method exhibited higher damaged cells (with membrane damage) compared to those produced through FD. This can be attributed to the high temperatures applied during SD, resulting in rapid water removal, loss of cell permeability, and protein denaturation (Kamil et al., 2020). According to our results, damaged cells in SD microcapsules were 34.4, 21.6 and 18.3% ($P \leq 0.05$) in “MD”, “MD/Cas a” and “MD/Cas b” formulations, respectively. However, damaged cells in FD microcapsules were 12.2, 3.77, and 2.77 % ($P \leq 0.05$) in “MD”, “MD/Cas a” and “MD/Cas b” formulations, respectively. Cas-based microcapsules (“MD/Cas a” and “MD/Cas b”) exhibited lower membrane damage compared to the control (MD) ($P \leq 0.05$), with reductions between 8.5% and 16.1%. However, no significant difference was detected between both Cas formulations ($P > 0.05$). Previous studies have reported the low protective effect of MD (used alone) during drying compared to other protective agents (Gul & Atalar, 2019; Tantratian & Pradeamchai, 2020). The low protective effect of MD could be explained by its inability to adequately replace water molecules due to its higher molecular weight, thus resulting in higher injured cells (Gul & Atalar, 2019).

Based on the results obtained in this study, the presence of Cas, during microencapsulation of *E. durans* F21 at both pH 3 and pH 7, showed a positive effect in reducing cell membrane damage compared to the control. Although the protective effect of Cas was previously pronounced during drying at pH 3 (Section 3.4.1.), where higher cell viability was obtained compared to the control (but not to microencapsulated cells at pH 7), its presence at pH 7 also reduced membrane damage during drying compared to the control. Therefore, the explaining hypothesis could be the contribution of hydrophobic interactions between Cas molecules and F21 cells during drying at pH 7. Indeed, *E. durans* F21 is a hydrophobic strain (Section 3.1.) and Cas like

other proteins, have hydrophobic groups along the peptide chain capable of promoting association with apolar sites on the bacterial cell surface (phospholipids, intrinsic proteins) (Leonard, 2013). Furthermore, Cas are rich in sodium salts (Na^+), and these cationic counter-ions in solution could reduce repulsive interactions between Cas and F21 cells at pH 7, thus favouring other non-specific interactions such as hydrophobic interactions (Ly et al., 2008).

3.4.3. Survival of microencapsulated cells under acidic and bile salts conditions

Survival of free and microencapsulated *E. durans* F21 cells under varying acidic conditions (pH 2.5 and pH 3) and bile salts (0.3% and 1%) is presented in Fig. 6. According to our results, all microencapsulated cells at pH 3 lost their viability after exposure to all tested conditions. However, higher survival rates were observed for free and microencapsulated cells at pH 7 under similar conditions. Under all acidic and bile salt conditions, microencapsulated cells at pH 7 using FD method showed significantly higher viability compared with those produced through SD ($P \leq 0.05$). The lower cell survival in SD microcapsules compared to FD microcapsules can be attributed to their higher rate of injured cells (with membrane damage). Indeed, at acidity pH of 2.5 (Fig. 6 A), survival rates in FD microcapsules ranged from 43.34% to 98.80%, while those in SD microcapsules varied from 25.52% to 88.42%. At pH 3 (Fig. 6 B), FD microcapsules showed survival rates ranging from 60.45% to 98.50%, meanwhile those of SD microcapsules ranged from 48.79% to 89.50%.

At 0.3% bile (Fig. 6 C), survival rates in FD microcapsules varied from 66.75% to 97.34%, while those in SD microcapsules ranged from 60.45% to 95.68%. At 1% bile (Fig. 6 A), survival rates in FD and SD microcapsules ranged from 30.58% to 65.62%, and from 28.63 to 42.06%, respectively.

Under all tested conditions, Cas-based formulations (“MD/Cas a” and “MD/Cas b”) produced at pH 7 showed significantly higher cell survivals (viability between 40% and 98%) compared to the control (MD) (viability between 25% and 66%) ($P \leq 0.05$). No significant difference was detected between both Cas formulations, indicating that Cas addition at 0.035% might be sufficient for the microencapsulation of *E. durans* F21 cells at pH 7. Furthermore, Cas-based formulations exhibited higher survival rates compared to free cells ($P \leq 0.05$). Survival rates in Cas-based microcapsules ranged from 85.75% to 98.50% at pH 2.5, 85.90%–98.80% at pH 3.0, 77.16%–97.34% at 0.3% bile, and 40.65%–65.36% at 1.0% bile. In contrast, under similar conditions, survival of free cells was 57.53%, 79.42%, 59.58% and 38.22% at pH 2.5, pH 3, 0.3% bile and 1% bile, respectively. The effectiveness of Cas-based formulations, produced at pH 7, as protective agents for bacterial cells against harsh conditions of exposure to acidity and bile salts, may support the contribution of hydrophobic interactions between Cas molecules and F21 cells during drying at pH 7 (Ly et al., 2008). We suggest that these hydrophobic interactions have exhibited an additional protective effect against high acidity and bile shock, resulting in higher cell resistance under these conditions. Our results are in agreement with other studies who reported higher survival rates of microencapsulated cells using Cas compared to free cells. Liu et al. (2016) have reported a significant protective effect of different MD/Cas ratios to protect probiotic *Lactobacillus zeeae* LB1 during exposure to simulated gastrointestinal fluid (SGF) at pH 2 and simulated intestinal fluid (SIF) at 0.08% bile for 2 and 4 h, respectively, compared to MD-only based microcapsules and free cells. Another study conducted by Zhou et al. (2023), has reported the positive influence of microencapsulation of *Bacillus coagulans* BC01 in Cas microcapsules through SD method to protect cells during exposure to *in vitro* gastrointestinal digestion, compared to free cells. The application of Cas for the microencapsulation of *Lb. acidophilus* through SD method significantly enhanced the viability of the strain during exposure to SGF and SIF for 2 and 6 h respectively (Dianawati et al., 2017). On the other hand, MD microcapsules (control) showed lower cell viability after exposure to all tested

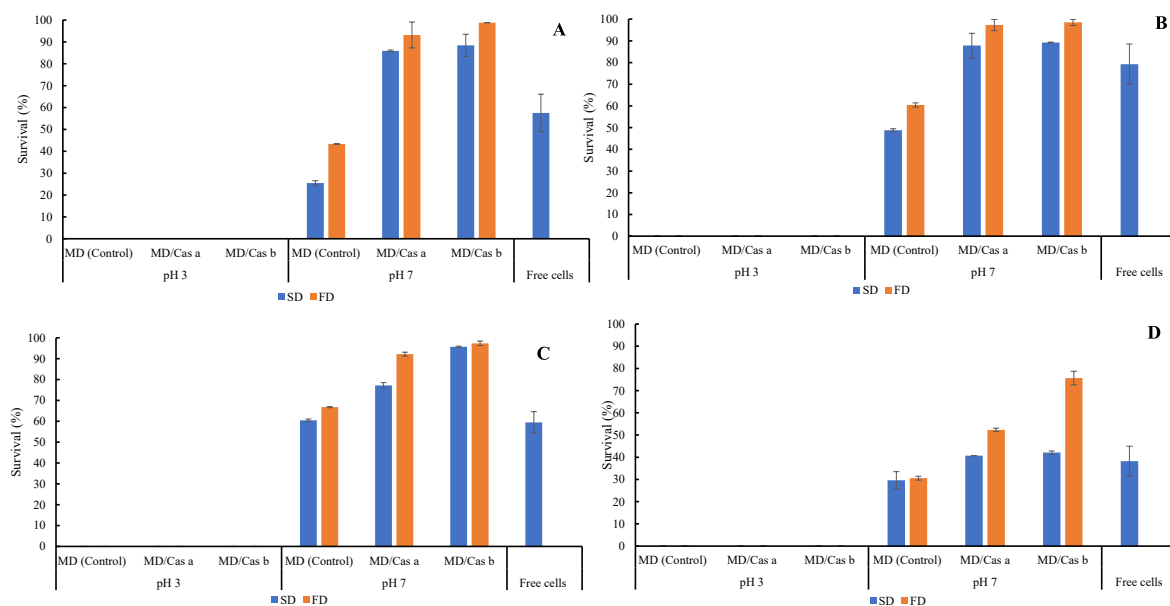


Fig. 6. Survival of free and microencapsulated *E. durans* F21 after exposure to acidic conditions of pH 2.5 (A) and pH 3 (B) and to bile salts conditions of 0.3% (C) and 1% (D).

conditions compared to free cells ($P \leq 0.05$), which can be attributed to the higher number of injured cells (with membrane damage) after drying process.

Regarding the other set of experiments conducted in this study, microencapsulated *E. durans* F21 cells at pH 3 lost all their viability following exposure to subsequent stress conditions, including high acidity and bile shock. Moreover, despite its positive effect as a bacterial cell protectant during drying at pH 3 compared to the control, the addition of Cas (at both concentrations) was ineffective in protecting cells under all the tested conditions. According to the literature, several studies have reported that pre-exposure of microbial cells to initial stress conditions, such as extreme temperatures, oxidative stress, or pH variations, can induce an adaptive cellular response, enhancing their resistance and survival under subsequent stress conditions (Guillén et al., 2021; Min et al., 2020; Tang et al., 2020). However, other studies have reported that initial exposure of cells to stress can compromise their physiological state, making them more susceptible to subsequent stress factors such as high acidity and the presence of bile salts (Barbosa et al., 2012; Garner et al., 2006; Samara & Koutsoumanis, 2009). Similarly, in this study, microencapsulated *E. durans* F21 cells at pH 3 exhibited increased sensitivity to subsequent stress conditions (high acidity and bile shock) after initial pre-exposure to stress conditions (pH 3 and dehydration steps), contributing to a complete loss of their viability.

3.4.4. Antimicrobial activity of microencapsulated cells

The antimicrobial activity of *E. durans* F21 could be attributed to a produced metabolite that has been identified as bacteriocin (Benkirane et al., 2023). The effect of the formulation, the pH and the drying methods on the antimicrobial activity of *E. durans* F21 was investigated. The results obtained by deferred antagonism assay to detect the ability of microencapsulated cells in the different microcapsules to inhibit *L. monocytogenes* B806 are presented in Table 2. Although there were injured cells within all microcapsules (and specifically in those produced at pH 3), microencapsulated *E. durans* F21 cells maintained their bacteriocin production ability, and the bacteriocin activity was retained against indicator strain. No significant differences were detected between microencapsulated and free cells ($P > 0.05$). These findings are in agreements with those previously reported for other dried preparations of LAB, where antimicrobial activity was maintained during drying conditions (Kamil et al., 2020; Silva et al., 2002).

Table 2

Antimicrobial activity of free and microencapsulated *E. durans* F21.

Drying method	Encapsulant formulation	Formulation pH	Antimicrobial activity (mm)
SD	MD (Control)	3	14.5
		7	14.5 ± 0.7
	MD/Cas a	3	14.5 ± 0.7
		7	15 ± 0.7
	MD/Cas b	3	15
		7	15
FD	MD (Control)	3	14.5 ± 0.7
		7	15
	MD/Cas a	3	14.5 ± 0.7
		7	15
	MD/Cas b	3	14.5 ± 0.7
		7	15
Free cells	-	-	15

3.4.5. Stability of powders

The viable cell counts of *E. durans* F21 microcapsules stored at 4 °C and room temperature were evaluated during 8 weeks of storage (Fig. 7). According to our results, all tested microcapsules displayed higher counts when stored at refrigeration temperature (4 °C) (Fig. 7 A, B) compared to ambient temperature ($P \leq 0.05$) (Fig. 7 C, D). Moreover, the stability of microcapsules produced at pH 7 (reduction of cell viability between 0.42 and 74.85%) (Fig. 7 A, C) was significantly higher than those obtained at pH 3 (cell reduction between 27.83 and 100%) ($P \leq 0.05$) (Fig. 7 B, D). Indeed, during storage at 4 °C, Cas-based formulations produced at pH 7 using FD method were the only ones to maintain the viability of the strain without any remarkable loss of cell viability upon the storage period (cell reduction between 0.42 and 1.72%, respectively), with no significant difference detected between Cas-based microcapsules (“MD/Cas a” and “MD/Cas b”) and the control ($P > 0.05$) (Fig. 7 A). Moreover, it is interesting to highlight that these microcapsules maintained their cell viability above 6 log CFU/g, which is generally recommended for probiotics to exert their beneficial effects (Terpou et al., 2019). In contrast, for SD powders, a significant loss in cell viability was observed throughout the storage period (cell reduction between 21.52 and 36.31%) ($P \leq 0.05$), which can be attributed to higher injured cells (with membrane damage) in these microcapsules (Fig. 7 A).

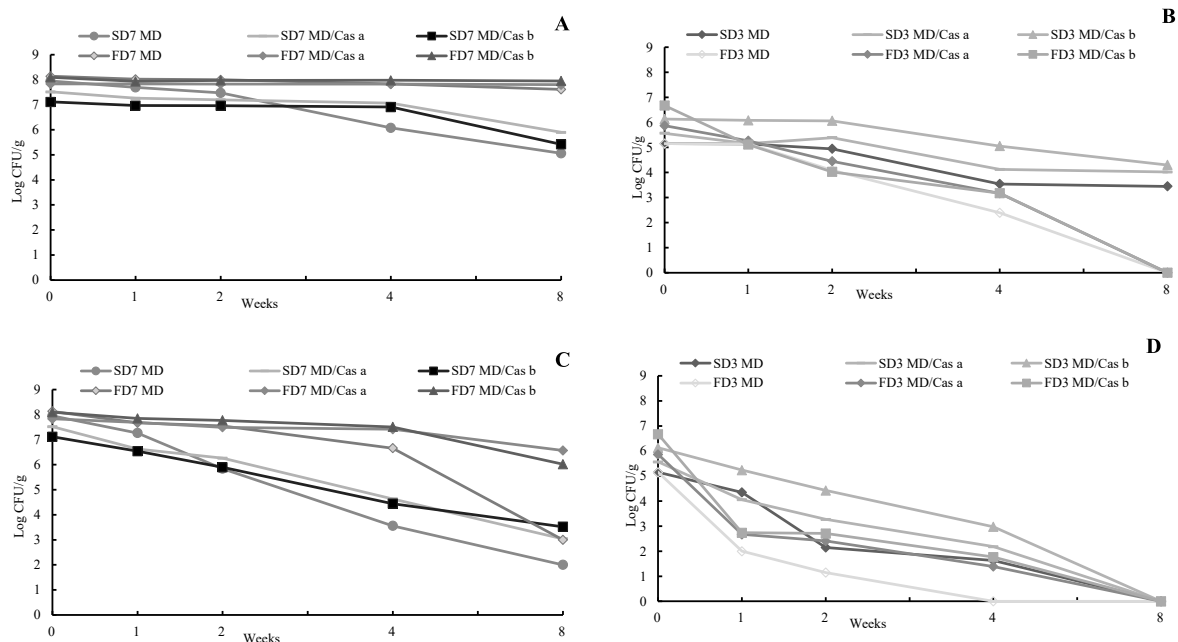


Fig. 7. Stability of microencapsulated *E. durans* F21 during 8 weeks of storage at 4 °C and room temperature. Microcapsules produced at pH 7 (A) and pH 3 (B) stored at 4 °C, and microcapsules produced at pH 7 (C) and pH 3 (D) stored at room temperature.

Concerning the microcapsules produced at pH 3 and stored at 4 °C (Fig. 7 B), the stability of *E. durans* F21 cells was significantly impacted during the storage period, and the viability loss was more pronounced compared to microencapsulated cells at pH 7. Notably, microcapsules produced through FD process were the most sensitive, with no viable cells being detected after the storage period (100% of cell reductions). Conversely, samples obtained through SD method were more resistant (reduction between 27.83 and 33.11%), which can be attributed to lower injured cells in these microcapsules compared to FD.

When the microcapsules were stored at room temperature (Fig. 7 C, D), *E. durans* F21 viability loss in all the microcapsules was more significant compared to refrigeration temperature ($P \leq 0.05$). For samples produced at pH 7 (Fig. 7 C), the FD process maintained higher cell viability compared to SD (with reduction varying between 16.18 and 63.14%, and between 50.5 and 74.85%, respectively). The Cas-based microcapsules obtained through FD process exhibited higher cell stability compared to the control ($P \leq 0.05$), with a maintained cell viability above 6 log CFU/g. The stability of Cas-based microcapsules can be attributed to lower damaged cells (with membrane damage) compared to the control. Moreover, no significant difference was detected between both Cas formulations (“MD/Cas a” and “MD/Cas b”), suggesting that 0.035% Cas concentration might be sufficient for the microencapsulation of *E. durans* F21 at pH 7. However, all microencapsulated cells at pH 3 revealed a complete loss (reductions of 100%) of their viability within 8 weeks of storage (Fig. 7 D).

Practically, retaining cell viability during storage period is challenging due to the combination of endogenous and exogenous factors (i. e., residual moisture, storage temperature, growth state of the culture, physical state of the microcapsules together with the presence of growth stimulate or oxygen scavengers) contributing to cell death. These factors are critical at influencing the storage stability of bacterial cells. Since the stability of microorganisms can be affected by storage temperature, the survival of microencapsulated cells at low temperatures such as refrigeration can be advantageous for prolonged storage (de Andrade et al., 2019). Under low temperatures, cells are stored in a latent state, avoiding the rearrangement of the wall material, thus preventing inadequate exposure of the microorganisms (de Andrade et al., 2019). Also, cold storage results in reduced rates of detrimental chemical reactions, such as fatty acid oxidation (Heidebach et al., 2010).

In this study, microencapsulation of *E. durans* F21 at pH 3 was ineffective in maintaining higher cell viability during storage conditions. Conversely, microencapsulated cells at pH 7 exhibited higher resistance, and addition of Cas (at 0.035% and 1%) displayed a positive effect on cell stability under similar conditions. Moreover, FD method proved to be the most effective to produce dry *E. durans* F21 microcapsules with higher viability and stability during the prolonged storage.

3.5. Physicochemical characteristics of powders

Physicochemical properties of powders have direct implications on shelf-life, quality, cell viability, and storage stability of dried microcapsules containing probiotic cells (Yoha et al., 2020). The physicochemical properties of the microcapsules containing *E. durans* F21 are presented in Table 3. The powders yield (%) is related to the efficiency of the applied drying methods. Both SD and FD methods resulted in satisfying product yields, which ranged from 79.42% to 83.28% for SD powders ($P > 0.05$) and from 81.41% to 85.87% for FD ($P > 0.05$). Despite SD powders exhibited lower yield compared to FD powders, no significant difference was detected between both drying methods ($P > 0.05$). The decrease in product yield in SD powders can be attributed to the adhesion of the powder particles to the cyclone (Kamil et al., 2020).

Drying conditions have an important effect on the residual water content of dried products. According to our results, the encapsulating materials or the used pH did not result in a significant difference in residual water content of microcapsules. However, a significant variation was observed between the two drying methods ($P \leq 0.05$). The FD powders showed higher moisture compared to SD powders ($P \leq 0.05$). The moisture content in SD powders was in the range of 2.05% and 3.50% ($P > 0.05$). Meanwhile, the moisture content in FD powders ranged between 5.50% and 5.75% ($P > 0.05$). It is obvious that the SD method produces low moisture content as high temperature was used. Also, the moisture content of all the samples was around 2–6% as generally recommended for the stability of probiotic powders during the prolonged storage (Misra et al., 2022). Moreover, an a_w below 0.6 is also recommended to ensure prolonged stability of probiotics during storage (Arepally et al., 2020). The a_w values of powders ranged between 0.30 and 0.45 with no significant difference between drying methods ($P >$

Table 3
Physicochemical properties of *E. durans* F21 microcapsules.

Drying method	Encapsulant formulation	Formulation pH	Yield (%)	Moisture content (%)	Water activity (a_w)	Hygroscopicity (%)	Particle mean size D [4,3] (μm)
SD	MD (Control)	3	79.64 \pm 4.12 ^a	2.5 \pm 0.7 ^a	0.34 \pm 0.00 ^a	15.8 \pm 2.8 ^a	7.9 \pm 0.8 ^a
		7	79.42 \pm 4.43 ^a	3.5 \pm 0.7 ^{ab}	0.39 \pm 0.004 ^a	13.85 \pm 3 ^a	8 \pm 0.8 ^a
	MD/Cas a	3	80.56 \pm 1.20 ^a	2.05 \pm 0.07 ^a	0.33 \pm 0.01 ^a	16 \pm 1.41 ^a	8.54 \pm 0.23 ^a
		7	82.15 \pm 1.95 ^a	2.5 \pm 0.7 ^a	0.37 \pm 0.006 ^a	13.5 \pm 2.12 ^a	7.8 \pm 0.41 ^a
	MD/Cas b	3	83.28 \pm 3.61 ^a	2.08 \pm 0.1 ^a	0.30 \pm 0.009 ^a	18 \pm 1.4 ^a	9.19 \pm 0.08 ^a
		7	82.77 \pm 0.44 ^a	3.11 \pm 1.2 ^a	0.33 \pm 0.06 ^a	15.5 \pm 0.7 ^a	9.13 \pm 0.02 ^a
FD	MD (Control)	3	84.38 \pm 2.58 ^a	5.5 \pm 0.7 ^c	0.39 \pm 0.003 ^a	13.4 \pm 2.2 ^a	165.6 \pm 7 ^d
		7	81.41 \pm 1.46 ^a	5.55 \pm 0.7 ^c	0.41 \pm 0.03 ^a	13.3 \pm 2.4 ^a	130.8 \pm 9.3 ^{bc}
	MD/Cas a	3	82.44 \pm 1.41 ^a	5.05 \pm 0.6 ^{bc}	0.41 \pm 0.04 ^a	13.5 \pm 0.7 ^a	135 \pm 4.3 ^{bc}
		7	82.69 \pm 1.06 ^a	5.05 \pm 0.6 ^{bc}	0.45 \pm 0.07 ^a	16 \pm 1.41 ^a	149.2 \pm 5.21 ^c
	MD/Cas b	3	85.42 \pm 1.34 ^a	5.5 \pm 0.7 ^c	0.34 \pm 0.04 ^a	12 \pm 1.4 ^a	136.6 \pm 9.2 ^c
		7	85.87 \pm 0.02 ^a	5.75 \pm 1 ^c	0.39 \pm 0.11 ^a	12 \pm 1.4 ^a	127.8 \pm 7.6 ^b

0.05). This ensures high cell stability during prolonged storage.

Hygroscopicity is the ability of powders to absorb moisture from the environment. It is related to environmental conditions (storage temperature and relative humidity), but also to structural aspects and encapsulating materials. As a direct consequence of the increase in water mobility, enzymatic or chemical reactions can take place, leading to a faster deterioration of the quality and modification of the physical properties of the final product (Soukoulis et al., 2014). In this study, the hygroscopicity of obtained powders was not significantly influenced by the drying method or the encapsulating material and the pH ($P > 0.05$). According to Nurhadi et al. (2016), the hygroscopicity of a powder is acceptable if it is below 20 g per 100 g of powder. The hygroscopicity of all samples ranged between 12 and 18 g/100 g of powder which meets this criterion.

Particle size is a crucial parameter in the characterization of dried products as it impacts the microcapsules appearance, irregularities, fluidity and overall texture (Agyare et al., 2022). Our results indicate that the encapsulating formulation and pH did not result in significant differences in microcapsules size ($P > 0.05$). However, a significant difference was detected between drying methods ($P \leq 0.05$). Microcapsules produced through the SD method exhibited sizes ranging from 7.80 μm to 9.19 μm ($P > 0.05$), whereas those produced by FD method were considerably larger (approximately 17 times larger) with a size range of 127.8 μm –165.6 μm . The difference in size between SD and FD microcapsules can be attributed to FD process, which leads to the formation of products with a physical appearance similar to a porous sponge. Consequently, a subsequent manual grinding process using a mortar or pestle is required to reduce their size to an appropriate level (Kamil et al., 2020).

Scanning electron microscopy (SEM), an embedding and microtomy method, was applied to study the morphology and internal structure of SD and FD microcapsules. The microscopic observations of the different microcapsules are shown in Fig. 8. Regardless of the formulation and pH, the SD powders (Fig. 8A–F) were characterized by a spherical and irregular shape with concavities and surface deflection, but without the appearance of signs of cracks. Similar surface morphology has been previously reported in several studies (Li & Zhong, 2023; Pius Bassey et al., 2024; Soukoulis et al., 2014). According to the literature, the surface morphology of SD microcapsules is affected by various factors such as the inlet temperature, the encapsulating material, and also the

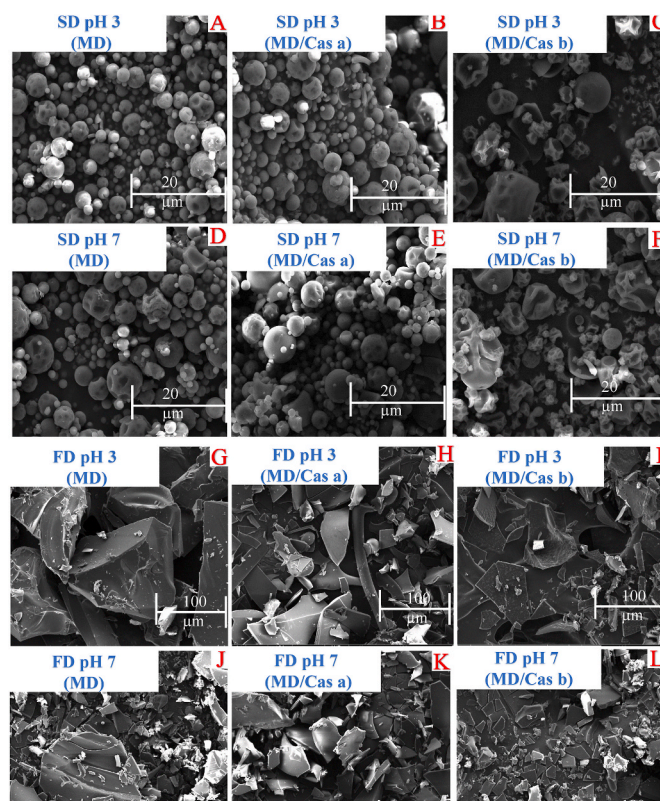


Fig. 8. Scanning electron microscopy (SEM) of *E. durans* F21 microcapsules obtained by SD (A–F) and FD (G–L) methods. SD microcapsules produced at pH 3 using MD (A), “MD/Cas a” (B) and “MD/Cas b” (C); and at pH 7 using MD (D), “MD/Cas a” (E) and “MD/Cas b” (F). FD microcapsules produced at pH 3 using MD (G), “MD/Cas a” (H) and “MD/Cas b” (I); and at pH 7 using MD (J), “MD/Cas a” (K) and “MD/Cas b” (L).

viscosity of the solution. Alamilla-Beltran et al. (2005) found that the application of higher inlet temperatures led to the formation of stiffer microcapsules with porous surfaces, which was our case (160 °C).

Furthermore, these morphological irregularities of the microcapsules may be related to the drying conditions, including heat penetration and rapid evaporation of water from the liquid droplet. Considering the effect of the formulation on the morphological aspects of the SD microcapsules, the addition of Cas at 1% ("MD/Cas b") resulted in more deflated microcapsules with deeper depressions and bumps (Fig. 8 C, F). Similar morphology was observed by Soukoulis et al. (2014), who obtained *Lb. acidophilus* encapsulated in the presence of MD and Cas with spherical conformation and formation of deep concavities, as well as Patil et al. (2023) who obtained SD particles with different sizes as well as irregular, spherical and concave surfaces. According to the observations of Li and Zhong (2023), the use of Cas in the encapsulation of a milk protein isolate (MPI) resulted in a hard crust in the caseinate micelle-rich surface layer upon drying of individual droplets, which collapsed when water was quickly removed. In contrast to "MD/Cas b" matrices, microcapsules containing MD (control) (Fig. 8 A, D) and Cas at 0.035% ("MD/Cas a") (Fig. 8 B, E) exhibited a similar microstructure, indicating that the minor variation in Cas concentration did not result in a significant difference. Regarding the FD powders (Fig. 8G–L), the obtained microcapsules presented an irregular shape like broken glass with some pores on the surface. The formation of pores and cavities on the surface of the microcapsules could be attributed to the sublimation of ice crystals (formed during the freezing process) (Misra et al., 2023). The composition of the encapsulation matrix and the drying rate, especially in the early stages, can affect the surface properties of the microcapsule (Elham et al., 2022). According to the obtained results, the structure of all FD samples was uneven and brittle. Microcapsules images obtained in the absence of Cas (Control) (Fig. 8 G, J) or in the presence of Cas (Fig. 8 H, K, I, L) were similar and all particles showed a smooth surface. The difference in the morphological aspect of FD powders compared to SD may be associated to mechanical grinding of the powders after the FD process (Kamil et al., 2020).

4. Conclusion

The main objective of this study was to develop *E. durans* F21 microcapsules, through SD and FD methods, with high potential for use as probiotics and bio-preservative agents in foods. *E. durans* F21 exhibited promising probiotic properties, including moderate auto-aggregation (47.7 %), high co-aggregation with pathogens (between 76% and 81%), moderate biofilm formation (OD_{590nm} of 0.162), and resistance to *in vitro* simulated gastrointestinal conditions. The encapsulation pH was shown to be a crucial factor affecting the viability of microencapsulated *E. durans* F21 cells. In fact, microencapsulation at pH 3 adversely affected cell viability after drying compared to pH 7. Although Cas (at 0.035 or 1%) exhibited a positive effect as a bacterial cell protectant during microencapsulation at pH 3-where higher cell viability and lower damaged cells were detected after drying compared to the control (but not to microencapsulated cells at pH 7)- no protective effect of Cas was pronounced following exposure to *in vitro* simulated gastrointestinal conditions or during prolonged storage. In contrast, microencapsulation at pH 7 was found to be most effective in maintaining higher cell viability during drying (viability between 89% and 96%). Moreover, the addition of Cas (at 0.035 and 1%) significantly reduced the rate of damaged cells during drying (reductions between 8.5% and 16.1%) compared to the control (MD), and enhanced cell viability during exposure to *in vitro* simulated gastrointestinal conditions (viability between 40% and 98%) and during storage (viability between 74.3% and 99.5%). Furthermore, FD was identified as the most effective method for maintaining higher cell viability.

In conclusion, the production of *E. durans* F21 microcapsules through FD method using the following formulation: 8 log CFU/mL cells, 0.035% Cas, 10% MD and at pH 7, could be a promising strategy for the production of highly viable F21 cells with appropriate properties for potential use as probiotic and bio-preservative agents in foods. Future research should be carried out to investigate the preservative potential

of these microcapsules in extending the shelf life of various food products.

CRedit authorship contribution statement

Ghita Benkirane: Writing – original draft, Methodology, Investigation. **Samir Ananou:** Writing – review & editing, Supervision, Funding acquisition. **Géraldine Agusti:** Investigation, Data curation. **Laila Manni:** Supervision. **Nour-Eddine Chihib:** Validation, Methodology. **Emilie Dumas:** Writing – review & editing, Supervision, Investigation, Funding acquisition. **Adem Gharsallaoui:** Writing – review & editing, Supervision, Funding acquisition.

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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