Innovative Food Science and Emerging Technologies 100 (2025) 103885

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

Unraveling the real potential of liquid whey as media culture and microencapsulation material for lactic acid bacteria

Cristian Mauricio Barreto Pinilla [a,*], Fabiana Galland [b], Maria Teresa Bertoldo Pacheco [b], Paula Janetti Bócoli [c], Daniele Fidelis Borges [c], Izabela Dutra Alvim [d], Leila Maria Spadoti [a], Adriana Torres Silva e Alves [a]

[a] Dairy Technology Center, Institute of Food Technology (ITAL), Campinas, São Paulo, Brazil
[b] Food Science and Quality Center, Institute of Food Technology (ITAL), Campinas, São Paulo, Brazil
[c] Packaging Technology Center, Institute of Food Technology (ITAL), Campinas, São Paulo, Brazil
[d] Cereal and chocolate Technology Center, Institute of Food Technology, Brazil (ITAL), Campinas, São Paulo, Brazil

ARTICLE INFO

Keywords:
Cheese whey
Lactic acid bacteria
Bioprocess
Drying

ABSTRACT

This study aimed to evaluate the circular use of plain whey as media culture and microencapsulation material lactic acid bacteria (LAB). For this, *Lacticaseibacillus paracasei* ItalPN16 grew in a bioreactor (1 L) at different controlled pH using plain whey, and the sugar consumption, biomass yield, and protein hydrolysis were monitored. As results, at the pHs evaluated (4.5. 5.5, and 6.5) bacterial counts above 10 Log CFU/mL and a consumption of around 30 % of the whey sugars were obtained. Protein analysis revealed different proteolysis patterns according to the pH, resulting in more immunoglobulins (IgG) hydrolysis at pH 6, and increased free amino acids. After bacterial drying using its own growth media, the range for encapsulation efficiencies was 72–90 % for spray-dried (SD) and 82–99 % for freeze-dried (FD) powders. In addition, Fourier transform infrared spectroscopy analysis (FTIR) revealed specific variations in the hydrophilic interactions (-OH) in the microcapsules, according to drying method. The microencapsulated *L. paracasei* using fermented whey at pH 6 as wall material showed notable tolerance to simulated gastrointestinal conditions. Regarding the powder's stability, the FD method proved to be more effective in protecting the *L. paracasei* during storage at 4 °C and 25 °C. Thus, using plain whey as a culture medium in pH-controlled fermentation, followed by microencapsulation through freeze-drying, has proven to be a sustainable, scalable, and economical alternative for producing commercial lactobacilli cultures.

1. Introduction

About 186 million tonnes (MT) of liquid cheese whey (CW) are globally produced by the dairy industry every year and from this amount, 66.5 MT of whey is not currently absorbed by the industry (Tsakali et al., 2010; Verma et al., 2023). However, over the past decades, several sustainable and economically efficient strategies of whey valorization have been studied, mainly regarding biotechnological products and food ingredients. These approaches have resulted in products such as whey powders, whey proteins with different levels of purification, lactic acid, bioplastics, and biofuels (Buchanan et al., 2023). In this context, whey has transitioned from a polluting waste to a highly valuable dairy co-product, and ongoing research aimed at discovering more feasible and environmentally friendly alternatives for whey utilization (Zandona et al., 2021). However, a large portion of the world's whey production is still discarded as effluent, mainly in small dairy industries that don't have an appropriate treatment system (Giulianetti de Almeida et al., 2023). Thus, the development of new methods for CW utilization is still relevant to add value to this by-product and contribute to their use reducing the environmental and economic effects of their discard.

In this scenario, applied technology approaches such as fermentation processes for biomass production and conversion of CW into high-value products, have emerged as potential ways for biotechnology whey utilization (Verma et al., 2023). One interesting approach is the use of CW as a culture medium for probiotic lactic acid bacteria (LAB), considering that CW being a low-cost source of carbohydrates, proteins, vitamins, and minerals (Krunić et al., 2019). Additionally, Aller et al. (2014)

* Corresponding author at: ITAL. Brasil Avenue 2880, PO Box 139, Campinas, SP 13070-178, Brazil.
E-mail address: cristianmaobarreto@gmail.com (C.M. Barreto Pinilla).

https://doi.org/10.1016/j.ifset.2024.103885
Received 2 August 2024; Received in revised form 29 November 2024; Accepted 1 December 2024
Available online 9 December 2024

reported that the branched-chain amino acids (BCAAs), l-isoleucine (Ile), l-leucine (Leu), and l-valine (Val) present in CW, enhance the growth of LAB such as *Lactococcus lactis* IL1403. Despite its potential as a media culture for LAB, whey has some drawbacks, including low levels of free amino acids and high concentrations of lactose, which represents a challenge for LAB metabolism.

LABs have fastidious nutritional requirements, which means that slight variations in the components of their cultivation media can affect their growth. Selecting the right growth medium for industrial-scale microorganism production is crucial for fermentation processes. This choice is particularly important because it constitutes a significant portion of the overall operational costs and influences both the cell concentration and the time needed to achieve optimal cell yields (Huang et al., 2016). Thus, the scale-up and creating a whey-based media with optimal performance is still a challenge. In this context, most of the research in the last years has been focused on developing different whey-based culture media adding additional nutrients (mainly sources of soluble protein) to favor the microorganism's growth (Huang et al., 2016; Krunić et al., 2019; Sharma et al., 2017). Research works that employ CW without supplementation, or CW without any protein concentration process (*e.g.*, whey permeates or whey protein isolate) as medium for biomass production of LAB are scarce. This highlights the need for further research and innovation in fermentation process with plain whey to overcome their physicochemical drawbacks and to obtain high biomass yields at low-cost.

After production, LABs are sensitive to food processing and storage, as well as passage through the gastrointestinal tract, thus, encapsulation technology using different matrixes is used to improve their functionality and viability (Vivek et al., 2022). Among the different wall materials used for LAB encapsulation, one of the most used is whey protein concentrate (WPC), due to its solubility, protein concentration, and low lactose content. Some authors studied the use of plain whey as wall material for encapsulating LAB through spray drying. They reported high bacterial survival after drying, but the stability of the powders stored at room temperature was low. (Aragón-Rojas et al., 2018; Rabaioli Rama et al., 2020; Senovieski et al., 2024). In our revision, we did not find any report of LAB freeze-drying using plain whey as a protective material.

In this context, our research group reported the growth and improved functional properties of *L. paracasei* ItalPN16 in plain sweet whey (SW) and acid whey (AW) at 37 °C, in free fermentation, however, the maximum biomass yield was 9.5 Log/CFU mL (Barreto Pinilla et al., 2023a). Thus, due to high concentrations of lactic acid may affect cell physiology causing growth interruption (Vinderola et al., 2019), CW fermentation with pH control close to neutrality, could support a higher growth rate and improve the biomass yield. In addition, the specific physicochemical features of the fermented whey under pH control, could favor their use as wall material for bacterial microencapsulation.

The industrial production of probiotic lactic acid bacteria (LAB) relies heavily on the effective bacterial growth, concentration, and preservation of biomass to ensure long-term viability and functional activity (Pramanik et al., 2023). Thus, this study aimed to scale up the use of unsupplemented cheese whey as a culture medium for the high-yield biomass production of *L. paracasei* ItalPN16 in a one-liter bioreactor, evaluating different pH levels during the fermentation process, and explored the potential of using the resulting fermented culture medium as part of the encapsulation material for spray-drying and freeze-drying.

2. Materials and methods

2.1. Bacterial strain and media

The strain *Lacticaseibacillus paracasei* ItalPN16 (GenBank accession number JALGQW000000000) from the culture collection of Dairy Technology Center (Tecnolat/ITAL) was maintained in a frozen stock culture of de Man, Ragosa and Sharpe broth (MRS. Merck, Darmstadt, Germany) containing 15 % (*v*/v) glycerol (Barreto Pinilla, Guzman Escudero, et al., 2023). The strain was incubated overnight in MRS media at 37 °C in anaerobic conditions before the experiments. Sweet whey (SW) was obtained from the pilot scale fabrication of Prato cheese in the Tecnolat/ITAL and conserved at −20 °C. Before using it for *L. paracasei* ItalPN16 growth, the whey was pasteurized at 65 °C for 30 min to avoid protein denaturation. After pasteurization, routine microbiological analyses were performed to confirm the sterility of whey before inoculation. The proximate composition of the SW used in this work was reported in a previous publication of our research group (Barreto Pinilla, Brandelli, et al., 2023).

2.2. Bacterial growth in bioreactor

Overnight cultures (MRS, 37 °C) of *L. paracasei* were inoculated (3 %, *v*/v) in 1 L of SW (previously pasteurized at 65 °C by 30 min) and incubated at 37 °C for 48 h with agitation of 100 rpm. Three different pH conditions were evaluated (pH 6.5, 5.5, and 4.5), controlling the acidification of the media with NaOH (1 M). These three treatments were selected to identify differences in the *L. paracasei* growth profile and changes according to the solubility of SW protein fractions. The concentration of sugars and the viable cell counts were determined at 0, 4, 8, 24, 32, and 48 h of fermentation by the DNS method (Miller, 1959) and using MRS agar plates incubated for 48 h at 37 °C under anaerobic conditions, respectively. After each fermentation, samples of media culture free from cells were collected to analyze the whey protein hydrolysis and the amino acids profile. The obtained fermented media and the bacterial biomass were used in the freeze-dried and spray-dried processes. The experiments were carried out in triplicate.

2.3. Hydrolysis of whey proteins

Whey protein degradation was determined after 48 h of fermentation. Before its determination, the whey protein was concentrated by centrifugation using Amicom ultra 3kD membrane filters, in order to eliminate the sugars and low molecular size nitrogen compounds. After this step, the native whey and the resultant media culture after fermentation at 6.5, 5.5, and 4,5 were diluted in a sample buffer (0.0625 M tris–HCl, pH 6.8, 2 % (*w/v*) SDS, 5 % (w/v) mercaptoethanol, 10 % (*v*/v) glycerol, 0.002 % (w/v) of bromophenol blue and subsequently boiled and centrifuged 10.000 *g* for 5 min. Protein content was measured by Lowry's method, using bovine serum albumin as standard. Equal amounts (20 μg) of protein and the molecular weight (MW) marker (Prestained protein ladder, Broad Range, 2–212 kDa, Sigma) were loaded separately. The SDS–PAGE was carried out on 12 % (w/v) polyacrylamide gels on vertical slab electrophoresis cells (BIO-RAD Mini PROTEANs 3 System, Hercules, CA, USA) for 1,5 h at 130 *V*. Coomassie brilliant blue R250 was used for staining the gels.

2.4. Total amino acids by high-performance liquid chromatography (HPLC)

The determination of total amino acids (AA) was performed as reported previously by Santos de Espindola et al. (2023). Briefly, the amino acids in the samples were obtained through acid hydrolysis for 22 h in a digester block at 120 °C, using a 6 N hydrochloric acid (HCl) and phenol solution. Then, the samples were treated with phenyl isothiocyanate (PITC) as a pre-column reaction. The separation and identification were performed in a liquid chromatograph equipped with a diode array detector (DAD) (Shimadzu, Brazil) and a C18 Luna-Phenomenex reversed-phase column (4.6 mm × 250 mm; particle size 5 μm) (Phenomenex Inc., Torrance, USA) using as mobile phase A of sodium acetate/ acetonitrile, and B, ultra-pure water/disodium EDTA, using a wavelength of 254 nm. The concentrations of each AA were obtained by comparing the results with the amino acid standard and the internal standard α-aminobutyric acid (AAAB).

2.5. Spray-dried and freeze-dried microencapsulation

Prior to the microencapsulation process, the cells were harvested and concentrated by centrifugation (4000 rpm, 4 °C, and 10 min). Then, the pellet was resuspended in half of its initial volume using the same culture media previously mixed 1:1 with an autoclaved solution of maltodextrin (20 %). Thus, the mixtures (fermented whey and maltodextrin) were used as wall material. Finally, the formulations were separated into two flasks. One part was dried using a spray dryer (SD) B290 (Büchi, Switzerland) with a 1.5 mm diameter atomizing nozzle, inlet temperature of 145 °C, atomizing air flux 0.15 MPa, feeding rate 250 mL/h, and outlet temperature controlled at 75 °C. The second part was frozen at −70 °C for 24 h and dried through lyophilization (FD) for 24 h (Liotop, São Paulo, Brazil). After microparticles production, samples were packed in enclosed plastic recipes to avoid humidification, sealed, and stored at 24 °C and 4 °C for the stability test. The remaining product was stored at −20 °C for the characterization analysis. The samples were identified according to the drying method and the pH used in the fermentation of SW (*e.g.* SD-pH 4.5 or FD-pH 4.5).

2.6. Powders characterization

2.6.1. Encapsulation efficiency

The encapsulation efficiency (EE %) was defined as the percentage of viable cells in the microparticles immediately after the microencapsulation process, compared to the viability of the culture in the liquid encapsulating materials before drying, according to the Eq. (1) (de Araújo Etchepare et al., 2020).

$$EE\% = \left(\frac{N}{N_0}\right) x100 \quad (1)$$

EE = encapsulation efficiency in %.

N_0= number of viable cells in the encapsulating matrix before spray drying.

N = number of viable cells in the powder.

2.6.2. Moisture and water activity

The A_w was determined using a digital water activity meter (AquaLab®), and the moisture was determined by direct drying in a laboratory oven at 105 °C for 24 h.

2.6.3. Scanning electric microscopy

The powders obtained for both drying processes were placed on carbon tape fixed on stubs and metalized in gold and palladium for 50s. Analyses were performed using a TESCAN VEGA LMU-SEM microscope (Tescan Analytics®, Brno, Czech Republic) at different magnifications, high vacuum, and 3–8 kV voltage.

2.6.4. Fourier-transform infrared (FTIR) spectroscopy

The FTIR spectra of maltodextrin and the microcapsules obtained from the encapsulations of *L. paracasei* by spray and freeze-dried were acquired using an Attenuated Total Reflectance Fourier Transform (ATR-FTIR) spectrophotometer (Perkin Elmer, Spectrum 100). The spectra were recorded at a resolution of 2 cm^{-1} and 16 scans per min over the wavenumber range of 650–4000 cm^{-1}.

2.7. Acid and bile resistance

Simulated gastric juice (SGJ) consisted of 9 g/L of sodium chloride containing 3.0 g/L of pepsin (Dinâmica, Indaiatuba, SP, Brazil) with pH adjusted to 2.0 using HCL. Before the analysis, the powder samples were hydrated (1:10) with sterile distilled water. Then, 0.2 mL of each suspension was mixed in 9.0 mL of SGJ and incubated for 60 and 120 min at 37 °C with constant agitation at 50 rpm. Surviving bacteria were enumerated by pour plate counts in MRS agar anaerobically incubated at 37 °C for 48 h. 0.2 mL of a standardized suspension (OD 600 of 0.5) of an overnight culture (MRS and 37 °C) of *L. paracasei*, harvested by centrifugation, and washed twice with sterile saline solution (8.5 g/L NaCl), were used as control of unencapsulated bacteria.

The survival percentage of the samples was calculated as follows:

%Survival = (CFU at the end of the test/Initial CFU inoculated)*100

The simulated intestinal juice (SIJ) was prepared by dissolving bile salts to final concentrations of 3.0 g/L in intestinal solution (Sigma, Poole, Dorset, UK) and adjusted to pH 7.5. The survivor index of *L. paracasei* to bile salts was determined using the same method related above for the SGJ.

The survival percentage of the samples was calculated as follows:

%Survival = (CFU at the end of the test/Initial CFU inoculated)*100

The simulated intestinal juice (SIJ) was prepared by dissolving bile salts to final concentrations of 3.0 g/L in intestinal solution (Sigma, Poole, Dorset, UK) and adjusted to pH 7.5. The survival of *L. paracasei* was determined using the same method related above for the SGJ.

2.8. Antioxidant activity

DPPH free radical scavenging activity of the microcapsules was determined according to Brand-Williams et al. (1995). Briefly, freshly prepared 0.06 mM mmol/L DPPH solution (1 mL) and 0.2 mL of the samples, previously hydrated with distilled water (1:10), were incubated in the dark for 30 min at room temperature. After incubation, the mixtures were centrifuged at 5000 ×*g* for 2 min at 20 °C, and then the optical density of the supernatant was read at 517 nm using a UV–Vis Spectrophotometer. DPPH radical scavenging activity (%) was calculated according to the Eq. (2):

$$\text{Inhibition } (\%) = \left(\left(\text{Abs}_{\text{control}} - \text{Abss}_{\text{ample}}/\text{Abs}_{\text{control}}\right) \times 100 \quad (2)$$

Where $\text{Abs}_{\text{control}}$ and $\text{Abs}_{\text{sample}}$ are the absorbances of the control and hydrated microcapsules, respectively.

The ABTS radical cation (ABTS•+) scavenging assay was performed as described by Arnao et al. (2001) with some modifications. Briefly, an ABTS• + stock solution was prepared by reacting 7 mmol/L ABTS with 2.45 mmol/L potassium persulfate. After 16 h of storage in the dark, the solution was diluted with 0.1 mol/L phosphate buffer (pH 7.0) to obtain an absorbance of 0.70 ± 0.02 at 734 nm. Then, the samples (50 μL) previously hydrated (1:10) with the same PBS 0.1 mol/L, were mixed with the ABTS• + working solution (1 mL), vortexed for 15 s, and incubated for 6 min in the dark. After this time, the mixtures were centrifuged at 5000 ×*g* for 2 min at 20 °C, and their absorbance at 734 nm, was determined. The scavenging activity of ABTS• + was calculated as a percentage inhibition using the eq. (2). The control for both analyses was a direct mixture 1:1 of native SW with a solution of maltodextrin 20 %.

2.9. Storage stability

Immediately after drying, each powder formulation was placed in hermetically sealed plastic tubes and stored at 4 °C and 25 °C in the dark for the stability analysis. The survival rate (SR%) of *L. paracasei* during storage was monitored for 45 days in both temperatures. The SR% was determined *via* the agar plate culturing technique. Briefly, 1 g of powder (FD or SD samples) was serially diluted in PBS (10 mM) and cultured on MRS agar plates. Then, the agar plates were incubated at 37 °C for 48 h in anaerobic conditions. The survival rate was calculated by the number of viable cells according to eq. 1, changing the EE% by SR%:

$$SR\% = \left(\frac{N}{N_0}\right).100 \quad (3)$$

N_0= number of viable cells (Log CFU/mL) immediately after drying;

N = number of viable cells (Log CFU/mL) in the powder during the storage.

2.10. Statistical analysis

The experiments were performed in triplicate and means were compared using Anova one-way followed by Tukey's test at a significance level of 95 % ($P < 0.05$). Statistica 10.0 software (Statsoft, Tulsa, OK, USA) was used for carried out theses analyses.

3. Results and discussion

3.1. Growth of L. paracasei ItalPN16 in SW

The choice of an appropriate growth medium for producing large-scale amounts of probiotic biomass is affected by the cost of culture media, efficiency of cell production, and ease of harvest. In this work, different controlled pH values were evaluated, for the fermentation of pasteurized SW by *L. paracasei* ItalPN16. As shown in Fig. 1A, after 24 h of fermentation, the viability count was around 9.6 Log CFU/mL in the three different pH studies. Then, at 48 h of fermentation, the bacterial counts continued to increase to 10.8, 10.3, and 11.2 Log CFU/mL, for the pH values 4.5, 5.5, and 6.5, respectively. These results indicate that SW is suitable for *L. paracasei* ItalPN16 growth at high yields at controlled pH during the fermentation.

In our previous work, we showed that *L. paracasei* ItalPN16, in a static culture at 37 °C by 48 h, stops its growth (around 9.4 Log CFU/mL) in SW when the media pH attempts the value of 3.8 (Barreto Pinilla et al., 2023), thus, under controlled pH and low agitation was obtained an increase of around 1.3, 0.8 and 1.7 Log CFU/mL (pH 4.5, 5.5. and 6.5, respectively), as compared with the static system. To our knowledge, this is the first report of bacterial growth above 11 Log CFU/mL using plain liquid SW under controlled pH conditions. In addition, no stationary phase was observed in any pH, indicating that higher times of fermentation could increase even more the biomass yield (Fig. 1A).

In the field of food science, some studies have utilized CW without any supplements to promote the growth of lactic acid bacteria (LAB). Their results indicate that CW is a suitable medium for this purpose, yielding final counts ranging from 8.5 to 9.5 Log CFU/mL (Lavari et al., 2014; Pescuma et al., 2012; Soriano-Perez et al., 2011). More recently, Rama et al. (2019) reported viability counts above 12 Log CFU/mL using CW and RW (Ricotta whey) for the growth of *L. paracasei* ATR6 in a bioreactor, without pH control. The differences between the reports reinforce the idea that the biomass yields obtained in CW fermentations are related to the strain-dependent properties, such as tolerance of high concentrations of lactose, low availability of proteins, and the ability to resist low pH (Pescuma et al., 2015). However, the main factor that affects the LAB growth in CW at high yields is the production and release of lactic acid in the media. This metabolic process lowers the cytoplasmic pH, which can inhibit the growth of cells and may even lead to death (Wu et al., 2011). Thus, alternatives such as pH control and the production of a microaerophilic environment through agitation, can be used to overcome the growth inhibition produced by lactic acid (Othman et al., 2017).

As shown in Fig. 1B, the *L. paracasei* ItalPN16 growth was followed by a sugar consumption of around 18 % in the first 24 h and 40 % at the end of the process (48 h) for the fermentations at pH 4.5 and 5.5. The higher sugar consumption (68 %) was observed at pH 6.5 at the end of the fermentation time. (Fig. 1B). Thus, although the growth was satisfactory at the three pHs, *L. paracasei* ItalPN16 was unable to consume all the available lactose. Similar results were reported by other authors (Rabaioli Rama et al., 2020; Secchi et al., 2012). Usually, during the LAB fermentation of CW, the decrease in lactose consumption is closely related to the bacterial stationary phase and the pH reduction during the fermentation (Rama et al., 2019). However, our results showed slow but continuous lactose consumption, in agreement with the bacterial growth curve under pH-controlled conditions.

3.2. Whey protein hydrolysis

Hydrolysis of whey proteins by the *L. paracasei* ItalPN16 was further analyzed by SDS gel electrophoresis, and changes in the whey amino acids profile produced after fermentation were determined by HPLC. As shown in Fig. 2, most of the whey proteins were unaffected after fermentation at different pH. The main changes were observed in the relative concentrations of lactoferrin, bovine serum albumin (BSA), and IgG heavy chains. The higher reduction of these macromolecules was observed in the SW fermented at pH 6.5. In addition, lower intensities of the α-LA bands (around 15KD) were observed in the three treatments.

Similar to our results, Pescuma et al. (2012), reported the hydrolysis of β-LG, α-LA, BSA, and immunoglobulins, of reconstituted whey powder by the strains *L. delbrueckii* subsp. *bulgaricus* CRL 454 and *L. acidophilus* CRL 636, during fermentation. However, differences were observed in the hydrolysis pattern using *Str. Thermophilus*, indicating a strain-dependent hydrolyzation capability. A more recent study reported the degradation of lactoferrin, BSA, and IgG fractions during the fermentation of liquid whey by its native LAB at various incubation temperatures. According to the authors, the most significant proteolytic activity was observed within the temperature range of 35 to 42 °C. (Mazorra-Manzano et al., 2020).

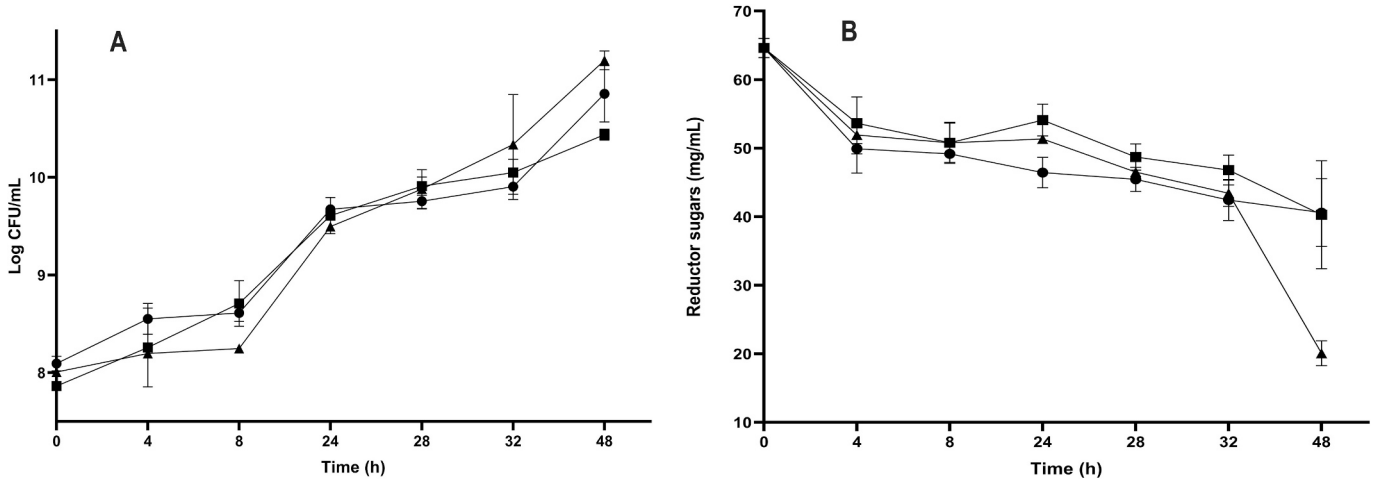

Fig. 1. A: Growth curve of *L. paracasei* ItalPN16 during 48 h at 37 °C in during controlled pH. pH 4.5 (circle), pH 5.5 (square) and 6.5 (triangle). B: Reductor sugars in each point, for the same sample, bars represent the standard deviation of three independent experiments.

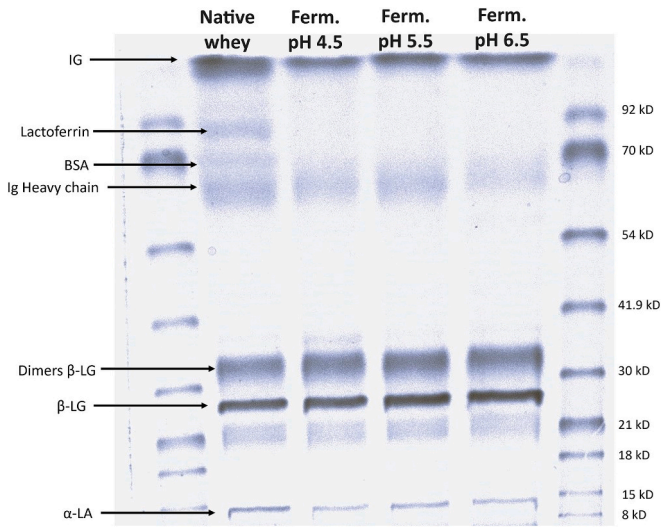

Fig. 2. Degradation of whey proteins during fermentation by *L. paracasei* ItalPN16 at 37 °C, after 48 h of incubation and controlled pH conditions. Lines: 1 and 6, broad range weight protein marker (2–212 kDa); line 2, non-fermented (native) sweet whey.

Cheese whey is a complex combination of globular proteins with different isoelectric points (IP), comprising β-LG (~50 % wt/wt, IP ~ 5), α-LA (~20 % wt/wt, IP ~ 4.2), immunoglobulins (IgG; ~10 % wt/wt, IP around 6), and BSA (~6 %, wt/wt) and others protein or peptide in minor amounts, including lysozyme, lactoferrin, lactoperoxidase, and growth factors (De Wit, 1998). In this work, the SW was pasteurized at 65 °C prior to fermentation due to moderate heating temperature improve the solubility of whey proteins (Teo et al., 2016). At temperatures above 70 °C, irreversible changes in protein structure are observed, including the formation of Maillard compounds, protein aggregation, or cross-linking, which alter negatively the functionality of the whey proteins and affect the growth of LAB (Zhang et al., 2021). In addition, in the conditions of the fermentations (pH 4.5, 5.5, or 6.5) the main protein fractions (β-LG, α-LA, and IgG) were at pH values close to their isoelectric point, favoring their solubility and stability.

Although LABs are usually considered to be weakly proteolytic, they can cause different degrees of proteolysis in fermented dairy products (Liu et al., 2010). LAB possesses a variety of proteolytic enzymes that enable them to utilize milk proteins as nitrogen sources and to ensure their growth during fermentation (Kieliszek et al., 2021). However, although several proteolytic enzymes (exopeptidases and endopeptidases) have been identified in several lactobacilli species, most of the current research has been focused on casein hydrolysis (Kieliszek et al., 2021). Thus, there is still little information about whey-related LAB proteases, the mechanism of action, expression, regulation and activation. Overall, based on our results, fermentation at pH 6.5 favored a higher hydrolysis of lactoferrin, BSA, and IgG heavy chain, proteins with IP close to 6, indicating a higher proteolytic activity and selectivity at this pH. However, more studies must be conducted to identify if this effect is the result of an optimal pH for enzymatic activity, the increased protein solubility, or both.

The analysis of amino acids (AA) present in the whey after the *L. paracasei* fermentations revealed increased values of almost all the AA evaluated, as compared with the native SW (Fig. 3). The most notorious increase was in the concentrations of Leucine (Leu), Glutamine (Glu), Proline (Pro), and Valine (Val). However, we found differences in the free AA profile, according to the pH of fermentation. At pH 6.5 were found significant ($P < 0.05$) higher concentrations of Proline (Pro), Glycine (Gly), and Lysine (Lys); at pH 5.5, the main result was the concentration of Leucine (575.3 mg/Kg) 10 times higher than the native SW (55.9 mg/Kg); and at pH 4.5 the concentrations of Serine (Ser) and Threonine (Thr), Histidine (His), and Alanine (Ala) were higher, as compared to the native whey and the other treatments. Only the Tyrosine (Tyr) showed a decreasing trend in the treatments after the fermentation. Thus, the different AA patterns produced from the SW hydrolysis at different pH of fermentation suggest different proteinase and aminopeptidases specificities from the *L. paracasei* ItalPN16. In addition, from a nutritional point of view, the fermentation increased the concentration of essential AA that enhances the nutritional value of

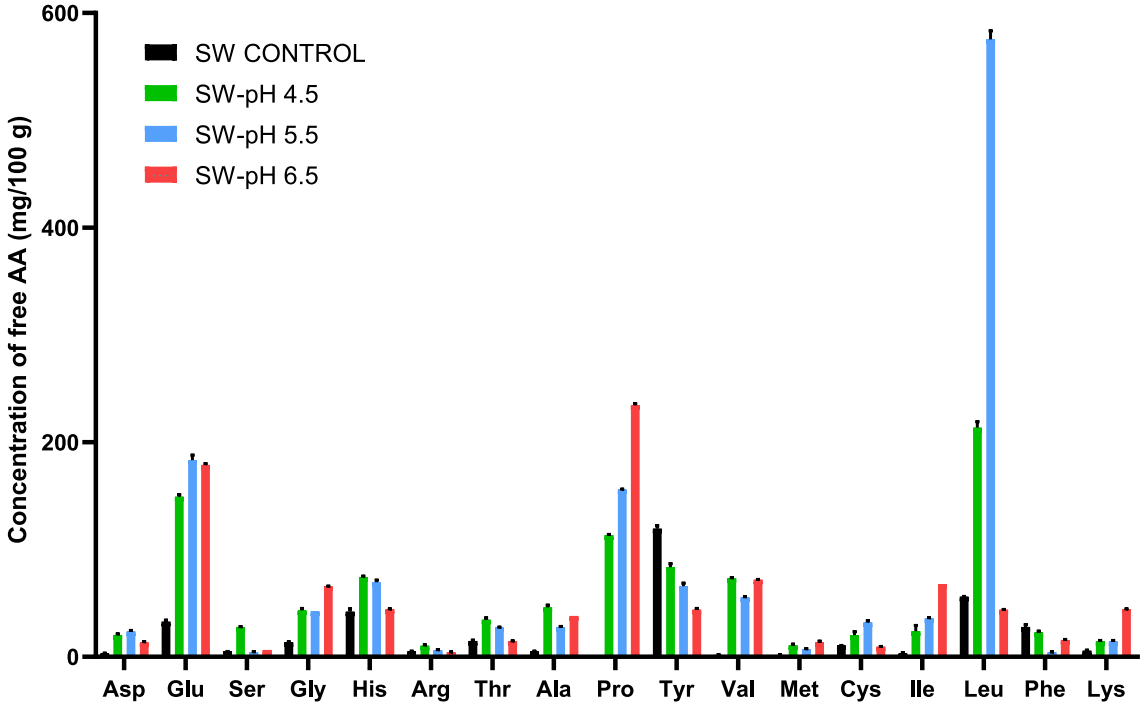

Fig. 3. Amino acid profile and concentration (g/100 g protein) of SW after 48 h fermentation by *L. paracasei PN16*, at different pH conditions. Control: Native SW prior fermentation. Bars represent the standard deviation of three independent experiments.

whey. Similar to our results, Mazorra-Manzano et al. (2020) reported that *L. delbrueckii* subsp. *bulgaricus*CRL 454 favored the release of Leu, Val, and His, during the fermentation of liquid whey. In addition, Olvera-Rosales *et al*, (2023) reported the increase of free AA by the hydrolysis of reconstituted whey (10 %) by *L. rhamnosus GG* after 21 h of fermentation at 41 °C. According to the authors, the proteolytic effects on whey protein may be driven by the cell envelope proteinases (PrtR), which acts in the first fractionation of the whey proteins, resulting in peptides that are incorporated into the cells through the system of transporters Opp, DtpT and Dpp (Griffiths & Tellez, 2012). Afterward, these peptides are broken down into AA by intracellular peptidases and aminopeptidases until satisfy the bacteria auxotrophies, and finally, the excess of peptides and AA are released into the medium (Olvera-Rosales, Cruz-Guerrero, García-Garibay, et al., 2023).

LAB requires many free amino acids to grow and develop; however, the available amount of such nitrogen compounds in cheese whey is usually low; hence, the main function of the LAB nitrogen metabolism proteases and peptidases, during whey fermentation, is to produce these nutrients by decomposing whey proteins (Kieliszek et al., 2021). Therefore, in response to the low bioavailability of these nutrients, LAB can regulate the production of extracellular proteases to supply the cells with essential amino acids (Brown et al., 2017). Besides the lack of information about the expression, regulation, and biosynthesis of specific proteolytic enzymes by LAB during whey fermentation, our results indicated that specific proteases and aminopeptidases are regulated according to the pH. These findings are important considering the potential use of LAB for whey protein hydrolysis, enabling the production of bioactive peptides and essential amino acids (Olvera-Rosales, Cruz-Guerrero, Jaimez-Ordaz, et al., 2023).

3.3. Drying and powder characterization

After 48 h of bacterial growth, the *L. paracasei* ItalPN16 was freeze-drying (FD) and spray-drying (SD). Maltodextrin and the fermented SW were used as wall materials in both methods, to investigate the impact of using various fermented SW on microencapsulation. As shown in Table 1, the drying process resulted in a high EE% for the FD method (83 % on average) as compared with the SD (92 % on average). Significant higher ($P < 0.05$) EE% values were obtained in the FD-pH 4.5 (99.30 %) and FD-pH 6.5 (95.26 %), followed by the SD-pH 4.5 and SD-pH 6.5, with EE of 89.96 % and 88.28 %, respectively. The lower values were found in the samples SD-pH 5.5 (72.15 %) and FD-pH 5.5 (81.56 %).

Our results of EE% are very similar to previous reports of LAB microencapsulation by FD and SD. Beldarrain-Iznaga et al. (2020) reported that the microencapsulation of L. *casei* achieved 98 % survival after the freeze-drying process, using a combined double emulsion and ionic gelation approach. Tang and colleagues (2020) reported survival rates of 70–77 % for *Lactobacillus acidophilus* FTDC 3081 after it was spray-dried and freeze-dried using various protectants, including skim milk, sucrose, maltodextrin, and corn starch. According to the authors, the powders produced with 10 % (*w/v*) maltodextrin and dried by either spray-drying or freeze-drying demonstrated the highest viability, reaching 77.2 % after 40 days of storage.

Given that lactose (present in fermented whey) and maltodextrin are considered suitable as cry and thermal protectants of LAB (Tang et al., 2020; Cui et al., 2022), their combined effect could result in a high EE% of *L. paracasei* ItalPN16, after the SD and FD. In this regard, it was suggested that the effectiveness of some wall materials such as dairy products and natural polymers in protecting cell viability during drying is closely related to their interactions with the bacterial membrane, preventing leakage during water removal (Maciel et al., 2014). Thus, the mixture of lactose and maltodextrin could favor the survival of *L. paracasei* ItalPN16, however, the thermal stress during the spray drying resulted in lower values of EE%.

Table 1 presents the results of water activity (Aw) and moisture contents (M %) in the produced microcapsules. The Aw values were below 0.28 and 0.16 for the SD and FD microparticles, respectively. Similar values of Aw for SD products were reported by Parlindungan et al. (2019), Alves Gragnani Vido et al. (2023), and Misra et al. (2023), who reported water activity values from 0.073 to 0.406 for spray and freeze drying of probiotic bacteria. In particular, Misra and coworkers compare the FD and SD techniques for microencapsulation of *Lactococcus lactis* SKL 13. They observed lower values of Aw, higher moisture content, and an increased survival rate when using FD compared to SD, which is similar to our findings (Misra et al., 2023). Dried microcapsules with Aw below 0.2, will result in good stability of the dried product, and prolonged shelf life of encapsulated LAB, due to the limitation of water available for microbial multiplication, however, values below 0.11 could produce cell death during storage (Dianawati et al., 2016).

Concerning the moisture content (M %), significant ($P < 0.05$) differences were observed between the different dried methods and the pH of the SW. The higher moisture values (3.48 % and 3.25 %) were found in the SD-pH 4.5 and FD-pH 6.5 microcapsules (Table 1), while the lower moisture value (1.54 %) was obtained in the SD-pH 5.5 sample. Ilha et al. (2015) reported values close to 4 % in dried *L. paracasei* using skim milk and cheese whey as wall material, obtaining satisfactory viable cell stability after drying. Recently, was reported a moisture content of 3.67 % and 2.1 % in the SD and FD microencapsulation of a mixture of LAB (Lactoferm ABY-6) using whey protein concentrate and sodium alginate as wall materials (Obradovic et al., 2022). Controlling the moisture content of dried probiotic cultures is crucial for achieving long-term storage stability. It is recommended to maintain moisture levels below 5 %. This low moisture content ensures that there is less water available for degradative reactions and minimizes the solubilization and mobility of components within the formulation (Peighambardoust et al., 2011). In dairy-based emulsions designed for encapsulation, blends of carbohydrates and milk proteins exhibit excellent film-forming and emulsifying properties. The proteins in these mixtures contribute to the stability of the mixture, while the carbohydrates serve as matrix-forming agents. This combination results in highly stable emulsions that minimize the adverse environmental effects on the functionality of both proteins and carbohydrates (Afzal et al., 2024a, 2024b). Thus, the different moisture values in the SD in the FD powders could be related to the different interactions between lactose, whey proteins, and maltodextrin, which resulted in powders with different water affinity.

Table 1
L. paracasei ItalPN16 microparticles. Encapsulation efficiency (EE %), water activity (A_w) and moisture (M %) from two types of drying and using bio-processed whey at different pHs.

Treatment						
	SD-pH 4.5	SD-pH 5.5	SD-pH 6.5	FD-pH 4.5	FD-pH 5.5	FD-pH 6.5
EE (%)	89.96 ± 0.05^{c}	72.15 ± 0.39^{e}	88.28 ± 0.02^{c}	99.30 ± 0.09^{a}	81.56 ± 0.17^{d}	95.26 ± 0.36^{b}
Aw	0.236 ± 0.001^{c}	0.208 ± 0.026^{c}	0.279 ± 0.078^{c}	0.087 ± 0.010^{a}	0.154 ± 0.013^{b}	0.149 ± 0.002^{b}
M (%)	3.48 ± 0.25^{a}	1.54 ± 0.51^{c}	2.16 ± 0.36^{b}	2.51 ± 0.50^{ab}	2.87 ± 0.88^{ab}	3.25 ± 0.61^{a}

Values are the mean ± standard deviation of 3 (three) independent experiments. Different superscript letters denote significant differences ($p < 0.05$) in the same line. SD: Spray drying; FD: Freeze drying.

3.4. Surface morphology

The morphology and surface appearance of the microparticles produced by SD and FD were observed by scanning electron microscopy (SEM), and the resulting images at 8 Kx and 3 Kx of magnification are presented in Fig. 4. The microcapsules produced by the SD process showed a spherical collapsed shape with smooth surface, in all the treatments. These observations are consistent with previous studies of

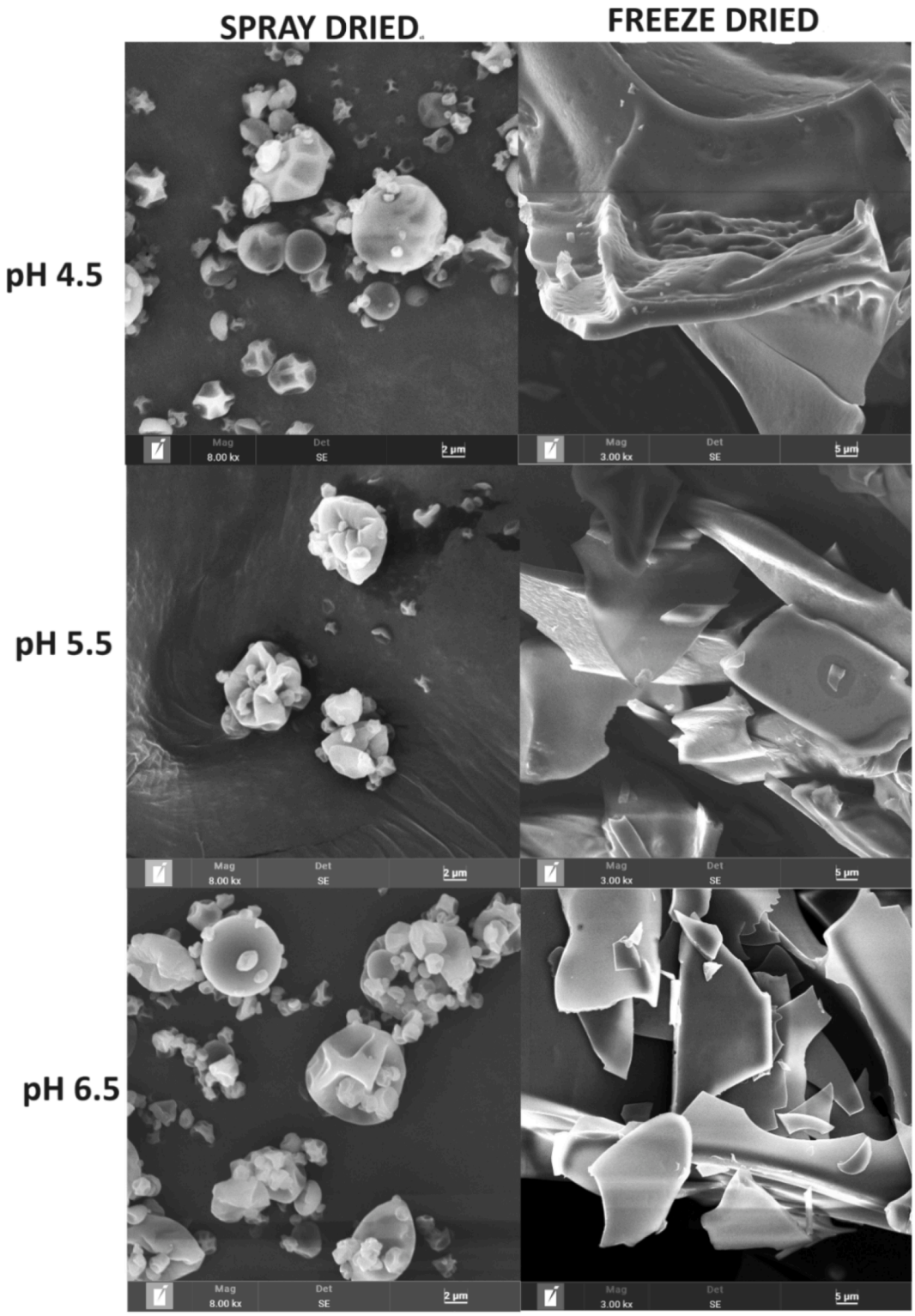

Fig. 4. Scanning electron microscopy (SEM) micrographs of spray-dried (8Kx magnification) and freeze-dried microcapsules (3Kx magnification).

SD microencapsulation of LAB (Bağdat et al., 2024; Bustamante et al., 2020), which reported the formation of small-sized microcapsules with spherical structures and concavities on the surface. This morphology in the SD microparticles results from the shrinkage of the particles during the rapid evaporation stage at higher temperatures and low solids in the solution (Okada et al., 2020). In addition, no signs of cracks or fractures were observed on the surfaces, which indicates good structural integrity of the capsules, favoring their protective behavior (Yonekura et al., 2014). According to Aragón-Rojas et al. (2020), maltodextrin molecules can bind whey protein, inhibit protein aggregation by generating steric hindrance, and create a smooth micro-structure.

The FD samples presented an irregular shape with a wide range of particles with low porosity and soft surfaces. No bacterial cells were observed on the surface, indicating randomly distributed bacterial cells within the wall matrix. Similar morphologies were reported by previous studies, in SEM observations of freeze-dried LAB microparticles

(Massounga Bora et al., 2018; Rajam & Anandharamakrishnan, 2015).

3.5. Fourier-transform infrared (FTIR) spectroscopy

The interaction between chemical bonds in the encapsulated *L. paracasei* ItalPN16 by SD and FD was analyzed using the Fourier transform infrared (FTIR) technique. Due to the presence of lactose on the microparticle's surface, the powders obtained by FD (Fig. 5A) presented a broad-spectrum band in the carbohydrate fingerprint region (800–1200 cm^{-1}), with higher intensities as compared with the single maltodextrin. In addition, the asymmetric elongation of amide-type I at 1601 cm^{-1} from the whey protein was observed in the powders FD-pH 5.5 and FD pH 6.5. The FD samples also presented a board spectrum band at 3268 cm^{-1} with a higher intensity as compared with the single maltodextrin, which is related to stretching vibrations of -OH groups and their interactions with -NH2 groups, at different energy levels (Patel et al., 2021). The increased intensity of - NH, -OH, and CH_2 stretching vibrations indicated the superposition and low molecular packing of the whey components (mainly lactose) and maltodextrin on the powder's microstructure. Similar results were reported by Zhang et al. (2018), evaluating the microencapsulation of xylooligosaccharides (XOS) by spray drying, using maltodextrin as wall material.

On the other hand, the powders obtained by SD (Fig. 5B) showed lower -OH stretching (3268 cm^{-1} band) and lower intensity in the carbohydrate fingerprint (800–1200 cm^{-1}) as compared with the FD. The samples SD pH 4.5 and SD pH 5.5 presented shorter -OH stretching intensity, as compared with the single maltodextrin, indicating the formation of a complex between the polysaccharides and the whey proteins present in the formulations at low pH. Reduced absorbance of the stretching band of C—H after spray drying of maltodextrin and whey protein was reported by Maqsoudlou et al. (2020), they suggested that the reduction of the intensity of the -OH band in this system could indicate rearrangements of the hydrogen bonds in the microcapsule's surface. This effect is related to a higher degree of homogeneity in the intermolecular interactions, which results in an increase of conformational selectivity due to the low dispersion of vibrational levels.

Overall, the results indicate a higher exposure of hydrophilic compounds, mainly carbohydrates in the FD surface powder and a more packed structure in the SD microcapsules. In addition, the composition and pH of the fermented whey used in the microencapsulation resulted in changes in the 3000–3600 cm^{-1} (-OH stretching) and the carbohydrate fingerprint (800–1200 cm^{-1}) to relatively lower intensities, these changes were more evident in the SD microcapsules (Fig. 5B).

3.6. Acid and bile resistance

The gastrointestinal environment is the main barrier to the viability of probiotic microorganisms due to their exposure to low pH, proteolytic enzymes, and bile salts (Ilha et al., 2015). Therefore, acid and bile salt tolerance are crucial features for probiotic microorganisms because this property helps them to survive during the harsh conditions of the digestive process. The survival microencapsulated *L. paracasei* ItalPN16 in simulated gastrointestinal conditions was studied after 1 h and 2 h of exposure, resulting in a high survival percentage, with values above 90 % (losing less than 1 Log CFU/g) in almost all the microcapsules. The lower values were observed in the control (non-protected) and the sample SD-pH 5.5, with survival of 72 % and 77 % after 2 h, respectively. The presence of bile salts showed an important effect on the strain survival, resulting in values below 70 % in all the samples, however, the sample FD-pH 6.5 presented significant ($p < 0.05$) higher survival to bile salts (65 %) after 2 h (Fig. 6B), as compared with the other treatments and de control. The antimicrobial action of bile salts (0.3 %) was less on the microencapsulated *L. paracasei* ItalPN16 than the control after 1 h. Still, after 2 h, the sample FD-pH 4.5 presented a notable survival reduction with no significant difference ($p < 0.05$) as compared with the control (around 32 % of final survival).

Overall, the best results observed for acid tolerance were found in *L. paracasei* ItalPN16 dried by freeze-drying (FD). This may be linked to the rapid solubilization of lactose and other whey components in the formulation during the test. These components provide ATP to the F0F1-ATPase through bacterial metabolism, which helps expel protons, thereby enhancing bacterial survival under gastric conditions. (Corcoran et al., 2005). In particular, the best survival of *L. paracasei* on acid and bile salts solutions was observed in the samples that used fermented whey at pH 6.5 as wall material. This indicates a strong effect of the whey composition on the bacterial survivor after drying. In this regard, it has been reported that some intact milk proteins including βlactoglobulin (βlg) and bovine serum albumin (BSA) can bind bile salts, reducing its biological activity (Bellesi & Pilosof, 2021; Naso et al., 2019). Thus, it is possible that specific structural conformation of βlg, IgG and BSA in the fermented whey at pH 6.5 favor the binding of bile salts, reducing its antimicrobial activity. However, most studies must be conducted to verify this hypothesis since the acid and bile resistance is highly strain-dependent, and also the strains could improve their survivor to an adverse environment according to the media culture composition or exposure to low pH during the growth (Barreto Pinilla, Brandelli, et al., 2023; Wendel, 2022). In this context, Gul and Atalar (2018), reported that after exposure to 3 % bile salt, the survival of spray

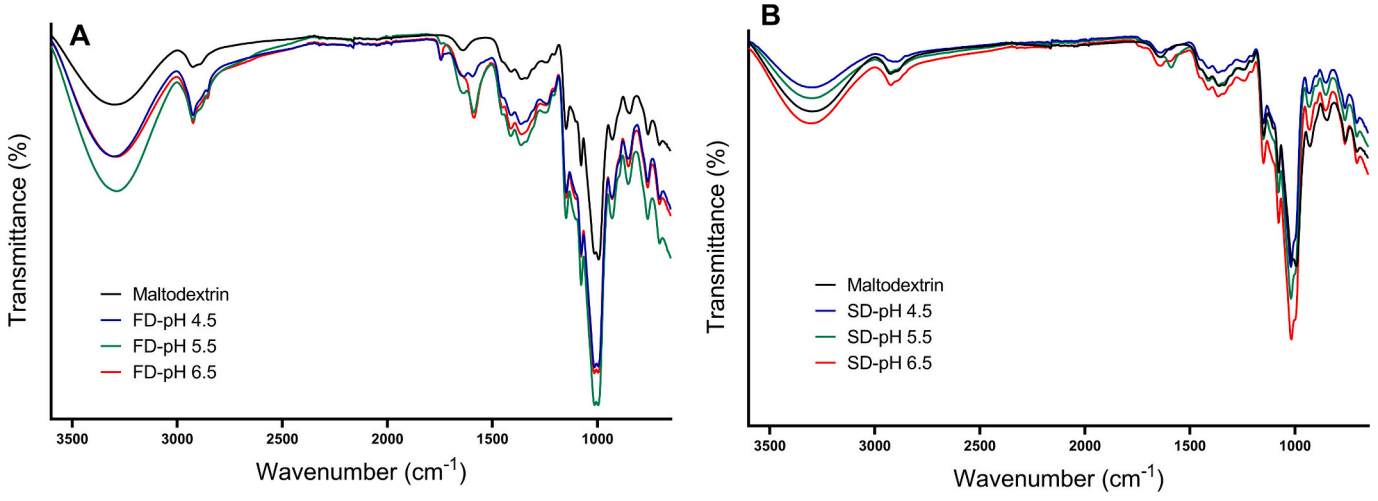

Fig. 5. Structural bond analysis of encapsulated probiotic powder by FTIR. A: Maltodextrin and freeze dried (FD) *L. paracasei* ItalPN16 including SW fermented at pH 4.4, 5.5 or 6.5. B: Maltodextrin, spray dried (SD) *L. paracasei* ItalPN16 including SW fermented at pH 4.4, 5.5 or 6.5.

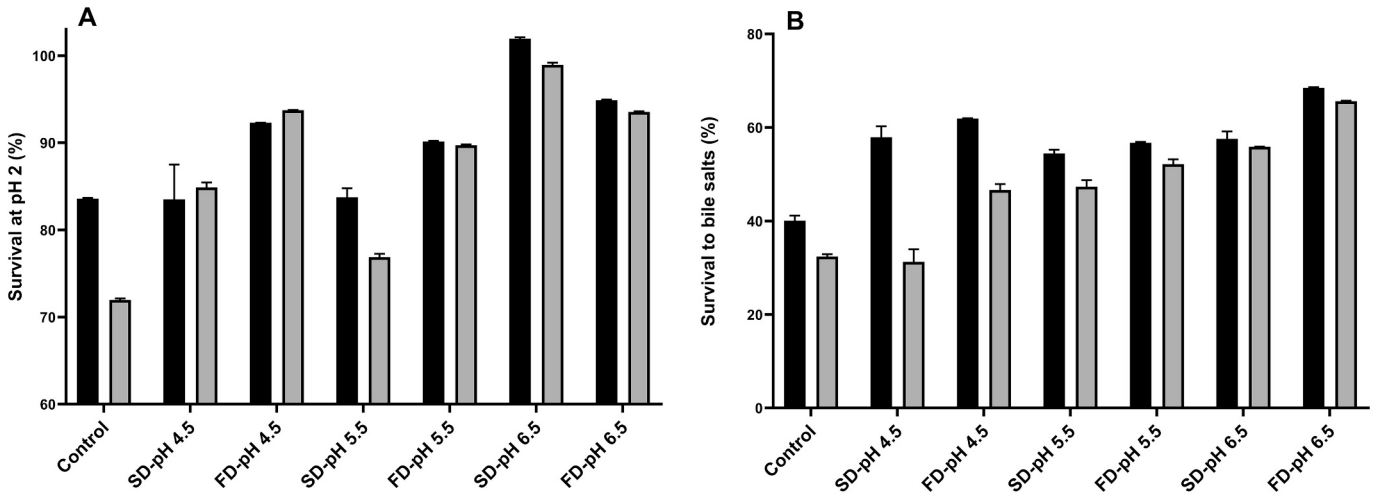

Fig. 6. Survivor index of *L. paracasei* ItalPN16 at pH 2(A) and bile salts (B). Bars represent the standard deviation of three independent experiments. Black columns represent the values after 1 h of treatment and grey columns represent the values after 2 h of treatment.

and freeze-dried microencapsulated *Lactobacillus casei* Shirota decreased by almost 2.5 Log CFU/g; they observed that the best results of cell protection against acidic pH, high temperature and bile salts, were related to the microencapsulation matrix. In contrast, Buahom et al. (2023), reported that an acid environment (pH 2.0) led to a significant decrease (1–2.8 Log CFU/mL) in the survival rate of 5 probiotic strains microencapsulated by freeze- and spray-dried using different wall materials, according with the authors, the survivability of dried probiotics during exposure to gastrointestinal conditions, were more depended on the bacterial species, than the drying method or the wall material.

3.7. Antioxidant activity

The antioxidant activity (AA%) of microencapsulated *L. paracasei* ItalPN16 was estimated using the ABTS and DPPH radicals. As shown in Fig. 7, the main differences were observed between the different fermented whey (pH 4.5, 5.5, and 6.5) used as wall material, and no significative differences ($p < 0.05$) were observed between the same whey component in the two drying methods (Fig. 7). In the ABTS method, the higher values of AA% were observed in the samples that contained fermented whey at pH 4.5 and pH 6.5 (around 52.%) in both drying methods. For DPPH were observed significant ($p < 0.05$) higher values of radical scavenging in the sample SD-pH 4.5 (59.71 %) and FD-pH 4.5 (59.59 %).

The protection against oxidative stress and the capacity to decrease the risk of accumulation of reactive oxygen species (ROS) is another beneficial effect presented by probiotic preparations (Martarelli et al., 2011). Probiotic bacteria usually possess redox systems associated with antioxidant enzymes and oxidative damage repair systems to avoid the effect of free oxygen radicals (Feng & Wang, 2020). However, due to the bacterial drying processes resulting in a high oxygen exposition, the addition of antioxidant compounds such as polyphenols and lignin as wall material has been evaluated to reduce the bacterial exposure to oxygen and therefore, resulting in higher survival and increased antioxidant capacity of the microcapsules (Celik & O'sullivan, 2013). Thus, considering that whey protein is recognized as a source of antioxidant peptides and one of the methods for the obtention of these bioactive compounds is bacterial hydrolysis (Brandelli et al., 2015), the antioxidant compounds produced during the fermentation and present in the dried formulation, resulted in improved antioxidant activity of the powders, besides the low amount of whey protein in the formulations (around 0.5 %). This effect was more notorious in the SW fermented at pH 4.5 considering the ABTS and DPPH results, indicating higher production of antioxidant peptides during growth of *L. paracasei* ItalPN16 at

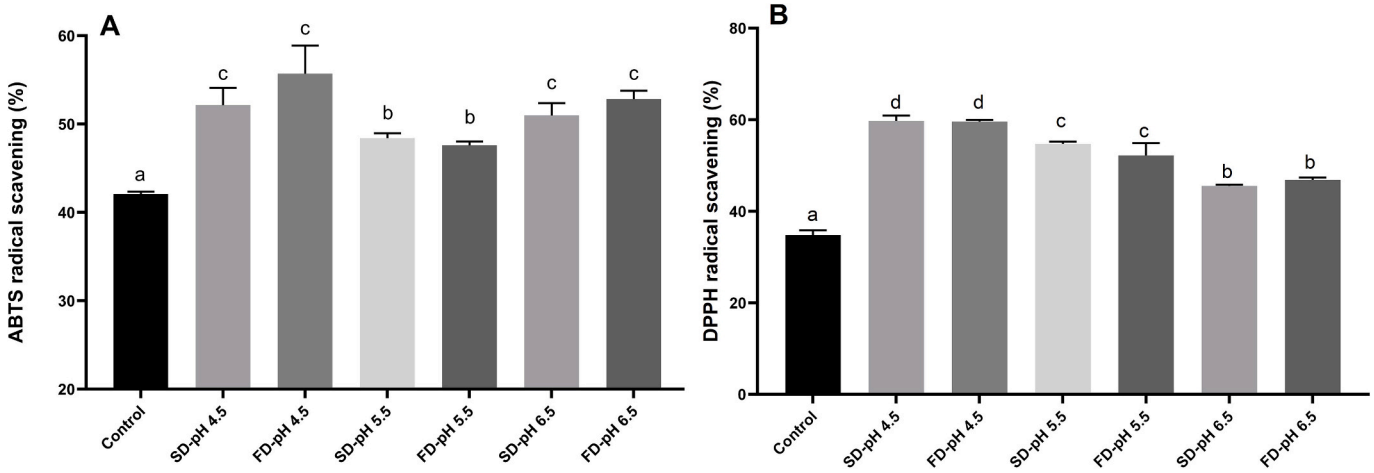

Fig. 7. Antioxidant activity of microencapsulated *L. paracasei* ItalPN16. A) ABTS; and B) DPPH radical scavenging. Control: native whey and maltodextrin (10 %) at 1:1 volume ratio. Values are the mean ± standard deviation of 3 (three) independent experiments. Different superscript letters denote significant differences ($p < 0.05$).

this pH.

3.8. Storage stability

As shown in Fig. 8A, after 45 days under refrigerated storage (4 °C), the survival rates (SR%) of FD microencapsulated *L. paracasei* ItalPN16 maintained values above 95 %. A similar result was observed in the sample SD-pH 5.5, in the first 30 days of storage. In contrast, the samples SD-pH 4.5 and SD-pH 6.5 presented a decrease of 12 % of survival, after the first 15 days. The best results in the refrigerated storage were for the samples FD-pH 4.5 and SD-pH 5.5 with SR% of 97.32 % and 98.23 %, respectively. In a similar trend, as shown in Fig. 8B, at 25 °C the SR% decreased rapidly in all the SD samples, with a reduction of more than 50 % in the first 15 days, and around 70 % at the end of the test (45 days). The best SR% at 25 °C was observed in the sample FD-pH 6.5 with a survival of 71.23 % (7.63 Log CFU/mL) after 45 days. Overall, the FD method allowed higher survival of the *L. paracasei* ItalPN16 at the two conditions of temperature evaluated.

In this study, the SR% of *L. paracasei* ItalPN16 after spray and freeze-drying was highly dependent on the storage temperature, and the drying method (Fig. 8). Similar to our results, it was reported that the stability of dried *L. acidophilus* FTDC 3081 at room temperature was higher after freeze-dried microparticles, as compared with the spray-dried method, using maltodextrin as the main wall material (Tang et al., 2020a, 2020b). More recently, Gagneten et al. (2024) conducted a study evaluating the response of two LAB strains to different environmental stresses associated with FD and SD processes. They found a strain-dependent response to osmotic, mechanical, and thermal stresses, and also reported that the SD results in low storage stability at mild ambient temperatures with high functionality loss, due to membrane damage.

In addition, fermentation parameters such as medium composition, temperature, pH, and harvesting time, influence the physiological state of the dried probiotic cultures affecting their performance and storage tolerance (Liu et al., 2019). In particular, pH has a significant influence on lactobacilli survival during the storage, affecting differentially each bacterial strain (Shafiee et al., 2010). Regarding the pH of LAB fermentation and its influence on storage survival, some evidence pointed that a low pH affects negatively the survivor rates of LAB, as related by Senovieski et al. (2024), who reported that the pH of fermentation of spray-dried *Bifidobacterium animalis* subsp. *lactis* INL1 reduce their survival during storage at 22 °C. However, strain-specific characteristics, environmental stress responses, and the wall material, can also increase the SR% of dried LAB, as showed by Gaudreau et al. (2006) and Wang et al. (2005), for *Lactococcus lactis* ssp. *Cremoris* and *Lactobacillus acidophilus* RD758, respectively. In our work, considering the two methods of drying, the higher SR% at 4 °C and 25 °C was achieved in the samples containing fermented whey at pH 5.5 and 6.5, respectively, indicating that low pH in the wall material affects the bacterial SR% during storage.

Overall, our results demonstrate that plain whey can be effectively used as a culture medium for achieving high biomass yields of LAB. Additionally, the fermented whey can serve as a wall material for the microencapsulation of the same bacteria in a single process. Some of the best results were observed in the fermentation at pH of 6.5 and utilizing spray drying (SD) as the drying method. This innovative technology could be a tool for a more sustainable use of whey and the development of cheaper media cultures for LAB production.

4. Conclusions

In this work, liquid SW was used as media culture for *L. paracasei* ItalPN16 in a bioreactor under controlled pH, resulting in bacterial counts above 10 Log CFU/mL after 48 h of fermentation. *L. paracasei* ItalPN16 was able to hydrolyze the IG (heavy chain) protein fraction of the whey, according to the pH of fermentation. This protein hydrolysis increased the free amino acid content in the fermented whey, indicating a pH-dependent activity of proteases and aminopeptidases. The residual medium plus maltodextrin was used as wall material for microencapsulation by FD and SD. Was observed that the drying method affected the microparticles values of M% and Aw e EE% and FTIR patterns. On the other hand, composition and pH of the wall material had a strong effect on the bacterial survival in simulated gastrointestinal fluids and the survival rate during storage. Thus, this study confirmed that pH control of SW is a suitable strategy for LAB biomass production at high yields, and as a wall material for microencapsulation in the same process. This low-cost approach could be industrially exploited to produce starter or adjunct cultures more sustainably and open new opportunities for cheese whey valorization. However, more studies must be conducted to reduce the time of fermentation, investigate the bioactive properties of whey after fermentation, and improve the powder stability after drying.

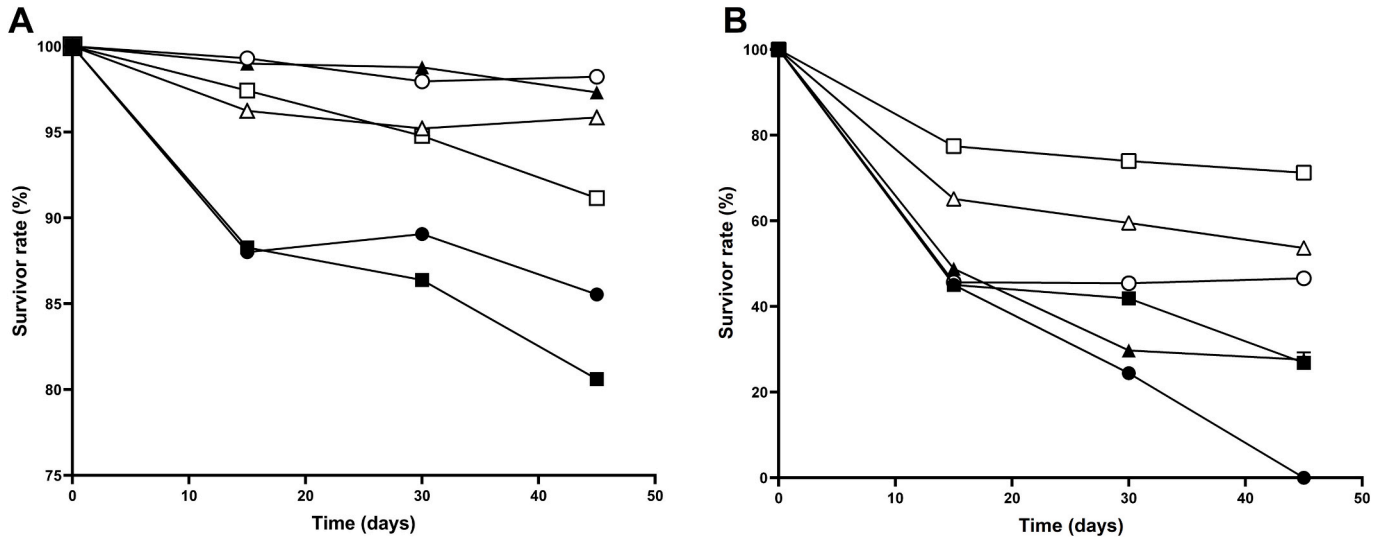

Fig. 8. Survival rate of *L. paracasei* ItalPN16 using as wall material maltodextrin and whey fermented at different pH, during storage at 4 (A) and 25 °C (B). Continued lines represent the mean of three different experiments. Treatments SD-pH 4.5 (●), FD-pH 4.5 (○) SD-pH 5.5 (▲), FD-pH 5.5 (Δ), SD-pH 6.5 (■), FD-pH 6.5 (□).

Funding

This work was supported by São Paulo Research Foundation (FAPESP) [grant number 2021/04987–0 and 2019/01896–4].

CRediT authorship contribution statement

Cristian Mauricio Barreto Pinilla: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Fabiana Galland:** Writing – review & editing, Methodology, Formal analysis. **Maria Teresa Bertoldo Pacheco:** Methodology, Formal analysis, Data curation. **Paula Janetti Bócoli:** Methodology, Formal analysis, Data curation. **Daniele Fidelis Borges:** Methodology, Formal analysis, Data curation. **Izabela Dutra Alvim:** Methodology, Conceptualization. **Leila Maria Spadoti:** Validation, Resources, Funding acquisition, Data curation. **Adriana Torres Silva e Alves:** Writing – review & editing, Visualization, Supervision, Project administration.

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.

References

Afzal, A., Afzaal, M., Saeed, F., Shah, Y. A., Raza, M. A., Khan, M. H., & Teferi Asres, D. (2024a). Milk protein based encapsulation of probiotics and other food material: Comprehensive review. *International Journal of Food Properties, 27*(1), 245–262.

Afzal, A., Afzaal, M., Saeed, F., Shah, Y. A., Raza, M. A., Khan, M. H., & Teferi Asres, D. (2024b). Milk protein based encapsulation of probiotics and other food material: Comprehensive review. *International Journal of Food Properties, 27*(1), 245–262.

Aller, K., Adamberg, K., Timarova, V., Seiman, A., Feštšenko, D., & Vilu, R. (2014). Nutritional requirements and media development for Lactococcus lactis IL1403. *Applied Microbiology and Biotechnology, 98*, 5871–5881.

Alves Gragnani Vido, M., Dutra Alvim, I., Vinderola, G., Isabel Berto, M., de Sá Blumer Zacarchenco Rodrigues, P., Mauricio Barreto Pinilla, C., & Torres Silva e Alves, A. (2023). Microencapsulation of Limosilactobacillus reuteri (DSM 23878) for application in infant formula: Heat resistance and bacterial viability during long-time storage. *Food Research International, 173*(Pt 2), Article 113378.

Aragón-Rojas, S., Quintanilla-Carvajal, M. X., & Hernández-Sánchez, H. (2018). Multifunctional role of the whey culture medium in the spray drying microencapsulation of lactic acid Bacteria. *Food Technology and Biotechnology, 56*(3), 381–397.

Aragón-Rojas, S., Yolanda Ruiz-Pardo, R., Javier Hernández-Álvarez, A., & Ximena Quintanilla-Carvajal, M. (2020). Sublimation conditions as critical factors during freeze-dried probiotic powder production. *Drying Technology, 38*, 333–349.

de Araújo Etchepare, M., Nunes, G. L., Nicoloso, B. R., Barin, J. S., Moraes Flores, E. M., de Oliveira Mello, R., & Ragagnin de Menezes, C. (2020). Improvement of the viability of encapsulated probiotics using whey proteins. *LWT - Food Science and Technology, 117*, Article 108601.

Arnao, M. B., Cano, A., & Acosta, M. (2001). The hydrophilic and lipophilic contribution to total antioxidant activity. *Food Chemistry, 73*(2), 239–244.

Bağdat, E.Ş., Akman, P. K., Kutlu, G., & Tornuk, F. (2024). Optimization of spray-drying process parameters for microencapsulation of three probiotic lactic acid bacteria selected by their high viability rate in sucrose and fructose levels and high temperatures. *Systems Microbiology and Biomanufacturing, 4*, 687–698.

Barreto Pinilla, C. M., Brandelli, A., Galland, F., Spadoti, L. M., & Torres Silva e Alves, A. (2023). Improved functional properties of the potential probiotic *lacticaseibacillus paracasei* ItalPN16 growing in cheese whey. *Letters in Applied Microbiology, 76*, ovad075.

Barreto Pinilla, C. M., Guzman Escudero, F., Torres Silvae Alves, A., Spadoti, L. M., & Brandelli, A. (2023). Draft genome sequence and comparative genome analysis reveal potential functional properties in Lacticaseibacillus paracasei ItalPN16. *Current Microbiology, 80*.

Beldarrain-Iznaga, T., Villalobos-Carvajal, R., Leiva-Vega, J., & Armesto, E. S. (2020). Influence of multilayer microencapsulation on the viability of Lactobacillus casei using a combined double emulsion and ionic gelation approach. *Food and Bioproducts Processing, 124*, 57–71.

Bellesi, F. A., & Pilosof, A. M. (2021). Potential implications of food proteins-bile salts interactions. *Food Hydrocolloids, 118*, Article 106766.

Brandelli, A., Daroit, D. J., & Correa, A. P. (2015). Whey as a source of peptides with remarkable biological activities. *Food Research International, 73*, 149–161.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology, 28*, 25–30.

Brown, L., Villegas, J. M., Elean, M., Fadda, S., Mozzi, F., Saavedra, L., & Hebert, E. M. (2017). YebC, a putative transcriptional factor involved in the regulation of the proteolytic system of *Lactobacillus*. *Scientific Reports, 7*(1), 8579.

Buahom, J., Siripornadulsil, S., Sukon, P., Sooksawat, T., & Siripornadulsil, W. (2023). Survivability of freeze- and spray-dried probiotics and their effects on the growth and health performance of broilers. *Veterinary World, 16*(9), 1849–1865.

Buchanan, D., Martindale, W., Romeih, E., & Hebishy, E. (2023). Recent advances in whey processing and valorisation: Technological and environmental perspectives. *International Journal of Dairy Technology, 76*, 291–312.

Bustamante, M., Laurie-Martínez, L., Vergara, D., Campos-Vega, R., Rubilar, M., & Shene, C. (2020). Effect of three polysaccharides (inulin, and mucilage from chia and flax seeds) on the survival of probiotic Bacteria encapsulated by spray drying. *Applied Sciences, 10*, 4623.

Celik, O. F., & O'sullivan, D. (2013). Factors influencing the stability of freeze-dried stress-resilient and stress-sensitive strains of bifidobacteria. *Journal of Dairy Science, 96*(6), 3506–3516.

Corcoran, B. M., Stanton, C., Fitzgerald, G. F., & Ross, R. P. (2005). Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. *Applied and Environmental Microbiology, 71*(6), 3060–3067.

Cui, S., Hu, M., Sun, Y., Mao, B., Zhang, Q., Zhao, J., … Zhang, H. (2022). Effect of Trehalose and lactose treatments on the freeze-drying resistance of lactic acid Bacteria in high-density culture. *Microorganisms, 11*, 48.

De Wit, J. N. (1998). Nutritional and functional characteristics of whey proteins in food products. *Journal of Dairy Science, 81*, 597–608.

Dianawati, D., Mishra, V. K., & Shah, N. P. (2016). Survival of microencapsulated probiotic Bacteria after processing and during storage: A review. *Critical Reviews in Food Science and Nutrition, 56*, 1685–1716.

Feng, T., & Wang, J. (2020). Oxidative stress tolerance and antioxidant capacity of lactic acid bacteria as probiotic: A systematic review. *Gut Microbes, 12*, 1801944.

Gagneten, M., Passot, S., Cenard, S., Ghorbal, S., Schebor, C., & Fonseca, F. (2024). Mechanistic study of the differences in lactic acid bacteria resistance to freeze- or spray-drying and storage. *Applied Microbiology and Biotechnology, 108*, 1–16.

Gaudreau, H., Fliss, I., & Champagne, C. P. (2006). Stability of autolytic lactococci during starter production and storage in commercial whey-based media. *International Dairy Journal, 16*, 200–206.

Giulianetti de Almeida, M. P., Mockaitis, G., & Weissbrodt, D. G. (2023). Got whey? Sustainability endpoints for the dairy industry through resource biorecovery. *Fermentation, 9*, 897.

Griffiths, M. W., & Tellez, A. M. (2012). *Lactobacillus helveticus*: The proteolytic system. *Frontiers in Microbiology, 4*, 30.

Gul, O., & Atalar, I. (2018). Different stress tolerance of spray and freeze dried *Lactobacillus casei* Shirota microcapsules with different encapsulating agents. *Food Science and Biotechnology, 28*(3), 807–816.

Huang, S., Cauty, C., Dolivet, A., le Loir, Y., Chen, X. D., Schuck, P., et al. (2016). Double use of highly concentrated sweet whey to improve the biomass production and viability of spray-dried probiotic Bacteria. *Journal of Functional Foods, 23*, 453–463.

Ilha, E. C., Silva, T. D., Lorenz, J. G., Oliveira Rocha, G., & Sant'Anna, E.S.. (2015). Lactobacillus paracasei isolated from grape sourdough: Acid, bile, salt, and heat tolerance after spray drying with skim milk and cheese whey. *European Food Research and Technology, 240*, 977–984.

Kieliszek, M., Pobiega, K., Piwowarek, K., & Kot, A. M. (2021). Characteristics of the proteolytic enzymes produced by lactic acid Bacteria. *Molecules, 26*, 1858.

Krunić, T.Ž., Obradović, N. S., & Rakin, M. B. (2019). Application of whey protein and whey protein hydrolysate as protein based carrier for probiotic starter culture. *Food Chemistry, 293*, 74–82.

Lavari, L., Páez, R., Cuatrín, A., Reinheimer, J. A., & Vinderola, G. (2014). Use of cheese whey for biomass production and spray drying of probiotic lactobacilli. *Journal of Dairy Research, 81*, 267–274.

Liu, H., Cui, S. W., Chen, M., Li, Y., Liang, R., Xu, F., & Zhong, F. (2019). Protective approaches and mechanisms of microencapsulation to the survival of probiotic bacteria during processing, storage and gastrointestinal digestion: A review. *Critical Reviews in Food Science and Nutrition, 59*, 2863–2878.

Liu, M., Bayjanov, J. R., Renckens, B., Nauta, A., & Siezen, R. J. (2010). The proteolytic system of lactic acid bacteria revisited: A genomic comparison. *BMC Genomics, 11*, 36.

Maciel, G. M., Chaves, K. D., Grosso, C. R., & Gigante, M. L. (2014). Microencapsulation of Lactobacillus acidophilus La-5 by spray-drying using sweet whey and skim milk as encapsulating materials. *Journal of Dairy Science, 97*(4), 1991–1998.

Maqsoudlou, A., Sadeghi Mahoonak, A., Mohebodini, H., & Koushki, V. (2020). Stability and structural properties of bee pollen protein hydrolysate microencapsulated using maltodextrin and whey protein concentrate. *Heliyon, 6*, Article e03731.

Martarelli, D., Verdenelli, M. C., Scuri, S., Cocchioni, M., Silvi, S., Cecchini, C., & Pompei, P. (2011). Effect of a probiotic intake on oxidant and antioxidant parameters in plasma of athletes during intense exercise training. *Current Microbiology, 62*, 1689–1696.

Massounga Bora, A. F., Li, X., Zhu, Y., & Du, L. (2018). Improved viability of microencapsulated probiotics in a freeze-dried Banana powder during storage and under simulated gastrointestinal tract. *Probiotics and Antimicrobial Proteins*, 1–10.

Mazorra-Manzano, M. A., Mora-Cortes, W. G., Leandro-Roldan, M. M., González-Velázquez, D. A., Torres-Llanez, M. J., Ramírez-Suárez, J. C., … Vallejo-Cordoba, B. (2020). Production of whey protein hydrolysates with angiotensin-converting enzyme-inhibitory activity using three new sources of plant proteases. *Biocatalysis and Agricultural Biotechnology, 28*, Article 101724.

Miller, G. L. (1959). Use of Dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31*, 426–428.
Misra, S., Pandey, P., Panigrahi, C., & Mishra, H. N. (2023). A comparative approach on the spray and freeze drying of probiotic and gamma-aminobutyric acid as a single entity: Characterization and evaluation of stability in simulated gastrointestinal conditions. *Food Chemistry Advances, 3*, Article 100385.
Naso, J. N., Bellesi, F. A., Pizones Ruiz-Henestrosa, V. M., & Pilosof, A. M. (2019). Studies on the interactions between bile salts and food emulsifiers under in vitro duodenal digestion conditions to evaluate their bile salt binding potential. *Colloids and surfaces B, Biointerfaces, 174*, 493–500.
Obradovic, N., Volić, M., Nedović, V. A., Rakin, M., & Bugarski, B. (2022). Microencapsulation of probiotic starter culture in protein–carbohydrate carriers using spray and freeze-drying processes: Implementation in whey-based beverages. *Journal of Food Engineering., 321*, Article 110948.
Okada, S., Ohsaki, S., Nakamura, H., & Watano, S. (2020). Estimation of evaporation rate of water droplet group in spray drying process. *Chemical Engineering Science, 227*, Article 115938.
Olvera-Rosales, L., Cruz-Guerrero, A. E., Jaimez-Ordaz, J., Pérez-Escalante, E., Quintero-Lira, A., Ramírez-Moreno, E., … González-Olivares, L. G. (2023). Differences in the proteolytic system of lactic acid Bacteria affect the release of DPP-IV inhibitory peptides from whey proteins. *Dairy, 4*(3), 515–526.
Olvera-Rosales, L. B., Cruz-Guerrero, A. E., García-Garibay, J. M., Gómez-Ruíz, L. C., Contreras-López, E., Guzmán-Rodríguez, F., & González-Olivares, L. G. (2023). Bioactive peptides of whey: Obtaining, activity, mechanism of action, and further applications. *Critical Reviews in Food Science and Nutrition, 63*(30), 10351–10381.
Othman, M., Ariff, A. B., Wasoh, H., Kapri, M. R., & Halim, M. (2017). Strategies for improving production performance of probiotic *Pediococcus acidilactici* viable cell by overcoming lactic acid inhibition. *AMB Express, 7*, 215.
Parlindungan, E., Dekiwadia, C., May, B. K., & Jones, O. A. (2019). Nutrient stress as a means to enhance the storage stability of spray dried microencapsulated *Lactobacillus plantarum* B21. *LWT, 111*, 204–210.
Patel, S. S., Pushpadass, H. A., Franklin, M. E., Battula, S. N., & Vellingiri, P. (2021). Microencapsulation of curcumin by spray drying: Characterization and fortification of milk. *Journal of Food Science and Technology, 59*, 1326–1340.
Peighambardoust, S. H., Tafti, A. G., & Hesari, J. (2011). Application of spray drying for preservation of lactic acid starter cultures: A review. *Trends in Food Science and Technology, 22*, 215–224.
Pescuma, M., Hebert, E. M., Bru, E., Font de Valdez, G., & Mozzi, F. (2012). Diversity in growth and protein degradation by dairy relevant lactic acid bacteria species in reconstituted whey. *Journal of Dairy Research, 79*, 201–208.
Pescuma, M., Valdez, G. F., & Mozzi, F. (2015). Whey-derived valuable products obtained by microbial fermentation. *Applied Microbiology and Biotechnology, 99*, 6183–6196.
Pramanik, S., Venkatraman, S., Karthik, P., & Vaidyanathan, V. K. (2023). A systematic review on selection characterization and implementation of probiotics in human health. *Food Science and Biotechnology, 32*(4), 423–440.
Rabaioli Rama, G., Kuhn, D., Beux, S., Jachetti Maciel, M., & Volken de Souza, C. F. (2020). Cheese whey and ricotta whey for the growth and encapsulation of endogenous lactic acid Bacteria. *Food and Bioprocess Technology, 13*, 308–322.
Rajam, R., & Anandharamakrishnan, C. (2015). Microencapsulation of Lactobacillus plantarum (MTCC 5422) with fructooligosaccharide as wall material by spray drying. *LWT - Food Science and Technology, 60*, 773–780.
Rama, G. R., Kuhn, D., Beux, S., Maciel, M. J., & Volken de Souza, C. F. (2019). Potential applications of dairy whey for the production of lactic acid bacteria cultures. *International Dairy Journal, 98*, 25–37.
Santos de Espindola, J., Ferreira Taccóla, M., Nunes, S., da Silva, V., Delazari dos Santos, L., Cesar Rossini, B., … Galland, F. (2023). Digestion-resistant whey peptides promote antioxidant effect on Caco-2 cells. *Food Research International, 173*, Article 113291.
Secchi, N., Giunta, D., Pretti, L., García, M., Roggio, T., Mannazzu, I., & Catzeddu, P. (2012). Bioconversion of ovine scotta into lactic acid with pure and mixed cultures of lactic acid bacteria. *Journal of Industrial Microbiology & Biotechnology, 39*, 175–181.
Senovieski, M. L., Loyeau, P. A., Puntillo, M., Binetti, A. G., & Vinderola, G. (2024). Cheese whey for production of breast milk-derived bifidobacteria: Influence of fermentation conditions on the survival to spray drying and storage. *International Journal of Dairy Technology, 77*, 853–861.
Shafiee, G., Mortazavian, A. M., Mohammadifar, M. A., Koushki, M., Mohammadi, A., & Mohammadi, R. (2010). Combined effects of dry matter content, incubation temperature and final pH of fermentation on biochemical and microbiological characteristics of probiotic fermented milk. *African Journal of Microbiology Research, 4*, 1265–1274.
Sharma, P., Trivedi, N., & Gat, Y. (2017). Development of functional fermented whey-oat-based product using probiotic bacteria. *3 Biotech, 7*, 272.
Soriano-Perez, S., Flores-Vélez, L. M., Alonso-Dávila, P. A., Cervantes-Cruz, G., & Arriaga, S. (2011). Production of lactic acid from cheese whey by batch cultures of Lactobacillus helveticus. *Annals of Microbiology, 62*, 313–317.
Tang, H. W., Abbasiliasi, S., Murugan, P., Tam, Y. J., Ng, H. S., & Tan, J. S. (2020a). Influence of freeze-drying and spray-drying preservation methods on survivability rate of different types of protectants encapsulated *Lactobacillus acidophilus* FTDC 3081. *Bioscience, Biotechnology, and Biochemistry, 84*(9), 1913–1920.
Tang, H. W., Abbasiliasi, S., Murugan, P., Tam, Y. J., Ng, H. S., & Tan, J. S. (2020b). Influence of freeze-drying and spray-drying preservation methods on survivability rate of different types of protectants encapsulated *Lactobacillus acidophilus* FTDC 3081. *Bioscience, Biotechnology, and Biochemistry, 84*(9), 1913–1920.
Teo, A., Goh, K. K., Wen, J., Oey, I., Ko, S., Kwak, H. S., & Lee, S. J. (2016). Physicochemical properties of whey protein, lactoferrin and tween 20 stabilised nanoemulsions: Effect of temperature, pH and salt. *Food Chemistry, 197 Pt A*, 297–306.
Tsakali, E., Petrotos, K., D'Allessandro, A., & Goulas, P. A. (2010). Review on whey composition and the methods used for its utilization for food and pharmaceutical products. In *Proceedings of the 6th international conference on simulation and modelling in the food and bio-industry, Braganca, Portugal, 24–26 June 2010* (pp. 195–201).
Verma, S. K., Iram, D., Sansi, M. S., Pandey, K. K., Vij, S., & Sood, S. K. (2023). Sustainable utilization of dairy waste paneer whey by *Pediococcus pentosaceus* NCDC 273 for lactic acid production. *Biocatalysis and Agricultural Biotechnology, 47*, Article 102588.
Vinderola, G., Champagne, C. P., & Desfossés-Foucault, É. (2019). *The production of lactic acid bacteria starters and probiotic cultures. Lactic Acid Bacteria. Microbioloical and functional aspects. 20* pp. 317–336). Boca Raton, FL, USA: CRC Press, Taylor & Francis Group.
Vivek, K., Mishra, S., Pradhan, R. C., Nagarajan, M., Kumar, P. K., Singh, S. S., … Gowda, N. (2022). A comprehensive review on microencapsulation of probiotics: Technology, carriers and current trends. *Applied Food Research, 3*, Article 100248.
Wang, Y., Corrieu, G., & Béal, C. (2005). Fermentation pH and temperature influence the cryotolerance of Lactobacillus acidophilus RD758. *Journal of Dairy Science, 88*(1), 21–29.
Wendel, U. (2022). Assessing viability and stress tolerance of probiotics—A review. *Frontiers in Microbiology, 12*, Article 818468.
Wu, C., Zhang, J., Chen, W., Wang, M., Du, G., & Chen, J. (2011). A combined physiological and proteomic approach to reveal lactic-acid-induced alterations in *Lactobacillus casei* Zhang and its mutant with enhanced lactic acid tolerance. *Applied Microbiology and Biotechnology, 93*, 707–722.
Yonekura, L., Sun, H., Soukoulis, C., & Fisk, I. D. (2014). Microencapsulation of Lactobacillus acidophilus NCIMB 701748 in matrices containing soluble fibre by spray drying: Technological characterization, storage stability and survival after in vitro digestion. *Journal of Functional Foods, 6*, 205–214.
Zandona, E., Blažić, M., & Režek Jambrak, A. (2021). Whey utilisation: Sustainable uses and environmental approach. *Food Technology and Biotechnology, 59*, 147–161.
Zhang, L., Zeng, X., Fu, N., Tang, X., Sun, Y., & Lin, L. (2018). Maltodextrin: A consummate carrier for spray-drying of xylooligosaccharides. *Food Research International, 106*, 383–393.
Zhang, L., Zhou, R., Zhang, J., & Zhou, P. (2021). Heat-induced denaturation and bioactivity changes of whey proteins. *International Dairy Journal, 123*, Article 105175.