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Unravelling the functional and technological potential of soy milk based microencapsulated *Lactobacillus crispatus* and *Lactobacillus gasseri*

Margherita D'Alessandro [a], Flavia Pisanu [d], David Baldo [b], Carola Parolin [c], Gianfranco Filippini [b], Beatrice Vitali [c], Rosalba Lanciotti [a,d], Francesca Patrignani [a,d,*]

[a] Department of Agricultural and Food Sciences, University of Bologna, p.zza Gabriele Goidanich 60, 47521 Cesena (FC), Italy
[b] Department of Agricultural and Food Sciences, University of Bologna, Viale Giuseppe Fanin 46, 40127 Bologna (BO), Italy
[c] Department of Pharmacy and Biotechnology, University of Bologna, via San Donato 19/2, 40127 Bologna (BO), Italy
[d] Interdepartmental Center for Industrial Agri-food Research, University of Bologna, via Quinto Bucci 336, 47521 Cesena (FC), Italy

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ABSTRACT

The main aim of this study was to evaluate the potential of microencapsulation with soy milk as a carrier, conducted by spray-drying, on three mixed cultures of functional vaginal lactobacilli, *Lactobacillus crispatus* (BC1, BC3, and BC4) and *Lactobacillus gasseri* BC9, that could be further exploited as an adjunct in health foods. The i) physicochemical properties (using scanning electronic microscopy), ii) viability of encapsulated bacteria over time, and iii) functional features of powders containing the encapsulated bacteria were characterised considering different storage conditions. All microbial mixtures exhibited high viability for 90 d of storage, regardless of the storage conditions. All microencapsulated microbial combinations, excluding *L. crispatus* BC3 + *L. gasseri* BC9, exhibited hydrophobicity values exceeding 60%. Moreover, regardless of the considered combination, the *in vitro* digestion results exhibited a more significant decrease in cell viability for non-encapsulated microbial combinations than that of the encapsulated strains.

1. Introduction

Members of the FAO/WHO Expert Panel and FAO/WHO Working Group, together with the International Scientific Association for Probiotics and Prebiotics (ISAPP), have agreed to define probiotic bacteria as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). Owing to the increasing awareness of the importance of food on human health, consumer demand for delivering bioactive ingredients through everyday foods has rapidly increased. The global functional food industry is estimated to exceed USD 260 billion, and is predicted to reach USD 380 billion in sales by 2024 (Eratte et al., 2018). However, the maintenance of adequate levels of probiotic cultures in food and their functional properties for the full shelf-life of food is highly challenging, considering their fate during the digestive process. When included in a food product, probiotic strains and their viability are affected by several factors, such as their sensitivity to process conditions (low pH, oxygen, fermentation temperature), the effect of the food matrix (water activity, pH, presence of natural antimicrobials), and storage conditions, which can affect their performance and viability (Patrignani et al., 2017). Moreover, during digestion, the low pH of the stomach or presence of bile salts in the small intestine can further contribute to the loss of viability of the strains (Barbosa & Teixeira, 2017). Therefore, the development of suitable technologies for the maintenance of an adequate number of viable probiotic bacteria (>7 log colony-forming units [CFU] /g of product) is a key step (Espitia et al., 2016; Sarao & Arora, 2017). Microencapsulation using a spray-dryer is one of the most promising and widely used techniques, which offers a valuable option for encapsulating heat-sensitive nutrients and probiotic microorganisms (Vivek et al., 2021). Several studies have demonstrated the potential of this technique in developing probiotic powders with different carriers that can preserve the functionalities (Anekella & Orsat, 2013; Mestry et al., 2011; Muzaffar et al., 2016; Paim et al., 2016; Pereira et al., 2014).

In recent years, probiotics have been proposed to improve genital health of women, and microbial strains with beneficial properties can be used to prevent or treat vaginal dysbiosis and genital infections. In

* Corresponding author at: Department of Agricultural and Food Sciences, University of Bologna, p.zza Gabriele Goidanich 60, 47521 Cesena (FC), Italy.
E-mail addresses: margheri.dalessandr3@unibo.it (M. D'Alessandro), flaviapisanu@yahoo.it (F. Pisanu), david.baldo@unibo.it (D. Baldo), carola.parolin@unibo.it (C. Parolin), gianfranco.filippini@unibo.it (G. Filippini), b.vitali@unibo.it (B. Vitali), rosalba.lanciotti@unibo.it (R. Lanciotti), francesca.patrignani@unibo.it, flavia.pisanu2@unibo.it (F. Patrignani).

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particular, members of the *Lactobacillus* genus, which are generally healthy human vaginal microbiota, can be administered locally or orally, as probiotics can reach the genital apparatus because of anatomic proximity once they colonise the gut (Heczko et al., 2015; Reid et al., 2001). Recent studies have highlighted the potential role of some vaginal *Lactobacillus* strains in promoting well-being of women, as they possess antimicrobial and functional features. In particular, vaginal *Lactobacillus crispatus* and *Lactobacillus gasseri* strains exhibited activity towards several genital pathogens, including *Candida* (Calonghi et al., 2017; Parolin et al., 2015), *Chlamydia trachomatis* (Nardini et al., 2016; Parolin et al., 2018) *Neisseria gonorrhoeae* (Foschi et al., 2017) Group-B *Streptococcus* (Marziali et al., 2019), and HIV1 (Ñahui Palomino et al., 2019). The safety and technological properties of the same strains have also been tested, and they exhibited a good ability to grow in milk and produce specific volatile molecules, promoting their potential application as functional additional cultures in the dairy sector (Siroli et al., 2017). D'Alessandro et al. (2021) demonstrated that some of these strains have interesting functional features, including high hydrophobicity and auto-aggregation values (over 70%), even when compared to *Lacticaseibacillus rhamnosus* GG ATCC® 53103™, a commercial probiotic strain used as reference. In this context, the connection between these two parameters is positive; lactobacilli with a hydrophobic cell surface and strong auto-aggregation ability could have a greater chance of adhering to human cells. Therefore, the addition of an appropriate combination of such *Lactobacillus* strains to food matrices could aid in using food as a dietary strategy to improve the well-being of women. However, these strains could be particularly sensitive to different stresses encountered during food processing, as mentioned above. Therefore, microencapsulation could offer an advantage for the *Lactobacillus* strains under the different stresses encountered by food products throughout their shelf-life and digestion when used in combination.

Therefore, the main aim of this study was to evaluate the potential of microencapsulation by spray-drying, using lab-scale equipment on three mixed cultures of functional vaginal lactobacilli (*L. crispatus* BC1 + *L. gasseri* BC9, *L. crispatus* BC3 + *L. gasseri* BC9, and *L. crispatus* BC4 + *L. gasseri* BC9) for their further use as adjunctive cultures in functional fermented food, with soy milk as a unique carrier exploiting its natural composition in total solid and its functional features. *L. crispatus* BC1, BC3, and BC4 strains and *L. gasseri* BC9 were selected based on previous investigations in which the strains exhibited good technological and functional features (Siroli et al., 2017; D'Alessandro et al., 2021). The strain viability of three powders containing the encapsulated *Lactobacillus* strain combinations was characterised immediately after spray-drying and during storage at different temperatures (25, 4, and −20 °C). Moreover, the water activity and moisture content of the mixtures were tested immediately after spray-drying and after one year of storage at different temperatures. The obtained powders were also analysed by scanning electron microscopy (SEM). Some functional features of the encapsulated strain mixtures were also tested, such as hydrophobicity and resistance to a simulated stomach-duodenum passage.

2. Material and methods

2.1. Strains

In this study, four vaginal lactobacilli *(L. crispatus* BC1, *L. crispatus* BC3, *L. crispatus* BC4, *L. gasseri* BC9) strains belonging to the FABIT (Department of Pharmacy and Biotechnology of Bologna University) collection were used. The strains were isolated from the vaginas of premenopausal Caucasian women (aged 18–45 years old), with no symptoms of vaginal or urinary tract infection in accordance with the Ethics Committee of the University of Bologna (52/2014/U/Tess). The lactobacilli were cultured in de Man, Rogosa, Sharpe (MRS) broth (Oxoid Ltd., Basingstoke, UK) with 0.05% L-cysteine and incubated at 37 °C for 24 h under anaerobiosis (GasPak System; Oxoid Ltd., Basingstoke, UK). Working stocks of cultures were maintained in a 20% glycerol suspension frozen at −18 °C. The experimental study was conducted using three bacterial culture mixtures to evaluate the synergistic and functional interactions between the selected *L. crispatus* and *L. gasseri* strains.

2.2. Cell culture preparation and spray-drying process

L. crispatus BC1, *L. crispatus* BC3, *L. crispatus* BC4, and *L. gasseri* BC9 were individually cultivated overnight at 37 °C in 1 L of MRS broth + 0.05% L-cysteine under anaerobiosis to obtain a final concentration of at least 10^9 CFU/mL for each strain. The strain cell loads were determined after serial dilutions in 0.9% NaCl isotonic solution by plating on MRS agar with 0.05% L-cysteine and incubating at 37 °C for 48 h under anaerobiosis. One litre of each strain was centrifuged at 8200 rpm for 15 min at 4 °C (Avanti J-26 XP with Ja A-10 rotor, Beckman Coulter). After removing the supernatant, the microbial pellet was washed with 1 L of 0.9% NaCl isotonic solution and then resuspended in 500 mL of commercial soy milk. The conditions were then set for the creation of mixtures 1, 2, and 3, which were composed of *L. crispatus* BC1 + *L. gasseri* BC9 (ratio 1:1 v/v), *L. crispatus* BC3 + *L. gasseri* BC9 (ratio 1:1 v/v), and *L. crispatus* BC4 + *L. gasseri* BC9 (ratio 1:1 v/v), respectively. Commercial soy milk with 9.04% total solids, 9.8 °Brix, pH 6.64, 1.8% fats, 2.8% carbohydrates, 3% proteins, and 0.4% fibre was used. Spray-drying was conducted using a mini spray-dryer (B191, Buchi - Labortechnik AG, Switzerland), which was a laboratory-scale spray-dryer equipped with a single fluid nozzle. For the formulation of each matrix, inlet and outlet air temperatures of 110 and 70 °C were selected, respectively. The pump rate was maintained between 19% and 36% aspiration, while the feed flow rate was 10 mL/min. For each mixed culture, 1 L of suspension was spray-dried to produce an average of 5.2 g of powder/100 mL of suspension. Spray-dried powder samples were collected from the cyclone, mixed gently, and vacuum-packed in nylon/polyethene, 102 μm high-barrier plastic bags (Tecnovac, San Paolo D'Argon, Bergamo, Italy) using an S100-Tecnovac device. Samples were then stored at 25, 4, and −20 °C.

2.3. Determination of powder moisture content and water activity

The moisture content was determined from the weight loss after drying 2 g of powder at 103 °C for 3 h, as described by the International Dairy Federation standard (IDF, 2004). The water activities of the samples were measured using a water activity meter (Aqualab 4TE, Decagon Devices, Pullman, Washington, USA) at 25 °C.

2.4. Encapsulation yield after spray-drying

The encapsulation yield of the spray-dried samples was determined following the plate count method. The spray-dried powder was rehydrated with 0.9% NaCl isotonic solution, followed by stirring for 10 min, to reach the same solid content as that of the feed solution. Suitably diluted feed solution and rehydrated samples (1 mL each) were plated on MRS agar with 0.05% L-cysteine and incubated at 37 °C for 48 h under anaerobiosis. The encapsulation yield (%) of the spray-dried sample was calculated as $100 \times N/N_0$, where N is the number of viable cells (CFU/mL) released from the rehydrated sample and N_0 is the number of viable cells (CFU/mL) in the cell concentrate before spray-drying (Ilha et al., 2015; Patrignani et al., 2017).

2.5. Scanning electron microscopy

The morphologies of the powder material wall and encapsulate were observed using a scanning electron microscope (Hitachi S-510) with an accelerated voltage of 15 kV. The samples were sputter-coated with gold, prior to SEM analysis, for 3 min at 40 mA using an EMITEC Sputter Coater K500. Microencapsulated powders were collected with a spatula and fixed to a sample holder using conductive scotch (3MScotch Tape

465KP). Excess non-adherent powders were removed. Finally, images were acquired using a DISS5 system from Point Electronics. SEM was also conducted using a soy milk sample as a control. The average particle diameter was determined by measuring 120 randomly selected microcapsules directly from the SEM images (Acordi Menezes et al., 2018). The analysis was conducted using the ImageJ software.

2.6. Cell viability over time

To evaluate the resistance of the encapsulated strains stored at 25 °C, + 4 °C, and −20 °C over time, several samplings were conducted; after 7, 14, 30, 90, and 365 d, 1 g of the microcapsules from each sample was resuspended in 9 mL of 0.9% NaCl isotonic solution, followed by stirring for 10 min, according to the method proposed by Ilha et al. (2015) with some modifications. The cell load of each sample was determined by plating on MRS agar with 0.05% L-cysteine and incubating at 37 °C for 48 h under anaerobiosis.

2.7. Hydrophobicity

Hydrophobicity, defined as the ability of microbes to adhere to hydrocarbons, was assessed following the method reported by Vinderola and Reinheimer (2003) pre and post spray-drying with some modifications. The pre-spray-drying hydrophobicity tests were conducted on the three selected vaginal lactobacilli mixtures resuspended in soy milk, as described in Section 2.2. For this, 0.1 mL of each functional mixture was resuspended in 3 mL of 0.9% NaCl isotonic solution and then centrifuged at 6000 rpm for 10 min at 4 °C (Avanti J-26 XP with 25–50 rotor, Beckman Coulter). The pellet was resuspended in 0.9% NaCl isotonic solution and was subsequently diluted to reach an absorbance value of 1 at 560 nm using a spectrophotometer (model 6705, Jenway, ST15 OSA, UK). To evaluate the hydrophobicity after spray-drying, 0.1 g of each encapsulated mixture was resuspended as described previously. Three millilitres of each bacterial suspension obtained before and after spray-drying was then vortexed with 0.6 mL of n-hexadecane (Sigma, Milan, Italy) for 4 min. The two phases were then allowed to separate for 1 h at 37 °C. The aqueous phase was carefully removed, and the absorbance (A) at 560 nm was measured. Finally, the hydrophobicity percentage was calculated using the following formula: (A0-At)/A0 × 100, where A0 is the absorbance at time 0 and At is the absorbance at 560 nm after 1 h of incubation at 37 °C.

2.8. Post spray-drying tolerance to simulated digestion

To evaluate the resistance of the encapsulated strains to passage through the stomach and duodenum, also considering the potential effect of the food matrix to vehiculate the encapsulated bacteria, the method proposed by Vinderola et al. (2011) was followed, with certain modifications. Briefly, for each microbial combination, a sample containing 1 g of microbial powder after spray-drying and 9 mL of commercial soy milk was prepared as the initial inoculum with at least 9 log CFU/g, and 0.1 mL of the sample was collected for cell counting. The sample was mixed with the same volume of a 'saliva-gastric' solution containing $CaCl_2$ (0.22 g/L), NaCl (16.2 g/L), KCl (2.2 g/L), NaHCO3 (1.2 g/L) and 0.3% (w/v) porcine pepsin (Sigma, Milan, Italy). The pH of the sample was quickly adjusted to 2.5–3 with 1-M HCl and then transferred to a thermostatic bath for 90 min at 37 °C (WB-MF, Falc Instruments, Treviglio, Italy). Following this, 0.1 mL of the sample was collected for the second cell viability sampling. Additionally, 2 mL of the sample was centrifuged at 12000 rpm for 4 min at 4 °C (Himac CT 15RE, VWR). After removing the supernatant, the microbial pellet was centrifuged with 2 mL of 0.9% NaCl isotonic solution (12,000 rpm, 4 min, and 4 °C). The microbial pellet was then resuspended in 2 mL of bile-extract porcine solution (Sigma, Milan, Italy) at a concentration of 1% in saline phosphate buffer, which simulated hepatic bile. The sample was placed in a thermostatic bath at 37 °C for 10 min to simulate the duodenal shock phase of bile. Then, 0.1 mL of the sample was collected for the third sampling to verify the cell viability. The remainder of the sample was centrifuged at 12,000 rpm for 4 min at 4 °C. Once the supernatant was removed, the microbial pellet was resuspended in 1.9 mL of 0.9% NaCl isotonic solution and centrifuged under the same conditions. Subsequently, a third solution representing enteric stress was added, which consisted of 0.3% bile and 0.1% pancreatin from the porcine pancreas (Sigma, Milan, Italy) dissolved in saline phosphate buffer. The sample was incubated in the thermostatic bath for 90 min at 37 °C. Then, 0.1 mL of the sample was collected for the final sampling. The number of CFU/g of the sample was determined by plating on MRS agar with 0.05% L-cysteine and incubating at 37 °C for 48 h under anaerobiosis. The results are expressed in log CFU/g. An assay to determine the tolerance to the simulated digestion process assay was also conducted and included the same unencapsulated mixed bacterial cultures used as controls. *L. crispatus* BC1, BC3, and BC4, and *L. gasseri* BC9 were individually cultivated overnight at 37 °C in MRS broth + 0.05% L-cysteine under anaerobiosis to obtain a final concentration of at least 10^9 CFU/mL for each strain. The strain cell loads were evaluated after serial dilutions in 0.9% NaCl isotonic solution by plating on MRS agar with 0.05% L-cysteine and incubating at 37 °C for 48 h under anaerobiosis. Briefly, for each microbial combination, a sample containing 1 mL of each strain and 9 mL of commercial soy milk was prepared with at least 8–9 log CFU/mL as the initial inoculum. All unencapsulated mixed cultures used as controls were tested in the same manner as the encapsulated strains with a simulated digestion process.

2.9. Statistical analysis

All experimental data are expressed as the mean of three replicates. The data were statistically analysed using Statistica software (version 8.0; StatSoft, Tulsa, Oklahoma, USA) and underwent analysis of variance (ANOVA) and mean comparison tests according to Fisher's least significant difference (LSD). The significance level was set at $p < 0.05$ and indicated in the text.

3. Results and discussion

3.1. Moisture content, water activity, and encapsulation yield of the three strain mixtures

The three mixtures of *L. crispatus* and *L. gasseri* strains underwent spray-drying at inlet and outlet temperatures of 110 and 70 °C, respectively. The strains were encapsulated together since according to previous data (data not reported) the yields of the single cultures were lower with respect those of the mixture. The physicochemical properties and encapsulation yield of the three mixtures are listed in Table 1.

The selected process parameters resulted in powder water activity values of 0.221. These data are consistent with those of Corcoran et al. (2004) who recommended water activity values ranging between 0.150 and 0.300 for spray-dried microcapsules to ensure microbiological stability. Some researchers have suggested that the moisture content of spray-dried powders must be below 5% to ensure storage stability (Behboudi-Jobbehdar et al., 2013; Chávez & Ledeboer, 2007). Our data indicate moisture content values of approximately 4.4% for all of the encapsulated mixtures obtained which complies with the standard acceptable moisture levels for spray-dried powders. Low water activity values and residual moisture contents are also prerequisites for the commercial production of spray-dried powders with good handling characteristics, such as high flow ability, low stickiness and agglomeration, and maximum probiotic viability.

Table 1 also shows the cell load values obtained before and after spray-drying and the relative encapsulation yield of the three mixtures of *L. crispatus* and *L. gasseri*. The highest encapsulation yield was obtained for the mixture of *L. crispatus* BC1 + *L. gasseri* BC9. In contrast, the other tested combinations showed lower encapsulation yields and

Table 1
Physicochemical properties and encapsulation yield of the three selected encapsulated mixtures obtained in relation to adopted inlet and outlet temperatures (110 and 70 °C). Results are shown as average ± SD. Considering the columns, samples with different letters are significant different ($p < 0.05$).

Strain Mixture	Inlet T (°C)	Outlet T (°C)	Moisture content (%)	Water activity (after spray drying)	N_0* (CFU/ml)	N** (CFU/ml)	Encapsulation yield (%)***
L. crispatus BC1 + *L. gasseri* BC9	110 °C	70 °C	4.45[a] (±0.01)	0.221[a] (±0.001)	5.2×10^9	1.8×10^9	33.90 %
L. crispatus BC3 + *L. gasseri* BC9	110 °C	70 °C	4.46[a] (±0.01)	0.221[a] (±0.001)	3.0×10^9	1.1×10^9	37.42 %
L. crispatus BC4 + *L. gasseri* BC9	110 °C	70 °C	4.45[a] (±0.01)	0.221[a] (±0.001)	5.6×10^9	1.4×10^9	24.69%

* N_0 was the number of viable cells in the cell concentrate before spray-drying process.
** N was the number of viable cells released from rehydrated sample.
*** The encapsulation yield was expressed as a percentage according to the formula: $100 \times N/N_0$.

viabilities. The loss of probiotic viability during convective thermal processing is related to cellular injuries caused by the combined effects of heat and mechanical stress. The selection of an inlet temperature of 110 °C in this study was based on literature data suggesting that a lower inlet air temperature could increase the viability of lactobacilli. According to Behboudi-Jobbehdar et al. (2013), a lower inlet air temperature (tested between 120 and 160 °C) could enhance the viability of *Lactobacillus acidophilus*. Additionally, the outlet temperature of 70 °C was selected as the same authors reported significant changes in the viability of *L. acidophilus* viability when the outlet temperature was reduced from 91.5 to 60 °C.

Nunes et al. (2018) reported that the viability of microparticles containing *L. acidophilus* La-5 (ML) *and Bifidobacterium lactis* Bb-12 (MB) produced at different drying temperatures (100–140 °C) was highest at 110 °C and negatively affected by an increase in temperature. Bustamante et al., 2017 also reported an optimal inlet temperature of 110 °C, who used chia seed mucilage extracted *via* spray-drying to encapsulate *Lactiplantibacillus plantarum* subsp. *plantarum* and *Bifidobacterium infantis*, observed a decrease in viability with increasing temperature. However, Arslan et al. (2015) reported that an inlet temperature of 125 °C resulted in reduced viability and lower survival rates of *Saccharomyces cerevisiae* var. boulardii. Fávaro-Trindade and Grosso (2002) reported that different microbial strains could have different temperature tolerance thresholds for spray-drying and reported that the number of viable *B. lactis* cells remained almost the same with inlet and outlet air temperatures of 130 and 75 °C, respectively, while the population of *L. acidophilus* was reduced by two logarithmic cycles.

Regarding the species used in this study, only a few examples of the encapsulation of *L. crispatus* and *L. gasseri* by spray-drying have been reported in the literature. Zhang et al. (2013) reported that the efficiency of *L. crispatus* encapsulation in polymethacrylate salt intended for vaginal delivery ranged between 45.7% and 95.5% under different parameters, such as the polymer concentration, polymer and drug ratio, and inlet temperature. Lavari et al. (2014) reported significant reductions in cell counts before and after spray-drying for *L. gasseri* 37 resuspended in skim milk- and whey-starch at inlet temperatures ranging between 130 and 160 °C.

However, the literature data regarding the viability of bacteria after spray-drying are conflicting, suggesting strain-dependent behaviour regarding the tolerance thresholds for spray-drying in relation to the carrier used. Although spray-drying has a lower cost than freeze-drying and allows the production of large amounts of dried cells in a continuous process (Gardiner et al., 2000), cell dehydration by spray-drying requires harsher conditions than freeze-drying, which may cause membrane damage and inactivation, depending on the technological conditions applied and intrinsic resistance of the strain used. The effect of the carrier wall material on probiotic survival through spray-drying has been studied previously (Shori, 2017; Ying et al., 2012). The total solids concentration of the carrier aliquot, as well as the presence of ingredients that can induce a significant decrease in the melting temperature of the microparticles, critically affect the structural integrity of the cytoplasmic membranes and control the osmotic pressure that leads to membrane rupture. Therefore, materials with good encapsulation capacity and impart acceptable functional characteristics for powders (porosity, free-flowing ability, anticaking, and good wetting and dispersal properties) are often selected as the carrier wall material, in addition to other ingredients that exhibit thermo-protective features, such as disaccharides (lactose, sucrose, or trehalose), dextrose, or polyols (mannitol, sorbitol), or act as probiotic growth stimulants (fructo- and galacto-oligosaccharides) (Behboudi-Jobbehdar et al., 2013).

In this study, soy milk was used as a carrier as soy is considered the best substitute for animal proteins as a wall material due to its renewability, low cost, high nutritional value, and functional properties, such as its gelation and emulsification abilities. Additionally, soy protein is an alternative for vegan individuals or those with milk protein allergies. Moreover, the prevalence of soy protein allergies is five-fold lower than that of milk protein allergies (Dunlop & Keet, 2018; Kattan et al., 2011; Nesterenko et al., 2013; Tang & Li, 2013b), and all components of soy contain functional compounds, such as isoflavones and oligosaccharides (Nilufer-Erdil et al., 2012), that play stimulating and prebiotic roles for encapsulated microorganisms. However, in this study, soy milk was used as a single carrier to utilise its natural composition (9.8% total solids), although previous studies have used soy products in combination with maltodextrins to further decrease the glass transition temperature (Tg) of the mixture (Acordi Menezes et al., 2018). However, the use of carriers such as maltodextrins, starch, buttermilk and cocoa powder in formulations for encapsulation may result in positive or negative interactions between the carriers, food matrices, and food organoleptic and nutritional features (Čurda et al., 2006; Favaro-Trindade et al., 2010; Ricci et al., 2011; Frenzel et al., 2015). For example, the exclusion of maltodextrins would allow these powders to be available for the formulation of food products for individuals with type-2 diabetes. However, maltodextrins or starch could enhance the physical properties of the powders obtained, cell viability after drying, and resistance to simulated gastrointestinal digestion. From this perspective, further initial formulation optimisation could be considered to further increase the cell loads of lactobacilli strains after encapsulation. However, the final purpose of the obtained encapsulated bacteria is their use as adjuncts in functional foods (cheese or fermented milk products) at a level of at least 7 log CFU/g, which is achievable with the level of viability obtained in this study.

3.2. Scanning electronic microscopy of the selected strain mixtures

Fig. 1 presents micrographs of the three selected mixtures obtained with inlet and outlet temperatures of 110 and 70 °C, respectively and soy milk without bacteria under the same spray-drying conditions. No free cells were observed in any of the considered mixtures, confirming the microencapsulation of the bacteria.

In all cases, the obtained capsules exhibited a rounded shape and an uneven surface with concavities, which are characteristic of products subjected to spray-drying (Favaro-Trindade et al., 2010) and attributed to the type of encapsulating agent (flat ball effect) and shrinkage of the particles during drying (Lian et al., 2002; Saénz et al., 2009). The average Feret diameter obtained using ImageJ software was 5.44 μm (±1.08). These results agree with those reported by Porras-Saavedra

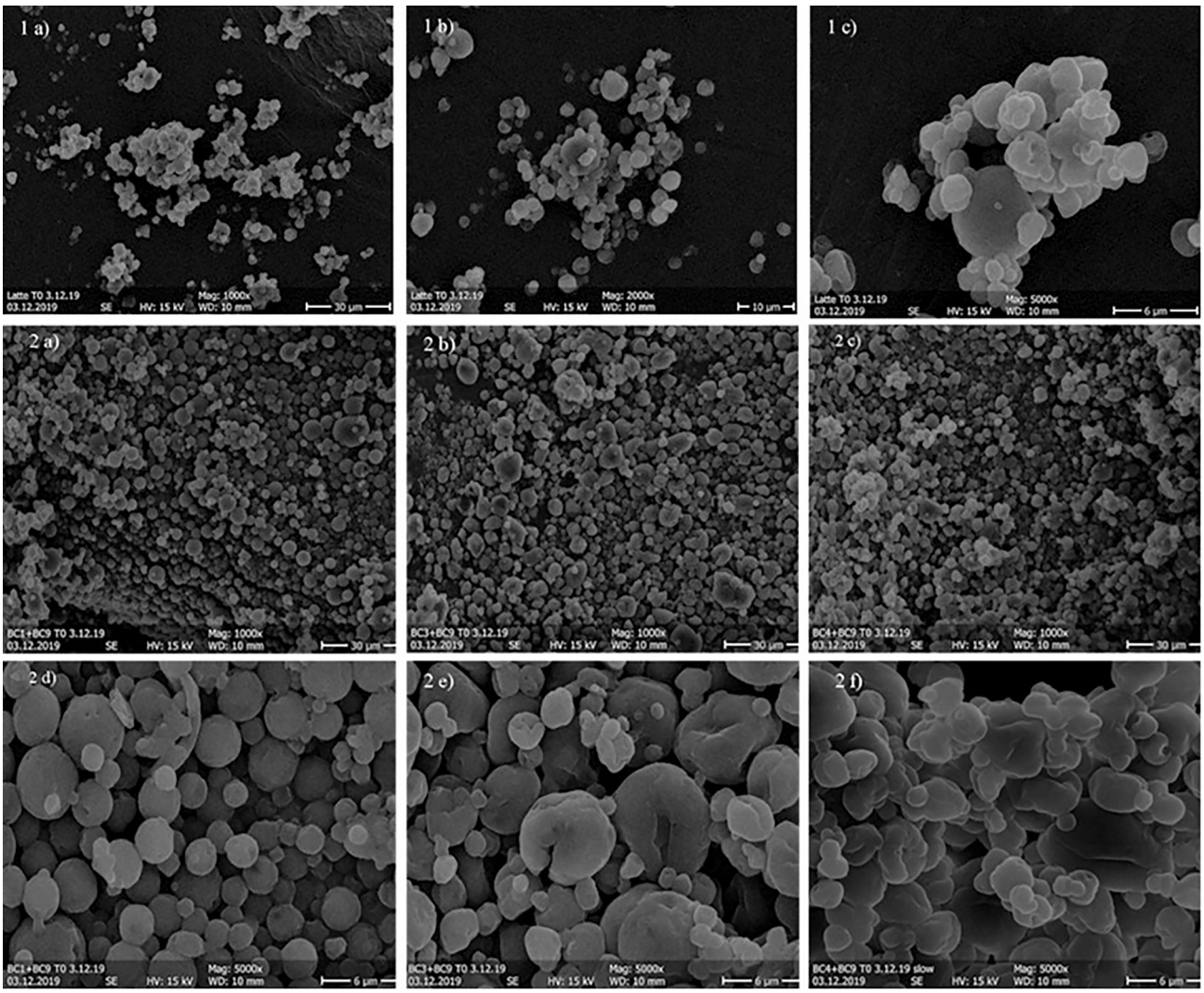

Fig. 1. Scanning electron microscopy (SEM) images of microparticles of soy milk: 1a) at ×1000 magnification, 1b) at ×2000 magnification, 1c) at ×5000 magnification and microparticles of *L. crispatus* BC1 + *L. gasseri* BC9, *L. crispatus* BC3 + *L. gasseri* BC9 and *L. crispatus* BC4 + *L. gasseri* BC9 carried by soy milk at ×1000 magnification (2a, 2b, 2c) at ×5000 magnification (2d, 2 e, 2f).

et al. (2015) who obtained microcapsules with diameters between 2 and 7 μm, and Acordi Menezes et al. (2018) who obtained capsules with diameters ranging between 4.97 and 8.82 μm.

The surface of the particles was not particularly rough, which could be related to the soy protein content, as reported by Tang and Li (2013) who found that an increase in the protein ratio could minimise shrinkage and, consequently, the concavities of the particle surfaces, in addition to preventing structural disruption. No significant differences among the three mixtures were observed. However, the limitations of roughness and particle features are highly important as they can affect consumer perception when using encapsulated bacteria in food formulation (Patrignani et al., 2017) particularly in dairy products (Ding & Shah, 2009; Iravani et al., 2015).

3.3. Cell viability of the strain mixtures over time

The bacterial viability of the three encapsulated mixtures was analysed based on the storage time and different storage conditions, that is, room temperature (25 °C), refrigeration (4 °C), and frozen storage conditions (−20 °C), to understand their technological features. Regardless of the combination and storage conditions, the viability of the encapsulated strain mixtures remained stable for 90 d of storage. As shown in Fig. 2, after 365 d of storage, the cell viability for mixture 1 (*L. crispatus* BC1 and *L. gasseri* BC9) significantly decreased ($p < 0.05$) for the sample stored at 25 °C. Moreover, as shown in Fig. 3, a decrease in cell viability was observed after 365 d of storage for mixtures 2 (*L. crispatus* BC3 and *L. gasseri* BC9) and 3 (*L. crispatus* BC4 and *L. gasseri* BC9) when stored at room temperature (25 °C), with reductions of 3 log (mixture 2) and 5 log (mixture 3). These data agree with literature data reporting a decline in the viability of lactobacilli species stored at 25 °C compared to those refrigerated at 4 °C (Behboudi-Jobbehdar et al., 2013). This could be because the higher temperature increased the metabolic rate of cells and other chemical or enzymatic reactions that may also occur, such as lipid oxidation, and also due to the molecular mobility of water. In addition to the intrinsic resistance of the strain to heat, the used carrier, presence (or absence) of the glass transition state, water activity, and residual moisture of the powders could be principal factors affecting the viability during storage. Moreover, the presence of oxygen and light can affect the encapsulated strains viability (Lavari et al., 2014). However, as shown in Table 2, the water activity and moisture content of the three mixtures after 365 d of storage were higher in the samples stored at 25 °C, confirming the decrease in viability.

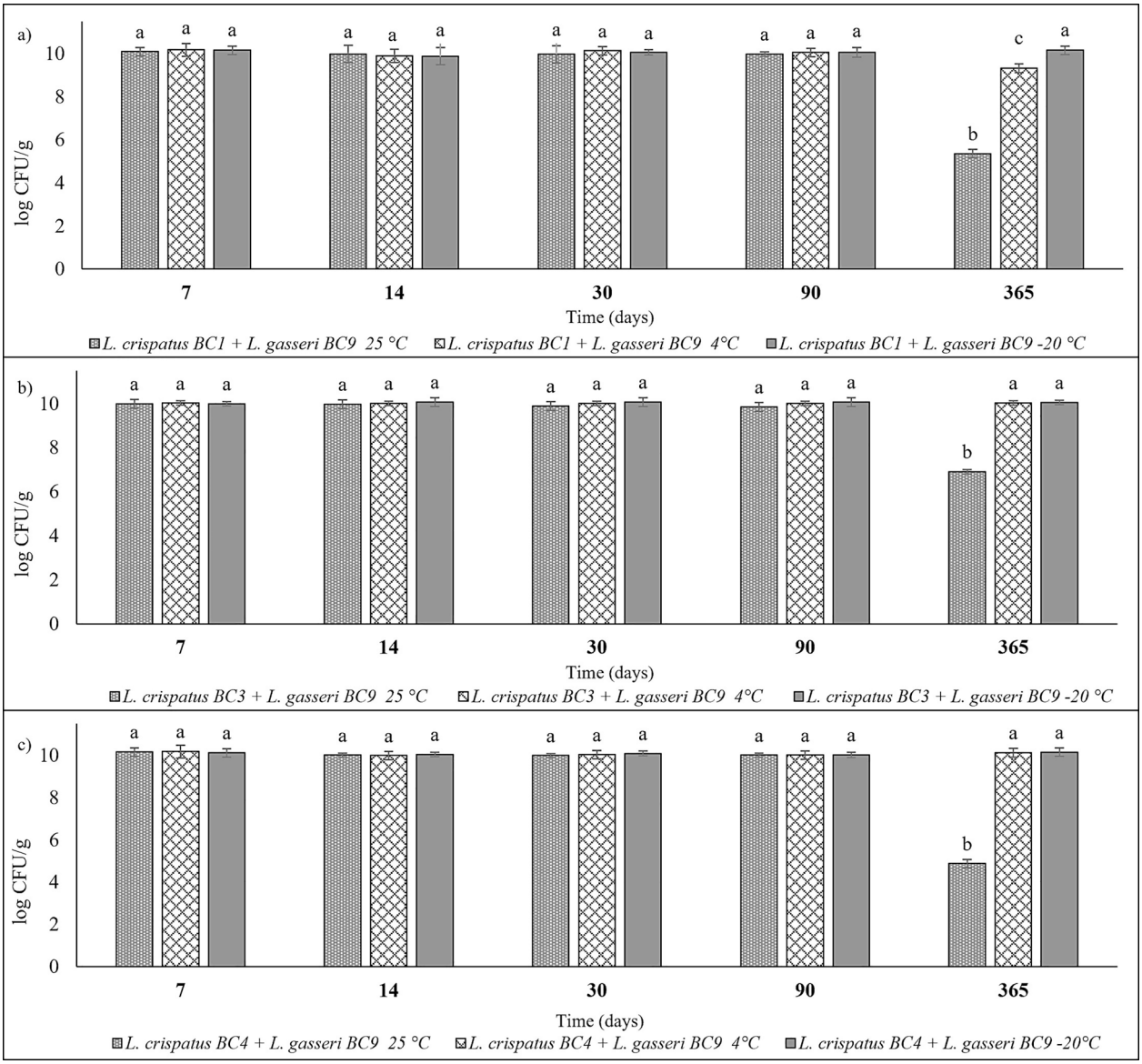

Fig. 2. Cell loads (log CFU/g) of *L. crispatus* BC1 + *L. gasseri* BC9 (a), *L. crispatus* BC3 + *L. gasseri* BC9 (b) and *L. crispatus* BC4 + *L. gasseri* BC9 (c) powders after 7, 14, 30, 90, 365 days of storage (25 °C, +4 °C and −20 °C). Results are shown as average ± SD. Samples with different letters are significantly different ($p < 0.05$).

3.4. Strain mixture hydrophobicity

The three mixtures, pre and post spray-drying, were subjected to hydrophobicity tests to evaluate the maintenance of some probiotic criteria (Fig. 3). The hydrophobicity of all considered mixtures decreased significantly ($p < 0.05$) after spray-drying, even if the *L. crispatus* BC4 + *L. gasseri* BC9 mixture exhibited fewer changes, which was characterised by the lowest initial values. Interactions between the soy milk composition and high temperatures applied during the process may lead to important variations in bacterial hydrophobicity, resulting in different exposure of sulphydrylic groups in the cell-wall proteins (Braschi et al., 2021).

The data obtained allow the functional properties of these strain combinations as the hydrophobic nature of the surface of microorganisms can be related to the attachment of bacteria to host tissues (Del Re et al., 2000; Mathara et al., 2008; Tabanelli et al., 2013), which offers them a competitive advantage and is important for its permanence in the human gastrointestinal tract (Schillinger et al., 2005; Tabanelli et al., 2013). Interaction with the carrier also appears to affect this probiotic index. Hydrophobicity is caused by the complex interactions between negatively and positively charged hydrophobic and hydrophilic components on the surfaces of microbial cells. Excluding mixture 3 after spray-drying, all microbial combinations exhibited hydrophobicity values exceeding 60%, which can be considered a threshold for this parameter (Hsiung et al., 2020).

3.5. Viability of strain combinations after simulated digestion

The different mixtures were subjected to stomach-duodenum simulated passage before and after spray-drying considering the effect of a potential food matrix as the encapsulated bacteria produced in this study are intended to be delivered as functional adjuncts. Both encapsulated and non-encapsulated bacteria were tested in soy milk. Regardless of the considered combination, Fig. 4 shows that the cell viability significantly decreased ($p < 0.05$) for the unencapsulated combinations, while that of the encapsulated strain mixtures remained stable throughout the simulated digestion process. In particular, the mixture composed of *L. crispatus* BC3, *L. gasseri* BC9, *L. crispatus* BC4, and *L. gasseri* BC9 was more sensitive to duodenal shock using bile salts when non-encapsulated. Generally, the low sensitivity of the encapsulated strains

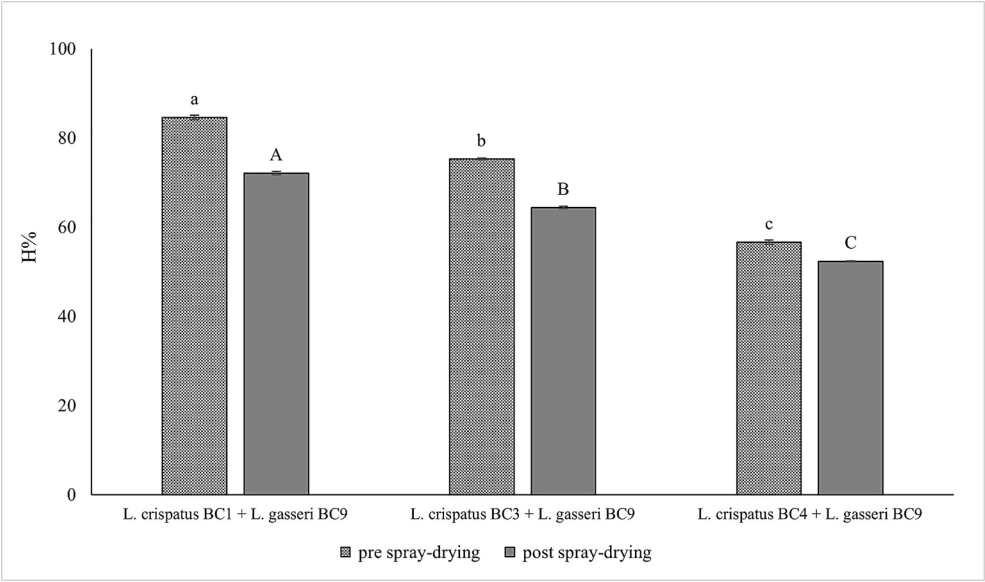

Fig. 3. Cell hydrophobicity of pre and post spray-drying samples. The hydrophobicity % was calculated with the following formula: (A0-At)/A0 × 100, A0 represents the absorbance at time 0 and At represents the absorbance at 560 nm after 1 h at 37 °C. Results are shown as average ± SD. Samples with different letters are significantly different ($p < 0.05$).

Table 2
Physicochemical properties (moisture content (%) and water activity) of the three selected encapsulated mixtures observed at 365 days in relation to different storage conditions. Results are shown as average ± SD. Considering the columns, samples of the same mixture with different letters are significant different ($p < 0.05$).

Strain Mixture	Storage conditions	Moisture content (%)	Water activity
L. crispatus BC1 + *L. gasseri* BC9	25 °C	4.75^{a} (±0.01)	0.331^{a} (±0.001)
L. crispatus BC1 + *L. gasseri* BC9	4 °C	4.74^{b} (±0.01)	0.288^{b} (±0.001)
L. crispatus BC1 + *L. gasseri* BC9	−20 °C	4.61^{c} (±0.02)	0.229^{c} (±0.001)
L. crispatus BC3 + *L. gasseri* BC9	25 °C	4.72^{a} (±0.01)	0.316^{a} (±0.001)
L. crispatus BC3 + *L. gasseri* BC9	4 °C	4.68^{b} (±0.01)	0.263^{b} (±0.001)
L. crispatus BC3 + *L. gasseri* BC9	−20 °C	4.56^{c} (±0.01)	0.223^{c} (±0.001)
L. crispatus BC4 + *L. gasseri* BC9	25 °C	4.95^{a} (±0.01)	0.347^{a} (±0.001)
L. crispatus BC4 + *L. gasseri* BC9	4 °C	4.67^{b} (±0.01)	0.275^{b} (±0.001)
L. crispatus BC4 + *L. gasseri* BC9	−20 °C	4.58^{c} (±0.01)	0.220^{c} (±0.001)

to bile salts, which are selective agents against gram-positive bacteria, is likely related to the protective effect of the encapsulating material, which acts as a barrier. However, the ability of the non-encapsulated bacteria to overcome this stress was related to their ability to deconjugate bile salts by bile salt hydrolase (BSH) enzyme activity, which is vital for the selection of functional strains as it can facilitate the reduction of bile toxicity via the deconjugation of bile salts into bile acids (D'Alessandro et al., 2021). Additionally, Vamanu (2017) reported significant reductions in different Lactic Acid Bacteria (LAB) strains in the presence of enzymes and bile salts related to small intestine stress when testing their viability using the GIS1 simulator. Furthermore, Bianchi et al. (2014) used SHIME® to evaluate the fate of *Lacticaseibacillus casei* Lc-01 inoculated in beverages, and observed a significant reduction in the viability of the strain under stomach and duodenum conditions. According to Xanthopoulos et al. (2000), bile salt resistance varies significantly among different lactic acid bacterial species and strains of the same species. According to Saarela et al. (2000) bile salts are toxic to microbial cells as they disrupt the cellular membrane structure; therefore, bile salt tolerance is one of the required characteristics for probiotic cultures.

4. Conclusions

The results of this study have highlighted the technological and functional features of the powders obtained by spray-drying different mixtures of vaginal *L. crispatus* and *L. gasseri* strains using soy milk as an encapsulating agent at the laboratory scale. The powders obtained produced microcapsules with suitable morphology, good technological features in terms of water activity and moisture content, and good strain viability, although the encapsulation yield needs to be further optimised also taking into consideration additional protective encapsulating material such as skim milk. The mixture with the highest viability consisted of *L. crispatus* BC1 + *L. gasseri* BC9, although it is difficult to understand the single-strain behaviour in relation to the spray-drying process. Therefore, for upscaling, the process conditions should be further optimized and some qPCR methods should be set up to understand the specific strain viability. However, the results obtained here are highly promising from the perspective of using encapsulated powders in food formulations to obtain novel functional foods.

Ethical statement

Hereby, I declare that all the data presented in the manuscript were produced by the Authors partnership and used with the permission of all the Authors.

CRediT authorship contribution statement

Margherita D'Alessandro: Investigation. **Flavia Pisanu:** Investigation. **David Balbo:** Investigation. **Carola Parolin:** Investigation. **Gianfranco Filippini:** Data Curation. **Beatrice Vitali:** Writing – review & editing. **Rosalba Lanciotti:** Writing – original draft. **Francesca**

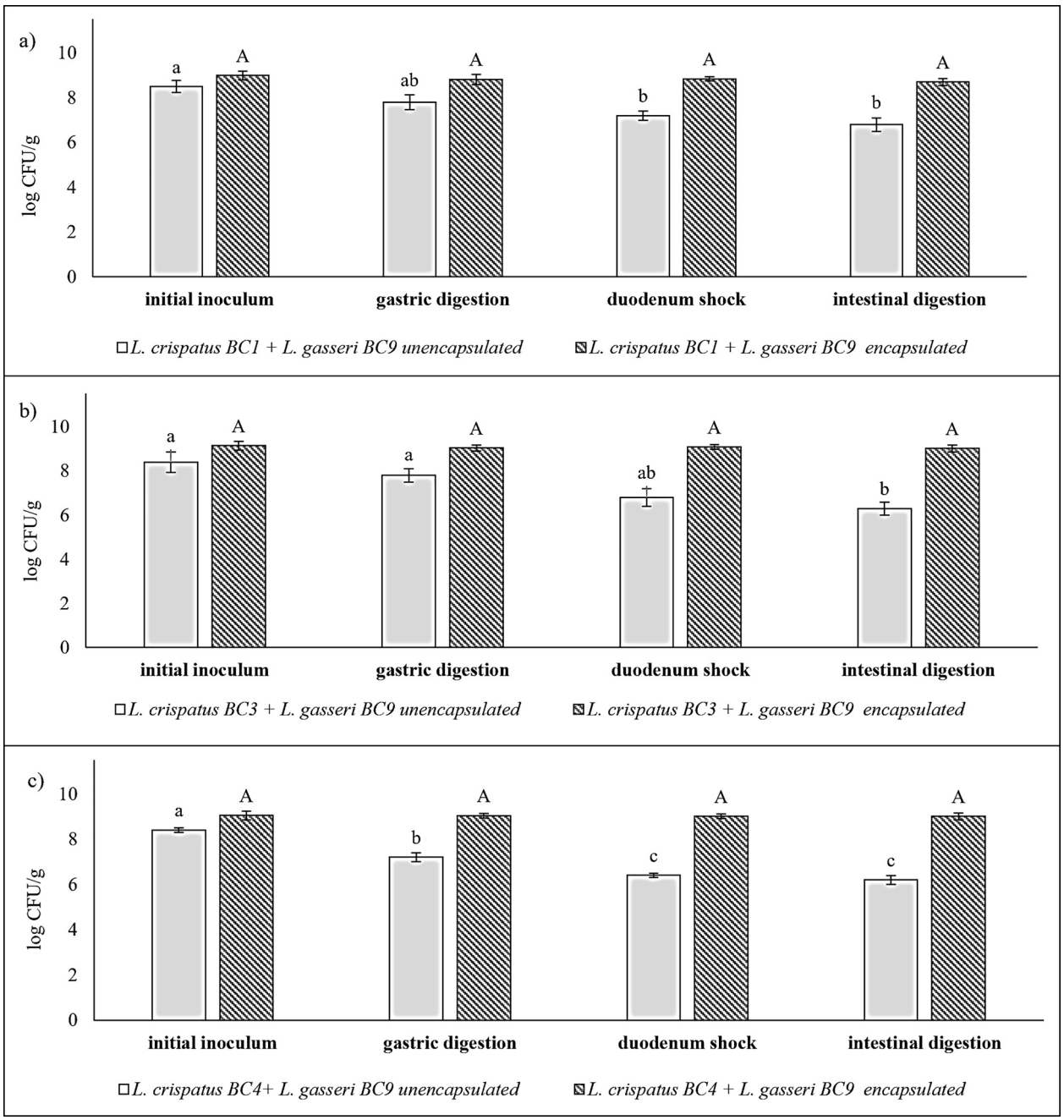

Fig. 4. Cell loads (log CFU/g) of *L. crispatus* BC1 + *L. gasseri* BC9 (a), *L. crispatus* BC3 + *L. gasseri* BC9 (b) and *L. crispatus* BC4 + *L. gasseri* BC9 (c) not encapsulated (control) and powders after the simulated stomach-duodenum passage. Results are shown as average ± SD. Samples with different letters are significantly different ($p < 0.05$).

Patrignani: Supervision, Writing – original draft.

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.

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