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Screening of main factors in microencapsulation of two *Bifidobacterium* strains by spray drying

Selección de factores principales de influencia en la microencapsulación de dos cepas de *Bifidobacterium* mediante secado por aspersión

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Abstract

Microencapsulation of *Bifidobacterium infantis* and *Bifidobacterium lactis* by spray drying has been studied. This work aimed to screen operational factors affecting the survival percentage, the probiotic powder yield, and activity water. Parameters such as air inlet temperature (AIT), air inlet flow rate (AIF), core material type (CM), core material concentration (CMC), and Lactic Acid Bacteria (LAB) were screened using a Taguchi design of experiments. The results indicated that the most influential parameters on survival percentage were LAB and AIF ($p <0.05$). On the other hand, all powders exhibited a high concentration of microorganisms (>8 log CFU/gds) and low activity water ($a_w <0.3$), establishing essential features for their use as probiotic powder and stability for their storage. Moreover, the results demonstrated that encapsulation by spray drying using skim milk reconstituted and β-cyclodextrin-Gum arabic effectively increased the survival of *Bifidobacterium* strains in the gastro-intestinal tract. Therefore, this microencapsulation process could allow better colonization into the intestine for probiotic effects assurance on consumer health.

Keywords: taguchi screening design, spray drying, microencapsulation, *Bifidobacterium*, cell viability.

Resumen

En esta investigación se evalúo la microencapsulación de *Bifidobacterium infantis* y *Bifidobacterium lactis* mediante secado por aspersión. El objetivo fue determinar los factores operativos que afectan el porcentaje de supervivencia, el rendimiento del polvo probiótico y la actividad del agua. Los parámetros como la temperatura de entrada de aire (AIT), el caudal de entrada de aire (AIF), el tipo de material del núcleo (CM), la concentración del material del núcleo (CMC) y las bacterias del ácido láctico (BAL) se examinaron utilizando un diseño de experimentos de Taguchi. Los resultados indicaron que los parámetros más influyentes en el porcentaje de supervivencia fueron BAL y AIF ($p <0,05$). Por otro lado, todos los polvos exhibieron alta concentración de microorganismos (>8 log UFC/gds) y baja actividad de agua ($a_w <0.3$), estableciéndose características esenciales para su uso como polvo probiótico y estabilidad para su almacenamiento. Además, los resultados demostraron que la encapsulación mediante secado por aspersión con leche descremada reconstituida y β-ciclodextrina con goma arábiga aumentó efectivamente la supervivencia de las cepas de *Bifidobacterium* en el tracto gastrointestinal. Por lo tanto, este proceso de microencapsulación podría permitir una mejor colonización en el intestino para garantizar los efectos probióticos en la salud del consumidor.

Palabras clave: diseño de cribado Taguchi, secado por aspersión, microencapsulación, *Bifidobacterium*.

1   Introduction

Functional foods promote health and well-being, which have recently increased the consumption of products with active compounds, such as probiotics and prebiotics (Pandey *et al.*, 2015). Nonetheless, these probiotic products, like functional foods, must be satisfactorily demonstrated to affect beneficially in the body beyond adequate nutrition (Sarao & Arora, 2017). Therefore, the number of viable cells of the probiotic products plays an essential role in functional contribution. In addition, scientific research has established that adequate amounts in the gastrointestinal tract benefit the host (FAO/WHO, 2002; Sarao & Arora, 2017).

Currently exist, several microorganisms are considered probiotics. However, *Lactobacillus* and *Bifidobacterium* are the main bacterial groups used extensively in probiotic products (Holzapfel *et al.*, 2001). Particularly, *Bifidobacterium* species have been documented with a broad array of beneficial properties on health, such as immunological system stimulation, antagonism against various pathogenic, reduction of food allergy, serum-cholesterol levels lowering, and decreased lactose intolerance. In addition, it has been reported that this probiotic does not affect the sensory properties of dairy products (Samona *et al.*, 1996). Thus, *Bifidobacterium* has been incorporated into food formulations such as dairy products, yogurts, ice cream, and cheeses. However, they are sensitive microorganisms to different stress types such as acidity, pH, storage time, temperature, and oxygen content (Doleyres & Lacroix, 2005). Many manufactured products such as fermented milk, yogurts, tablets, and granular powders have exhibited a lower probiotic load than stated on the label (Ibrahim & Carr, 2006; Lin *et al.*, 2006). Moreover, some free-cell products have shown a considerable decrease in viable cells when subjected to gastrointestinal tract conditions (Lin *et al.*, 2006). Therefore, an essential priority for the food industry is developing suitable technologies for producing and protecting probiotic foods.

In this context, encapsulation by spray drying is a process that can help protect probiotics against harsh environmental conditions, keeping a high density of viable cells for its functional contribution assurance (Riaz & Masud, 2013; Sarao & Arora, 2017). Moreover, a high number of viable probiotics for subsequent applications are desirable in spray-dried powder, generating a convenient storage and transportation presentation for its incorporation in functional food. However, this microencapsulation technique has different process parameters that need to be considered: flow configuration (co-current or counter-current), strain type and its pre-adaptation to the carrier material, carrier material, drying temperature, drying residence time and storage conditions (Lian *et al.*, 2002; Tonon *et al.*, 2009; Zuidam & Shimoni, 2010). Additionally, every coating material shows different structural behavior, and their ability to protect probiotics and bioactive substances varies (Tonon *et al.*, 2009). Reconstituted skim milk (SM) is a protector agent that improves cell viability survival during spray drying. Many studies have reported the SM in concentration from 10 to 20% (v/v), indicating a higher protective effect over Gum arabic, gelatin, starch, maltodextrin, and polydextrose (Lian *et al.*, 2002; Reddy *et al.*, 2009; Okuro *et al.*, 2013). Alternatively, a mixture of β-cyclodextrin and Gum arabic has shown to be an optimal material due to its ready availability, edibility, gel-forming ability, and economy. The β-cyclodextrin-Gum arabic mixture has been used as carrier material to encapsulate *L. acidophilus*, observing that 20% (w/v) was the best concentration to obtain good encapsulation efficiency (Okuro *et al.*, 2013). Thus, these encapsulating materials were chosen to encapsulate *Bifidobacterium* strains in this research.

This study aimed to identify the main factors affecting the encapsulation process of *Bifidobacterium lactis* and *Bifidobacterium infantis* using a Taguchi screening design (TSD). TSD is used to reduce the number of experiments necessary to evaluate the survival percentage (SP), the probiotic powder yield (PPY), and activity water (a_w). The process variables in this study included air inlet temperature (AIT), air inlet flow rate (AIF), core material (CM) (reconstituted skim milk and a β-cyclodextrin-Gum arabic mixture), core material concentration (CMC), and bacteria type (LAB). In addition, the best microcapsules obtained in the experiments were subjected to simulated gastrointestinal conditions. Finally, the powder stability was evaluated under the controlled atmosphere.

2   Materials and methods

2.1   Cultures and growth conditions for obtaining bacterial biomass

Fresh cultures of *Bifidobacterium infantis* SP37 (SACCO, Raff) (BI) and *Bifidobacterium lactis* BLC1 (SACCO, Raff) (BL) were activated by two successive transfers in MRSc (Man-Rogosa-Sharpe) broth supplemented with 0.05% (w/v) of L-cysteine (Sigma-Aldrich) at 37°C for 48h. Bacterial cultures in the late log phase were centrifuged at 3900 rpm for 10 min at 4°C (Sigma 3-18K ASPELAB) and washed with sterile phosphate-buffered saline (PBS) pH 7.4. The obtained bacterial biomass was mixed with the core materials.

Table 1. L8-Taguchi design of experiments for the spray drying process.

Exp.	AIT (°C)	AIF (mL/min)	CM	CMC (%)	LAB
1	160	5	SM	15	BI
2	160	5	SM	20	BL
3	160	10	BC-GA	15	BI
4	160	10	BC-GA	20	BL
5	180	5	BC-GA	15	BL
6	180	5	BC-GA	20	BI
7	180	10	SM	15	BL
8	180	10	SM	20	BI

AIT=air inlet temperature; AIF=air inlet flow rate; CM=core material (SM-reconstituted skim milk, BC-GA-β-ciclodextrin-Gum arabic); CMC=core material concentration; LAB=Lactic Acid Bacteria (BL-*Bifidobacterium lactis*, BI-*Bifidobacterium infantis*).

2.2 Preparation of bacterial suspension with a carrier material

According to Table 1, a quantify of the encapsulant agent was 100 g mixed sterile distilled. A heat-treated at 90°C for 10 min was realized to reconstituted skim milk (DIFCO, Detroit, Mich., USA) (SM) to remove undesirable microorganisms. On the other hand, β-cyclodextrin was mixed with Gum arabic in a ratio of 9:1 (w/w) (BC-GA) before its dissolution in water. Samples of the encapsulating agents were blended using a homogenizer (PRO260, Laboratory Homogenizer with Digital Speed Control, America PRO Scientific Company, USA) at 10,000 rpm for 5 min. For each experiment, 6 mL of bacterial biomass, 93.8 mL of a carrier material solution, and 0.2 mL of Tween 60 were added. The obtained suspension was subjected to a second stirring cycle in a homogenizer at 5,000 rpm for 10 min.

2.3 Encapsulation by spray drying

A laboratory spray dryer (BUCHI B-290, Switzerland was used for the bacterial suspension with carrier material operated at set conditions (Table 1). At 76 ± 2°C was the outlet temperature and a magnetic stirrer was used to agitate the carrier solution constantly. The dry microcapsules were collected in sealed glass vials and stored at 25°C.

The probiotic powder yield (PPY) was determined from solids introduced into the fed bacterial suspension before spray drying and the total powder recovered in the cyclone dryer. PPY was calculated with Equation (1):

$$PPY = \frac{P}{Pi} \times 100 \qquad (1)$$

where Pi is the weight of dry solids fed in the spray dryer, and P is the weight of the final spray-dried powder obtained.

2.4 Microcapsules stability assessment

The spray-dried powder's water activity (a_w) was measured using an Aqualab water activity meter (Aqualab, 3TE, Decagon, USA) according to the method proposed by Cai & Corke (2000) with some modifications. On the other hand, the hygroscopicity of the powders was determined. Samples (1 g) were placed in a glass container at 25°C with NaCl, KCl, and KSO$_3$ saturated solution, corresponding to 76% (w/v), 84% (w/v), and 98% (w/v) of relative humidity. Next, these samples were weighed until equilibrium (~2 weeks). Then, hygroscopicity was expressed as g of adsorbed moisture per 100 g of dry solids (g/100gds).

2.5 Viability determination and bacterial survival percentage after spray drying

The bacteria suspensions' total viable cells were determined by growth on the MRSc medium using the Miles-Misra technique (Picot & Lacroix, 2004). On the other hand, the spray-dried powders were re-suspended in PBS (pH 7.4) and homogenized for 2 min at room temperature to ensure encapsulating material complete dissolution. Samples were serially diluted before plating on MRSc medium. Plates were incubated at 37°C for 48 h under anaerobic conditions, and subsequently, the colony numbers were determined. Results were expressed as a logarithm of colony-forming units per gram of dry solids (log CFU/gds).

Bacterial survival percentage was determined using the total viable cells from the bacterial suspensions before spray drying and obtained powder after spray drying. Survival Percentage (SP) of the microencapsulation process was calculated with Equation (2):

$$SP = \frac{N_1}{N_0} \times 100 \qquad (2)$$

where N_0 is the number of viable bacteria per gram of dry solids (log CFU/gds) before drying, and N_1 is the

number of viable bacteria per gram of dry solids (log CFU/gds) in the powder.

2.6 Microscopic evaluation of microcapsules

Microcapsule particle size, morphology, and microstructure were observed on a scanning electron microscope (SEM) JEOL model JSM 6390 LV (JEOL, Tokyo, Japan) at an accelerating 10 and 15 kV. Samples were placed on adhesive paper and coated with gold particles with a vacuum sputtering coater (Leica, model EM SCD 500, Wetlar, Germany), as described by Lian *et al*. (2002). The diameter was determined with at least 120 particles from each of the different formulations of microcapsules measured.

2.7 Survival assessment of the encapsulated bacteria during the gastrointestinal conditions

Encapsulated and unencapsulated bacteria were exposed to a gastrointestinal simulation to evaluate encapsulation process parameters on the survival of bacteria, following the protocol described previously (Picot & Lacroix, 2004). The bacterial survival was evaluated sequentially exposed to gastric juice (pH 1.9) and small intestinal juice (pH 7.5) at 37°C. It monitored the total viable count changes periodically. The gastric juice was prepared using pepsin from porcine gastric mucosa 1:60, 000; (P7012, Sigma Aldrich®) in 0.1N HCl solution at a final concentration of 0.26 g/L, pH 1.9. The pancreatin (Hycel, México) in sterile sodium phosphate buffer (0.2M, pH 7.5) with a final concentration in the digestion mixture of 1.95 g/L, pH 7.5, was considered like pancreatic juice. Concentrated bile salt solution (150 g/L) was prepared using bile extract powder (bovine bile B3883, Sigma Aldrich®) in distilled water and subsequently sterilized by filtration. Before using the simulated pancreatic juice, gastric juice, and bile salt solution, were prepared to avoid eventual degradation.

The single samples of encapsulated and unencapsulated probiotics (1.0 g) were mixed with phosphate buffer (9 mL) in an Erlenmeyer flask. Each mixture resulting was added pepsin and incubated at pH 2.0 and 37°C for 30 min. The enzymatic reaction was stopped by increasing the pH to 7.5 with a 1 N NaOH solution. A sample of 1 mL was withdrawn for the viable cell number determination. Next, 5 mL of concentrated sodium phosphate buffer (0.5 M, pH 7.5) and 2.0 mL of bile salt solution were added. The mixture obtained was adjusted pH to 7.5 and the volume to 20 mL with sterile distilled water, and 10 mL of the pancreatic juice was added to get a final volume of 25 mL. At different times (1, 2, 3, and 6 h), 1 mL of the mixture was removed for bacteria enumeration. Viability at each stage is based on the powder sample mass, so the values were comparable.

2.8 Experimental design and statistical analysis

An L8 Taguchi design (n=8) was used with one replica for this design of experiments with air inlet temperature (AIT), inlet air flow rate (AIF), core material type (CM), core material concentration (CMC) and bacteria type (LAB) as parameters: the survival percentage (SP), the probiotic powder yield (PPY), and the water activity (aw) of the spray dried product as the response variables. An ANOVA followed by TMC (Tukey Means Comparison), with a confidence interval of 95% (p <0.05), was performed to evaluate the effects of different factors during the spray drying. All statistical calculations were performed using MODDE 7.1 software from Umetrics®. The factors and levels were selected and incorporated in Taguchi experimental design in base preliminary tests. The methodology of TSD is a tool that allows an optimal process through factor settings and makes them resistant to source variation. The Taguchi design proposes fewer experiments than the classic design of experiment methods, such as response surface methodology and custom design, in which a larger number of experimental runs are necessary for a set of process variables and levels. The diminution of process variation with TSD allows the creation and optimal compilation of levels by maximizing the signal/noise ratio by a fractional factorial design obtained from orthogonal arrays.

3 Results and discussion

3.1 Effects of experimental factors during the microencapsulation process

Spray-drying experiments were designed to survey the process parameters' effect on probiotic survival rate, the probiotic powder yield, and activity water (Table 2). All microencapsulated *Bifidobacterium* exhibited a survival range of 78.91% and 97.50 after spray drying. Water activity remained between 0.190 and 0.417, and the probiotic powder yield varied between 9.7% and 56.8%.

The Taguchi experimental design allowed the establishment of a first-order screening model, expressed by equation (3)

$$y = a_0 + \sum a_i x_i \qquad (3)$$

Where y represents the response variables, x_i is the experimental factors, a_0 is a constant, and a_i are the relative effects of each parameter.

Table 2. Experimental results. Effects of the spray-drying conditions on the response variables studied.

Exp	Log CFU/g$_{ds}$ before spray drying	Log CFU/g$_{ds}$ after spray drying	SP (%)	a_w	PPY (%)
1	11.57±0.02	11.26±0.06	97.34±0.53^a	0.215±0.004^d	14.7±1.2^f
2	12.72±0.03	10.29±0.08	80.84±0.79^{de}	0.190±0.007^e	56.8±1.8^a
3	11.26±0.06	10.94±0.01	97.21±0.57^a	0.417±0.009^a	27.8±2.3^e
4	11.30±0.07	8.92±0.01	78.91±0.51^e	0.219±0.008^d	33.8±1.6^d
5	11.48±0.08	10.51±0.02	91.50±0.58^c	0.195±0.005^e	27.8±1.6^e
6	11.58±0.01	11.29±0.08	97.50±0.67^a	0.405±0.011^b	9.7±0.8^g
7	12.43±0.06	9.93±0.01	79.89±0.32^d	0.249±0.002^c	40.3±1.5^c
8	10.16±0.09	9.68±0.02	95.31±0.76^b	0.198±0.008^e	51.3±1.4^b

Table 3. Statistical analysis of studied parameters and responses variables for the spray drying process.

Parameter	SP(%)	a_w	PPY(%)
R^2	0.973	0.933	0.957
R^2_{adj}	0.959	0.907	0.918
SD	40.17	0.124	22.91
RSD	4.76	0.023	3.95
RSD*	5.30	0.046	6.14
AIT			
160°C	24.6^a	0.2663^a	33.3^a
180°C	24.8^a	0.2733^a	33.3^a
MSD ($p <0.05$)	-0.2	-0.0070	1.0
AIF			
5 ml/min	20.7^a	0.2643^a	26.6^a
10 ml/min	28.7^b	0.2753^a	37.1^a
MSD ($p <0.05$)	-7.9	-0.0960	-10.5
CCM			
15%	22.2^a	0.2602^a	26.8^a
20%	27.2^a	0.2795^a	36.9^a
MSD ($p <0.05$)	4.9	-0.0192	-10.1
CM			
Skim milk	21.7^a	0.2177^a	40.0^a
β-ciclodextrin-Gum arabic	27.7^b	0.3220^b	23.7^b
MSD ($p <0.05$)	-6.0	-0.1042	-16.3
LAB type			
B. infantis	46.5^a	0.3143^a	25.1^a
B. lactis	3.0^b	0.2253^b	38.5^b
MSD ($p <0.05$)	43.5	0.0890	-13.4

$^{a-b}$means ± standard deviation, different superscript letters in the same line represent significant differences (p<0.05) between treatments; MSD=mean significant difference

Results from ANOVA and TMC tests for each response variable studied using Equation (3) are shown in Table 3, where R2 is the regression coefficient, R2adj is the value of R2 adjusted for degrees of freedom, SD is the standard deviation of the model, RSD is the standard deviation of the residuals, and RSD* is the standard deviation of residuals with its upper confidence level (95%), even if the standard deviation of the answers in figure 1, items B and C seem high, which is probably due to important variations in the experimental determinations. As can be seen from the ANOVA analysis, SD-model values are more significant than RSD*, and R2adj values are close to 1.0. Thus, the proposed model adequately describes the behavior of the response variable as a function of operating parameters within the studied range.
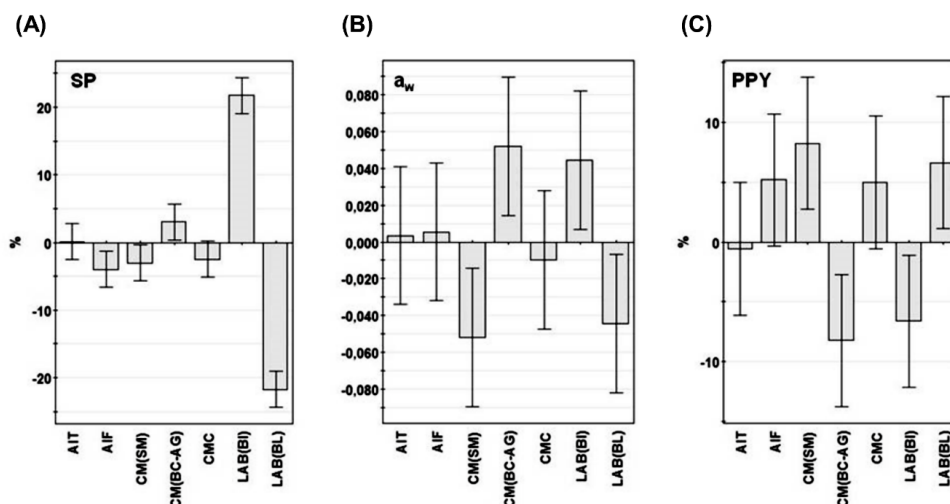
Figure 1. Main factors plot on survival percentage (SP), water activity (a_w) and probiotic powder yield using the L8 Taguchi design.

On the other hand, the practical effects on the response variables (SP, aw, PPY) were determined from experimental results, which are presented in Figure 1. The bar means the positive or negative impact of the operational parameter, while the height of the bar is proportional to the intensity of the effect. The coefficient was significant when the confidence interval did not cross zero.

The results from the TMC test are shown in Table 3, according to Figure 1, where AIF, CM, and LAB type statistically affected SP (Figure 1A), while only the last two statistically affected aw and PPY (Figure 1B and 1C). Overall, both *B. lactis* and *B. infantis* were moderately tolerant to the drying process, showing moderates SP values in all experiments. On the contrary, YPP was deeply affected by CM and LAB types. Therefore, YPP gained considerable importance for operation conditions selection.

3.2 Effects on probiotic powder yield

The microencapsulation yield is an important challenge of the drying process employing living microorganisms. In this experimental design, the probiotic powder yields PPY were significantly affected by the type of core material and microbial strain (Figure 1C). As shown in Table 2, powders-yield employing *B. lactis* varied between 27.8% and 56.8%, while *B. infantis* ranged from 9.7% and 51.3%. Concerning protector materials, SM presented the best yields (E2, E8, and E7) with moderate cell viability after drying. By contrast, BC-GA exhibited lower yields (E3, E5, and E6) with high cell viability. According to these results, YPP represents a critical parameter for the selection of optimal conditions of the drying process. Therefore, experiment 2 is the best because of keeping a balance between yield (56.8%) and cell viability (80.84%).

In other studies, SM has been recognized as an efficient core material because of its physicochemical properties that impact cell protection and high-power yields (Ananta *et al.*, 2005, Maciel *et al.*, 2014, Dimitrellou *et al.*, 2016). According to these authors, lactose and milk proteins may interact with the microbial cell membranes, preventing leaks during drying. On the other hand, the elevation glass transition-surface temperature gradient (dT)in materials such as plasticizers (sugars) generates an increase in the adherence of the surface of the materials stickiness (Adhikari *et al.*, 2005).

Thus, as the ΔT value increases, the droplets move toward the rubbery state sticking on the drying chamber surface, reducing the powder recovery yield.

However, this effect is subject to heat and mass diffusion rates because water is a macromolecule plasticizer (Barbosa-Canovas *et al.*, 2005). Thereby to achieve better yields, some parameters must be modified. In our study, the high air inlet temperature and air inlet flow rate, as shown in experiments 7 and 8 (Table 2), led to less particle stickiness. These results could be obtained because of the lower residual contents achieved by sufficient heat penetration and the core-to-droplet surface water diffusion rate.

3.3 Effect on water activity and hygroscopicity

In this experimental design, the water activity of *B. infantis* and *B. lactis* probiotic powders was significantly affected by the type of core material and bacterial strain (Figure 1B). As shown in Table 2, *B. infantis* powders ranged from 0.198 to 0.417, and *B. lactis* powders ranged from 0.190 to 0.249. Regarding coating materials, SM presented the lowest and BC-GA the highest values.

Table 4. Hygroscopicity (g/100 g$_{ds}$) of *Bifidobacterium* encapsulated obtained at different values of relative humidity.

Relative humidity (%)	Experiment			
	2 (SM^L)	3 (BC-GA^H)	5 (BC-GA^L)	7 (SM^H)
76	16.52±0.11^a	13.23±0.08^b	17.41±0.19^a	17.99±0.15^a
84	20.28±0.05^a	12.81±0.26^b	16.56±0.34^a	20.00±0.49^a
98	26.14±0.18^a	15.32±0.27^b	22.55±0.38^a	25.59±0.34^a

^{L,H}: experiments with the (L)owest and the (H)ighest a_w values for each core material.

The a_w indicate water free contained in the powders. In other words, water is not bound to molecules participating in enzymatic reactions, generating quality deterioration and changes in the powders' physical properties. Hence, low water activity (0.2-0.3) values are desirable for the spray-dried powders (Guillén-Velázquez *et al.*, 2022). In these conditions can be obtained probiotic powders with high flowability, low stickiness, low agglomeration, and maximum probiotic viability (Barbosa-Canovas *et al.*, 2005; Viernstein *et al.*, 2005). In addition, water mobility reduction through a glassy state can inhibit the cell metabolic activity of the bacterial cells, leading to extended shelf life (Fu & Chen, 2011; Ying *et al.*, 2012; Ananta *et al.*, 2005). In this context, most experiments presented good water activity characteristics, except for 3 and 6.

The hygroscopicity of powders with the lowest and highest a_w values for each core material were determined. Experiments 2 and 7 were considered for SM, whereas experiments 5 and 3 were for BC-GA. At high relative humidities (84 and 98%), SM capsules presented a higher hygroscopicity tendency (Table 4). Tonon *et al.* (2009) reported an increased hygroscopicity for encapsulating agents with lower molecular weight and shorter chains. In this sense, SM powder is mainly composed of casein (23,600 g/mol) and lactose (342 g/mol), whose components are more hygroscopic than Gum arabic (47,000-3,000,000 g/mol). Furthermore, Gharsallaoui *et al.* (2007) mentioned that increased water activity, solubility, and humidity occur when skimmed milk is spray dried; the hydrophilic groups (the mixture of lactose and milk proteins) were more exposed. Thus, the higher hygroscopicity of SM powders was probably due to these phenomena.

3.4 Effects on survival percentage of *Bifidobacterium* after the spray drying

The survival percentage (SP) was statistically affected by bacteria type (LAB), air inlet flow rate (AIF), and core material type (CM) (Figure 1A). The survival percentage of *B. infantis* and *B. lactis* using different conditions for drying are shown in Table 2. *B. infantis* exhibited a viability reduction of 0.28 to 0.47 log CFU/gds, while *B. lactis* of 0.97 to 2.5 log CFU/gds,

showing a maximum survival percentage of 97.50% and 91.50%, respectively. In both microorganisms, the highest survival was observed using 5 mL/min of air inlet flow rate and BC-GA as the protective agent during drying. On the contrary, the lowest survival was detected by employing 10 mL/min of air inlet flow rate and SM as the protective agent. Therefore, this behavior suggests that the air inlet flow rate and coating agents are essential to the *Bifidobacterium* survival.

It is well known that the diminution of probiotic survival during convective thermal processing is associated with cellular injuries caused by heat stress and mechanical. The denaturation of nucleic acids, damage to ribosomes, peroxidation of lipids, and cell membrane rupture have been reported (Fu & Chen, 2011; Corcoran *et al.*, 2008, Cortés-Rodríguez *et al.*, 2022). Therefore, heat and mass transfer kinetics variations at the air-solid interface should occur during drying. The effect of spray dryer heat on cell viability depends on the time residence of the particles in the system; therefore, particle size plays a crucial role. The larger particles have a shorter residence time because they are heavier than smaller ones, and the air has a less held effect on them (Jin & Chen, 2009). In this context, the hydrodynamic factors related to the rheological behavior of the encapsulation materials could contribute to microbial survival. Low-viscosity emulsions have been reported to facilitate droplet formation during spray dryer atomization, creating smaller particle sizes than those developed from a high-viscosity emulsion (Kim *et al.*, 2009). Regarding the system pressure, the pressure drop in the flow regime generates increasing particle size; on the contrary, an increase in pressure causes smaller particles (Pereyra-Castro *et al.*, 2018). On the other hand, the trajectory of the droplets inside the dryer chamber significantly influences heat and mass transfer. The inlet air cools over time and becomes more humid, so its heat and mass transfer capacity decreases (Moreira *et al.*, 2021). Likewise, as the droplet shifts away from the nozzle, its evaporation rate is substantially reduced (Tran *et al.*, 2017). Both aspects cause the particles not to achieve enough moisture loss throughout your trajectory. It is also important to mention that the temperature increase necessary for promoting the evaporation of the particle

depends on the particle's diameter. Hence, particles with a more significant size have a lower evaporation rate, retaining more moisture (Anandharamakrishnan *et al.*, 2010).

In this study was observed that experiments 3 and 6 with Gum arabic showed higher water activity and high survival percentages. This effect could be related to a larger particle size since Gum arabic is a polysaccharide with a high molecular weight that could generate highly viscous emulsions that oppose the formation of small particles. In addition, the high moisture content associated with the water activity was detected in the spray-dried powders (Table 2). This moisture retention could be related to the particle size. Therefore, this information suggests that these experiments developed large and heavy particles that triggered a shorter residence time in the drying chamber, benefiting the survival of microorganisms. On the other hand, using SM as an encapsulation material, experiments 1 and 8 showed the highest survival percentage; however, these did not present high moisture content linked with the water activity. Likely, these experiments developed small and light particles that encourage a longer residence time in the drying chamber; however, microbial viability was not compromised. Therefore, these findings could be linked protective mechanism of the encapsulating agent. When skim milk is used in drying processes, the fats in this material form a thin layer on the surface of the droplets, protecting cells from thermal effects and dehydration (Fu *et al.*, 2011; Anandharamakrishnan & Ishwarya, 2015).

Another important finding was the strain type employed. For example, both *B. lactis* and *B. infantis* using SM and BC-GA as protective agents showed different results in the survival percentage (Table 2). In other studies, it has also been observed that encapsulating agents and microorganisms type significantly impact cell viability during the drying process. For example, Lian *et al.* (2002) indicated that *B. longum* B6 encapsulated with SM presented better viability (82.60%) than those encapsulated with Gum arabic, soluble starch, and gelatin, while *B. lactis* exhibited low viability (<15.99%) using all encapsulating materials. On the other hand, in *L. acidophilus* and *B. bifidum* using *β*-cyclodextrin and Gum arabic as protective agents have observed a survival percentage of 93.51% and 82.02%, respectively (Arslan-Tontul & Erbas, 2017). Therefore, the intrinsic properties of microorganisms also play a critical role in the cell survival rate during the drying process. Sakata *et al.* (2002) noted different survival rates in several species of *B. longum* regardless of spray drying conditions and the protective material, showing an intrinsic microbial tolerance to heat. Interestingly, Simpson *et al.* (2005) found similar behavior among various

Bifidobacterium species in unencapsulated cells assays, assessing heat and oxygen tolerance. These heat stress tolerances could be associated with physiological and molecular mechanisms native to each *Bifidobacterium* strain. Proteins as chaperones (GRoEL, GroES, DnaK, and Dnaj), proteases (HtrA), and heat shock proteins (HSP) have been identified as part of a protection system in heat stress response (Ventura *et al.*, 2004, Sanchez, *et al.*, 2008). Therefore, we argued that the highest survival observed in *B. lactis* and *B. infantis* could be a synergic effect of intrinsic microbial mechanisms, core material, and adequate drying conditions that impact microbial thermal tolerance.

3.5 Morphology and particle size

Particle size analysis can assist in understanding the microparticles' behavior and make inferences regarding their stability. In this context, it also can contribute to understanding the effect of microbial viability. Scanning electron microscopy observed the spray-dried *B. lactis* and *B. infantis* powders (Figure 2). A spherical shape characterized both microcapsules with concavities and surface deflations but without evidence of cracks or fissures. The latter is fundamental for guaranteeing higher protection and lower permeability of gases which could lead to cell damage. Both microcapsules obtained in this study were of assorted sizes, between 7 to 15 mm (Figure 2). Such values are typical for microcapsules obtained through spray drying, which ranged from 10 to 100 mm, according to Fang & Bhandari (2010).

As observed in this study, the 'flat ball' effect has also been reported in previous studies. Wang & Mutukumira, (2022) encapsulated *L. reuteri* DPC16 in 10% reconstituted skim milk (SM) and 10% Gum arabic, observing squashed and wrinkled spherical capsules. Lucas *et al.* (2020) conducted experiments encapsulating curcumin with 1% Gum arabic. They also noted the capsules' spherical shape with concavities and surface deflations using low concentrations of Gum arabic. This effect is directly related to the conditions during spray drying, particularly heat penetration and water evaporation from the liquid droplet (Barbosa-Canovas *et al.*, 2005). Once the particles have been in contact with the hot air, the droplet surface remains dry, forming a layer around the dust particle that hinders internal water evaporation (Wei *et al.*, 2019). The extreme mechanical resistance of this shell could origin the droplet to suffer rapid inflation by boiling, stimulating it may grow or shrink depending on the airflow or encapsulating materials (Moreira *et al.*, 2021). Fang *et al.* (2012) demonstrated that intermediate inlet temperatures are associated with partially collapsed matrices and good mechanical strength and dissolution properties of the resultant powder.
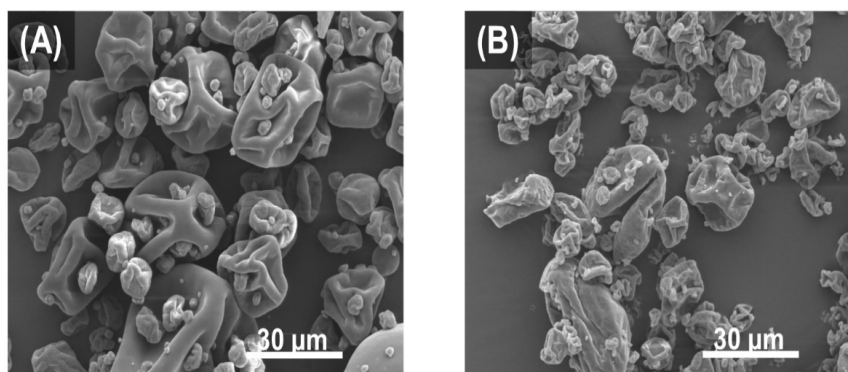
Figure 2. Micrographs of microcapsules produced: a) *Bifidobacterium infantis* (AIT 160°C, AIF 5 ml/min, CM Skim milk, CMC 15%), b) *Bifidobacterium lactis* (AIT 160°C, AIF 10 ml/min, CM BC-GA, CMC 20%).
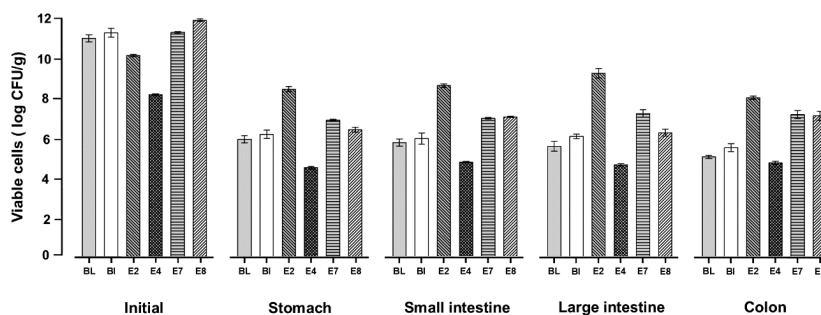

Figure 3. Assessment of cells viability under simulated GIT conditions. Controls (BL-*Bifidobacterium lactis*, BI-*Bifidobacterium infantis*).

On the other hand, this effect has also been associated with high-protein materials such as gelatin and skim milk since proteins migrate within particles during drying, destabilizing molecular interactions (Moreira *et al.*, 2021; Wang & Mutukumira, 2022).

3.6 Viability of probiotic microcapsules under simulated gastro-intestinal conditions

In probiotics selection, one of the essential criteria is their viability in the gastrointestinal system for their functional contribution assurance in the host; thereby, they must survive the acidic stomach and upper intestinal tract bile conditions (Argyri *et al.*, 2013). In this sense, encapsulation can safeguard the microorganisms against harsh environmental conditions, keeping a high density of viable cells. In this study, we evaluate the survival progression of *Bifidobacterium* encapsulated under the simulated gastrointestinal conditions, considering only the powders that presented high yields (2, 4, 7, and 8) (Figure 3). The unencapsulated cells (BL and BI) gave a low viability cell during gastrointestinal simulation (SP < 50%), reducing their cell load to 5 log CFU/g at the end. On the contrary, encapsulate cells (E2, E7, and E8) displayed moderate cell viability (SP >60%), obtaining a cell load of more than 7 logs CFU/g at the

end. Regarding coverage materials, microorganisms encapsulated with SM (E2, E7, and E8) showed more resistances than those encapsulated with BC-GA (E4), therein *B. lactis* (E2) exhibited the best results with maximum viability of 7.95 logs CFU/g (SP 79%) at the end.

In this study, unencapsulated and encapsulated cells exhibited more sensitivity to acidic stomach conditions than intestine conditions. A typical bacterial response to acidic conditions is the so-called acid tolerance response (ATR), wherein acid adaptation is achieved through an assemblage of inducible molecular mechanisms (Sakata *et al.*, 2002). According to Maus & Ingham (2003), *Bifidobacterium* enhances their survival after continuous exposition to sub-lethal acidic conditions by ATR induction. It has been proposed that the F0F1-ATPase membrane transport system exerts an essential contribution to the *Bifidobacterium* survival during pH acid exposure, expelling excess protons from the cytoplasm towards the outside (Matsumoto *et al.*, 2004; Collado & Sanz, 2006). Therefore, microencapsulation protects the microorganisms during their production chain and assures the release of metabolically active cells in the intestine.

Regarding the encapsulated microorganisms, the results agree with other studies that used SM as encapsulating agents and different *Bifidobacterium* strains, where 60 to 80% of microbial survival has

been observed (Lian *et al*., 2002; Fritzen-Freire *et al*., 2013). The trend of loss of viability during gastrointestinal simulation is due to the rate of degradation of the microcapsules in the gastric fluid. The main factors are linked to water solubility and the acid pH effect on encapsulation materials (Gharsallaoui *et al*., 2007). Gum arabic and skimmed milk used in this study are highly soluble in water. Therefore, encapsulated microorganisms are released and exposed to extreme stomach conditions within a period. On the other hand, it has been reported that protein denaturation in covering materials can affect the functional properties of the spray-dried powder (Millqvist-Fureby *et al*., 2001). In other words, the caseins in the skim milk could be denatured by the gastric fluid's acidic pH, destabilizing the capsules. In this context, many studies suggest combining encapsulation materials, modifying encapsulation materials by linking hydrophobic side chains or using other encapsulation strategies to achieve greater cell viability (Gharsallaoui *et al*., 2007). For example, Heidebach *et al*. (2009) used a method based on transglutaminase-catalyzed gelation of casein suspensions containing *L. paracasei ssp. Paracasei* F19 and *B. lactis* Bb12. Herein obtained spherical water-insoluble capsules that allowed a more remarkable survival of microorganisms. The transglutaminase-induced cross-links build a covalent network, making capsules highly resistant to dissolution at low pH. Therefore, this approach can protect these microorganisms from damage due to acid pH levels like the human stomach.

Conclusions

A Taguchi design of experiments was used to study the influence of the main parameters affecting microencapsulation by spray-drying two strains of *Bifidobacterium*. The results show that the primary variable affecting viability is the strain type, noting that *B. infantis* has a higher strength than *B. lactis*. The inlet flow rate is the only process variable statistically affecting viability within the range studied. The influence of the type of wall material was also found, showing that the BC-GA mixture favors cell viability. However, in all experiments, high cell counts (>8.9 logs CFU/gds) were obtained, which are suitable for formulating probiotic foods. On the other hand, hygroscopicity and water activity studies show that most powders have good storage stability (a_w <0.3). It was observed that SM powders were slightly more hygroscopic than those of BC-GA and, thereby, less stable in humid atmospheres. However, this higher hygroscopicity may also be related to improved solubility and hence the release of probiotics. In addition, statistical analysis showed that SM favors

the stability of the microcapsules since it effectively decreases the value of water activity. Experiments with SM also obtained the highest yields in the spray drying, obtaining values higher than 50%. Finally, this study demonstrated that encapsulation by spray drying using SM and a BC-GA mixture is an effective way to improve *Bifidobacterium*'s survival in the human stomach's harsh acidic conditions, allowing better colonization of the large intestine by these beneficial organisms.

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