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Lactose-free skim milk and prebiotics as carrier agents *of Bifidobacterium* BB-12 microencapsulation: physicochemical properties, survival during storage and *in vitro* gastrointestinal conditions behavior

Running title: Functional lactose-free spray-dried powders

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Summary

Bifidobacterium BB-12 was microencapsulated by spray drying using lactose-free milk, lactose-free milk and inulin, and lactose-free milk and oligofructose, resulting in powder 1, 2, and 3, respectively. The highest encapsulation yield (88.01%) and the highest bifidobacteria viability during 120 days of storage were noted for spray-dried powder 2. Spray-dried powders 1 and 3 showing a higher tendency to yellow color. After being submitted to in vitro simulated gastrointestinal conditions, the best probiotic survival rate result was found for spray-dried powder 3 (87.59%). Therefore, spray-dried powders containing prebiotics were the most appropriate combinations for microencapsulation of *Bifidobacterium* BB-12 and maintenance of cell viability during storage and gastrointestinal system, showing great potential to be used in lactose-free dairy products.

Keywords: Microencapsulation, free-lactose milk, inulin, oligofructose, bifidobacteria, gastrointestinal simulation

1. Introduction

Probiotic microorganisms are constantly studied because of their beneficial effects on human health, such as contribution to intestinal microbiota equilibrium and support for the immune

system. Thus, the recommended minimum daily intake of probiotic viable cells is between 10^6 and 10^7 CFU per g of product (food matrix) (Wang *et al*., 2020). However, probiotic microorganisms experience unfavorable conditions when incorporated in food (for example, change in pH, temperature, and water activity). Furthermore, Mei *et al*. (2014) reported a considerable loss of probiotic cells viability as they pass through the low pH of the stomach and the high bile salt conditions of the intestine. Nevertheless, Verruck *et al*. (2018) stated that the stability of the probiotic cell can be obtained using the microencapsulation process by spray drying, through a combined utilization of carriers agents, also called wall materials. As for such agents, protein, polysaccharides, or a combination thereof are widely used. Among the polysaccharides, those with prebiotic properties, such as inulin and oligofructose, are commonly employed. These carbohydrates are not digested by the enzymes of the gastric tract (soluble fiber), and thus selectively stimulate the growth of beneficial bacteria in the colon (Gibson *et al*., 2017). Both inulin and oligofructose are formed by a varying number of fructose moieties linked by β (2-1) glycosidic bonds. Our previous works have shown that, after stress conditions (heat treatments, storage, and simulation of gastrointestinal digestion), the highest cell survivals were found for spray-dried powders that contained inulin and/or oligofructose as wall materials (Fritzen-Freire *et al*., 2013; Pinto *et al*., 2015a; Pinto *et al*., 2015b; Verruck *et al*., 2017). Moreover, Verruck *et al*. (2018) reported the best thermal stability for probiotic microcapsules obtained from goat's milk and inulin. The measurement of such properties is extremely important, since heat treatments may be used in food processing. Apart from the works carried out by our group, the effectiveness of inulin in spray drying microencapsulation processes is already known and accepted by the scientific community. For example, Dos Santos *et al*. (2019) observed an improvement in *Lactobacillus acidophilus* La-5 survival after in vitro gastrointestinal stress when using inulin as a carrier agent compared to the use of the free probiotic.

Microencapsulated probiotic bacteria have been incorporated into a wide range of foods, among them, milk and milk products have been successfully employed (Gul, 2017; Pinto *et al*., 2017; Verruck *et al*., 2020). However, there is a significant segment of the adult world population (approximately 75%) showing permanent or temporary lactose intolerance (Suri *et al*. 2019). The prevalence of this inability to digest lactose may be associated with the genetically programmed reduction in lactase activity during adulthood, which can cause severe digestive disorders (Corgneau *et al*., 2017). Given this world scenario of lactose intolerance, coupled with the fact that milk is an excellent carrier agent for probiotics (Lee *et al*., 2015), it is necessary to develop

alternative mean that meets the demand formed by people who want to restrict lactose from their diet and consume probiotics. The microencapsulation of probiotics in a lactose-free milk matrix is an excellent choice since the addition of milk-based probiotics microcapsules in lactose-free dairy products constitutes a source of product contamination.

In this sense, our work seeks to evaluate the effect of lactose-free skim milk, oligofructose, and inulin as wall materials on the stability of *Bifidobacterium animalis* ssp. *lactis* BB-12, using the spray drying process. The stability of bifidobacteria entrapped was verified under in vitro simulated gastrointestinal conditions and during storage for 120 days at room temperature. The spray-dried powders were characterized concerning their physicochemical properties.

2. Materials and methods

2.1 Preparation of probiotics cells

According to the procedure described by Fritzen-Freire *et al*. (2013), a stock solution was obtained from 25 g of *Bifidobacterium animalis* spp. *lactis* BB-12 freeze-dried culture (Nu-trish® BB-12®, Chr. Hansen, Hønsholm, Denmark) rehydrated in 1 liter of sterile lactose-free milk with the following composition: 5.0 g 100 g^{-1} of carbohydrates, 3.2 g 100 g^{-1} of proteins, and 0.40 g 100 g^{-1} of lipids. After the rehydration step, the stock solution was frozen at − 18 °C into sterile glass bottles. The stock solution was defrosted, inoculated in MRS broth (Difco, Sparks, USA) (150 mL L^{-1}), and incubated at 37 °C for 48 h, under anaerobic condition using anaerobic jars with AnaeroGen® (Oxoid, Hampshire, UK). In sequence, this cell suspension was centrifuged (Nova Técnica, São Paulo, Brazil) at 1,000 x *g* for 10 min at 25 °C. The supernatant was discarded, and the precipitate, which contained probiotics cells; was washed with a sterile saline solution (0.85 g 100 mL^{-1}) three times. Finally, it was obtained probiotics cells precipitate freshly prepared.

2.2 Preparation of spray-dried powders with bifidobacteria microcapsules

Three spray-dried powders with bifidobacteria microcapsules were obtained from three feed solutions with different compositions (Table 1). Lactose-free skim milk powder was used as the basis for the three formulations. For this purpose, UHT lactose-free skim milk was purchased from the local market with the following composition: 5.0 g 100 g^{-1} of carbohydrates (2.5 g 100 g^{-1} of

glucose and 2.5 g 100 g^{-1} of galactose), 3.3 g 100 g^{-1} of proteins, 0.0 g 100 g^{-1} of lipids and 0.21 g 100 g^{-1} of ash. This milk was subjected to a spray drying process (B 290 mini spray dryer, Buchi, Flawil, Switzerland), resulting in the lactose-free skim milk powder (85.5 g total solids 100 g^{-1}, 32.5 g protein 100 g^{-1}, 0.0 g lipid 100 g^{-1}, 3.0 g ash 100 g^{-1} and 50.0 g carbohydrates 100 g^{-1}). By direct correlation with its fluid version, we associated 50% of the total carbohydrates as galactose (25.0 g 100 g^{-1}) and 50% of the total carbohydrates to glucose (25.0 g 100 g^{-1}).

In the feed solution 1, only the lactose-free skim milk powder was used as a carrier agent, resulting in the spray-dried powder 1. For the feed solutions 2 and 3, inulin (DP ≥10) (92.10 g inulin 100 g^{-1} and 7.90 g fructose + glucose + sucrose 100 g^{-1}) (Orafti® Gr, Orafti, Tienen, Belgium) and oligofructose (DP = 2−8) (96.90 g oligofructose 100 g^{-1} and 3.10 g fructose + glucose + sucrose 100 g^{-1}) (Orafti® P95, Orafti, Tienen, Belgium) were incorporated, respectively. All feed solutions were homogenized, heat-treated at 80 °C for 30 min, and left to cool down to room temperature (25 °C). In sequence, probiotic cells precipitate freshly prepared were added in feed solutions at a concentration of 100 mL L^{-1}.

Microencapsulation processes were performed with a laboratory-scale spray dryer (B-290 mini spray dryer, Buchi, Flawil, Switzerland), operating at the constant air inlet temperature of 150 °C and the outlet temperature of 44 °C. For this, the feed solutions were kept under magnetic agitation (MS-3000, BioSan, Riga, Latvia) at room temperature and fed into the main chamber through a peristaltic pump, with a feed flow of 12 mL min^{-1}, drying airflow rate of 35 m^3 h^{-1}, and compressor air pressure of 0.7 MPa. The spray-dried powders were collected from the cyclone base and placed under vacuum (200 B, Selovac, São Paulo, Brazil) in aluminum packaging. Three batches were produced and pooled for each one spray-dried powder type.

2.3 *Bifidobacterium* BB-12 encapsulation yield

According to Sheu *et al*. (1993) but with some modifications, for the entrapped bacteria release of microcapsules, 1 g of spray-dried powder was previously re-suspended in 9 mL of sterile phosphate buffer solution (0.1 mol L^{-1}, pH = 7), and mixed in a vortex (VTX-F-100, Biomixer, São Paulo, Brazil) during 10 min, at room temperature (25 °C). Mixtures and feed solutions were serially diluted in peptone water (Oxoid, Hampshire, UK) (0.1 g 100 mL^{-1}), and plated on MRS agar (Merck, Darmstadt, Germany) modified with the addition of lithium chloride (Vetec, Rio de Janeiro, Brazil) (0.2 g 100 g^{-1}) and sodium propionate (Fluka, Neu-Ulm, Germany) (0.3 g 100 g^{-1}), as the methodology described by Vinderola and Reinheimer (1999). The plates

were incubated in anaerobic jars containing AnaeroGen® at 37 °C for 72 h. After the incubation period, the count of viable probiotic cells was carried out and expressed as log colony-forming units per gram (log CFU g^{-1}). Therefore, the encapsulation yield was calculated as proposed by Chávarri *et al*. (2010) using the Eq. (1). All these determinations were realized in triplicate.

$$\text{Encapsulation yield (\%)} = \left(\frac{C}{C_0}\right) \times 100 \quad (1)$$

where C is the number of viable cells (log CFU) per gram in the spray-dried powders, and C_0 is the number of viable cells (log CFU) per gram in the feed solutions.

2.4 Spray-dried powders properties

The lactose content and physical properties of all spray-dried powders were realized in triplicate.

2.4.1 Lactose analysis

Spray-dried powders lactose analysis was realized according to the methodology established by Steinbach and Wille (2008). Before analysis, spray-dried powders samples were diluted 1:100 (v/v) with ultrapure water and placed in the sample vials upon the rack of the sample processor. The subsequent dialysis of samples, followed by the injection of the dialysate onto the separation column of chromatography with pulsed amperometric detection (Chromatograph 881 Compact IC Pro, Metrohm AG, Herisau, Switzerland) was realized to determine the lactose content. Instrument control, data acquisition, and processing were performed by Metrodata IC Net software (Metrohm AG, Herisau, Switzerland). Lactose was reagent grade and purchased from (Sigma Aldrich, Buchs, Switzerland). It was employed columns Metrosep Carb 1 and Metrosep CO_3 Trap 1, and the pre-column Metrosep Carb 1 Guard (Metrohm AG, Herisau, Switzerland). The mobile phase used was a solution of sodium hydroxide (5.0 mmol L^{-1}), with a flow rate of 1.2 mL min^{-1} at a temperature of 45 °C.

2.4.2 Moisture, water activity, and water solubility

The moisture content (g 100 g^{-1}) was determined by gravimetrically by oven drying under vacuum at 70 °C (Model TE-395, Tecnal®, Piracicaba, Brazil) until reaching constant weight, as described by AOAC (AOAC, 2005).

The water activity was measured in the Aqualab 4TE analyzer (Decagon Devices, Pullman, USA) at 25 °C, after the initial samples stabilization for 15 min, according to the method proposed by Fernandes *et al*. (2014), with some modifications. One gram of each spray-dried powder was weighed and stirred into 25 mL of distilled water for 5 min using a magnetic stirrer (HS-17, JoanLab®, Zhejiang, China) at a medium speed. The solution was then centrifuged (5430R, Eppendorf, Germany) at 760 x *g* for 10 min. An aliquot of 20 mL of the supernatant was transferred to a pre-weighed Petri dish and oven-dried at 105 °C overnight. The water solubility (%) was calculated as the percentage of dried supernatant about the amount of spray-dried powder originally added (1.0 g).

2.4.3 Bulk density and interstitial air

The density of spray-dried powders was measured both as loose and tapped bulk density, as proposed by Lebrun *et al*. (2012), but with some modifications. Approximately, 2 g of each spray-dried powder was freely poured into a glass tarred graduated cylinder without tapping and disturbance, and this was measured as loose bulk density of spray-dried powders, by the following equation:

$$\text{Loose bulk density} = \frac{\text{mass of spray} - \text{dried powder (g)}}{\text{bulk spray} - \text{dried powder volume } (cm^3)} \quad (2)$$

The spray-dried powders samples from loose bulk density evaluation were mechanically tapped, and after 100 taps were obtained the tapped bulk density, which was computed using the following equation:

$$\text{Tapped bulk density} = \frac{\text{mass of spray} - \text{dried powder (g)}}{\text{tapped spray} - \text{dried powder volume } (cm^3)} \quad (3)$$

Loose and tapped bulk densities values were used to determine the interstitial air value, as proposed by Chever *et al*. (2017), as follows:

$$\text{Interstitial air} = \left(\frac{1}{\text{Loose bulk density } (kg\ m^{-3})} - \frac{1}{\text{Tapped bulk density}(kg\ m^{-3})}\right) \text{X } 100000 \qquad (4)$$

2.4.4 Flow properties

Carr's index (CI) and Hausner ratio (HR) were used to evaluating the flowability and cohesiveness of spray-dried powders, respectively. Both Carr's index and Hausner ratio were calculated according to the following equations given by Reddy *et al*. (2014):

$$\text{Carr's index (CI)} = \frac{\text{tapped bulk density} - \text{loose bulk density}}{\text{tapped bulk density}} \text{ X } 100\ (\%) \qquad (5)$$

$$\text{Hausner ratio (HR)} = \frac{\text{tapped bulk density}}{\text{loose bulk density}} \qquad (6)$$

2.4.5 Color measurements

Color measurements (L*, a*, and b* values) were performed using a chromameter CR-400 (Konica Minolta, Osaka, Japan) with illuminant D65. The instrument was calibrated with a white reference tile before the measurements. The L*, a* (+, red; - , green) and b* (+, yellow; - , blue) color coordinates were determined according to the CIELab coordinate color space system. Color measurements were performed on spray-dried powders at room temperature. The total color difference (ΔE*) was calculated as described by Himmetagaoglu and Erbay (2019), as follows:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad (7)$$

where ΔL* is the difference of luminosity, Δa* is the difference of the parameter a*, and Δb* represents the difference of the parameter b*, between two types of spray-dried powders.

2.5 Effect of storage on microencapsulated probiotic viability

Spray-dried powders (1, 2, and 3) with bifidobacteria microcapsules, placed under vacuum in aluminum packaging were stored at room temperature (25 °C) for 120 days. The samples were withdrawn at thirty days intervals to monitor the viable cells counts of *Bifidobacterium* BB-12, as previously described in item 2.3. These results were also expressed in log colony-forming units per gram (log CFU g^{-1}).

2.6 Survival of bifidobacteria under *in vitro* simulated gastrointestinal conditions assay

Bifidobacterium BB-12 cells precipitate freshly prepared (free cells), and spray-dried powders (1, 2, and 3) were submitted to under *in vitro* simulated gastrointestinal conditions steps. The typical conditions prevailing in the human mouth, esophagus-stomach, duodenum, and ileum were sequentially simulated, as a traditional digestion step. Parameters (enzymes solutions, pH values, periods, and intensities of stirring in each part of the human digestive system) used to simulate the gastrointestinal conditions were realized exactly the protocol described by Verruck *et al*. (2017). As the control, all spray-dried powders with bifidobacteria microcapsules, and free cells samples were not also exposed to the simulated gastrointestinal conditions. The processing conditions used in each step of the simulated gastrointestinal are summarized in Figure 1. After each step condition, viable cell counts of *Bifidobacterium* BB-12 were done as also previously described in item 2.3. This assay was carried out in triplicate, and results were exhibited as log colony-forming units per gram (log CFU g^{-1}).

The survival (%) of bifidobacteria was calculated as proposed by Guo *et al*. (2009) using the equation (12):

$$\text{Survival (\%)} = \left(\frac{N}{N_0}\right) \text{x } 100 \qquad (8)$$

where N is the viable cell count of bifidobacteria (it expressed as log colony-forming units per gram [log CFU g^{-1}]) after exposure to each step of simulated gastrointestinal conditions, and N_0 is the initial viable cell count of bifidobacteria (it expressed as log colony-forming units per gram [log CFU g^{-1}]) before to simulated gastrointestinal conditions.

2.7 Statistical analysis

To determine significant differences ($P < 0.05$) between results, it was used one-way analysis of variance (ANOVA) and Tukey studentized range test. All statistical analyses were performed using STATISTICA 13.3 software (TIBCO Software Inc., Palo Alto, CA). All data were expressed as mean ± standard deviation.

3. Results and discussion

3.1 *Bifidobacterium* BB-12 encapsulation yield

The viable cell count for the bifidobacteria in the feed solutions and their respective spray-dried powders are shown in Table 2. All the spray-dried powders showed viable cell counts above 6 log CFU g^{-1}, which is the minimum recommended amount for the probiotic product claim. Furthermore, there were no significant differences ($P > 0.05$) between the powders for cell survival after spray drying. Similar behavior was observed by Verruck *et al*. (2019) and by Pinto *et al*. (2015a) for microcapsules produced with reconstituted full-fat goat's milk powder/prebiotic agents and whey concentrate/prebiotic agents, respectively. The highest values of encapsulation yield (EY) were noted for the spray-dried powders produced with inulin and oligofructose, consecutively. For these same carrier agents, some authors (Fritzen-Freire *et al*., 2012; Kingwatee *et al*., 2015; Rajam & Anandharamakrishnan, 2015) had already reported a thermoprotective effect on probiotic microorganisms during spray drying processes. On the other hand, according to Ananta *et al*. (2005), large chains polymers would not interact directly with the polar head groups of cell membrane phospholipids, reducing its protection during the drying process. Besides, the dehydration involved in the spray drying process generally results in injury or death to bifidobacteria cells (Verruck *et al*., 2020). However, our results were satisfactory, and according to Dias *et al*. (2018), this may be attributed to the low outlet temperature kept in the spray dryer equipment (44 ± 3 °C), in addition to the natural resistance of the microorganism, because as verified in the work of Verruck *et al*. (2017), the counts of free *Bifidobacterium* BB-12 remained above 6 log UFC g^{-1} when they were exposed to a temperature of 55 ºC for 15 min. Moreover, Verruck *et al*. (2019) affirm that due to the presence of milk proteins and lactose, dairy products are effective in cell protection during spray drying processes. During water removal, these compounds would prevent the membrane from breaking through their interaction with it. As there is no lactose in our powders, we associate the good viability, especially with proteins. The findings of Wang *et al*. (2020) corroborate with this since these authors found a more significant role of milk proteins than lactose in bacterium protection during dehydration. The authors also verified higher respiratory activity and membrane integrity of bacteria for treatments with more proteins. Furthermore,

according to Ying *et al*. (2013), the bovine whey protein creates a buffered environment within the particle obtained by spray drying.

We also highlight that our samples contain the monosaccharides glucose and galactose, given the previous enzymatic hydrolysis of lactose. In this context, Amaretti *et al*. (2007) studied kinetics and metabolism of *Bifidobacterium adolescentis* growing, and they found a greater growth rate and cellular yield when they were subjected to growth on galactose than glucose, lactose, and galactooligosaccharides. According to Prasanna *et al*. (2014), although some strains can grow in milk and utilize lactose as the substrate, the genus *Bifidobacterium* includes saccharolytic organisms and is characterized mainly by to ferment glucose, galactose, and fructose.

3.2 Spray-dried powders properties

The physicochemical characteristics of the spray-dried powders are shown in Table 3. The presence of lactose was not detected in any of the spray-dried powders by the method used in this work, thus ensuring that this product can be called lactose free. The moisture content of the spray-dried powders 2 and 3 was similar, as well as their water activity. In turn, the moisture content and water activity values of the spray-dried powder 1 were highest. It is known that glucose and galactose present in milk are highly reactive when compared to disaccharide lactose (Milkovska-Stamenova & Hoffmann, 2016). Besides, both glucose and galactose have lower glass transition temperature (T_g) than inulin and oligofructose (31, 30, 132, and 102 ºC, respectively) (Schuck *et al*., 2005; Silva *et al*., 2016; Hinrichs *et al*., 2001). In our case, the spray-dried powder 1 certainly had lower T_g than the other two samples, due to the T_g theory resulting from the composition of the product proposed by Couchman and Karasz (1978). A glass transition temperature close to room temperature facilitates water absorption (Juliano & Barbosa-Cánovas, 2010) and as a result, it can bring a series of problems to milk powder such as particle agglomeration, cacking, and rehydration difficulty. Also, the lower the degree of polymerization of a component, the more hygroscopic it will be (Jimenez-Sánchez *et al*., 2018), which also justifies the higher moisture value found for powder 1 since glucose and galactose are simpler carbohydrates compared to the prebiotics used in the other formulations. However, despite this higher value found for spray-dried powder 1, the results are still within the recommended range, because according to Riveros *et al*. (2009), the spray dryer operating conditions should be set to reach temperatures close to or below 60 °C at the air discharge point to assure the obtainment of a product with less than 10 g 100 g^{-1}

moisture. These results are like to that described by Rajam and Anandharamakrishnan (2015); they found a moisture content between 5.52 and 7.43 g 100 g^{-1} in the powders obtained in microencapsulation by spray drying of *L. plantarum* using as carriers agents fructooligosaccharide and whey protein isolate, and fructooligosaccharide and denatured whey protein isolate, with an outlet temperature of 55 °C. Ilha *et al*. (2015) report moisture content 4.30 g 100 g^{-1} for *L. paracasei* spray dried in reconstituted skim milk and cheese whey. Wang *et al*. (2020) suggested that ideal water activity for probiotic stability must be less than or equal to 0.4. Our water activity results were similar to those found by Fritzen-Freire *et al*. (2012), who microencapsulated *Bifidobacterium* BB-12 in reconstituted skim milk, inulin, and oligofructose and obtained water activity values between 0.21 and 0.27.

The solubility is a useful parameter for the application of the powders in various matrices, which is dependent on the affinity of the powders to water and hydrophilic components (Rodriguéz-Restrepo *et al*., 2017). Sadat *et al*. (2017) affirmed that the main factors affecting the solubility of milk powders are drying conditions and the physical characteristics of the feed liquid (e.g., viscosity). According to Himmetagaoglu and Erbay (2019), the composition of the powder also provides a considerable effect on this parameter. However, the solubility values found in our study did not vary significantly as a function of carrier agents used, remaining on average 63%. C. Kalita *et al*. (2018) also obtained solubility values in the range of 62 to 68% for symbiotic spray-dried powders from maltodextrin and fructooligosaccharide. Low solubility rates result in a slower release of microencapsulated cells since the rehydration of the powders also occurs more slowly (Pinto *et al*. 2015a). Therefore, the lowest solubility rates contribute to the longest microcapsule dissolution time. Pinto *et al*. (2015b) highlighted that by adding microcapsules in food products, it is expected that there is good control of the release of the probiotic cells when in contact with an aqueous solution, and a longer dissolution time assists in this regard.

The bulk density can be affected by some parameters such as moisture content, particle size distribution, and morphology and it is important for the processing, storage, and packaging of powders (Rajam & Anandharamakrishnan, 2015). It was possible to note, both for loose bulk density and for tapped bulk density, that the use of oligofructose as the carrier agent contributed to the lowest volume occupied of the spray-dried powder 3 (Table 3). Rajam and Anandharamakrishnan (2015) also found higher loose bulk density values for microcapsules with a higher content of fructooligosaccharide. They attributed this behavior to the fact that microcapsules with higher fructooligosaccharide ratio presented particle aggregation and less

interspace between particles. Furthermore, our values are similar to those found in other studies. For example, De Liz *et al*. (2020) found variations between 0.32–0.33 g cm^{-3}, and 0.54–0.55 g cm^{-3}, respectively for loose and tapped bulk densities of spray-dried powders with goat's whey freeze concentrate and inulin, and with only goat's whey freeze concentrate, respectively. Looi *et al*. (2019) found 0.36–0.45 g cm^{-3} and 0.52–0.64 g cm^{-3}, respectively for loose and tapped bulk densities for probiotic spray-dried powders from *Moringa oleifera* Lam. The change in specific volume between the loose and tapped bulks is recognized as interstitial air content (IA) (Wu *et al*., 2019). The IA was highest ($P < 0.05$) for the spray-dried powder 2 (with the addition of inulin), and the values were lower and without statistical differences ($P > 0.05$) for both spray-dried powders 1 and 3. As observed in the work of Chever *et al*. (2017), IA values tend to decrease proportionally with the increase of the agglomeration of a powder.

There were variations in the values of flowability and cohesiveness between the three spray-dried powders. According to Parthasarathi and Anandharamakrishnan (2016), these flow properties are important quality parameters for the industrial production of dried microcapsules. These same authors state that Carr's index (CI) results above 38% are classified as "very, very poor" flowability. They also state that a Hausner ratio greater than or equal to 1.25 indicates a powder with "poor" flow characteristics. Our spray-dried powders showed a flowability < 30% for all samples, and Hausner ratio < 1.25 for powders 1 and 3, and > 1.25 for powder 2. Thus, according to the tables of classification of the flowability and cohesiveness of powders arranged in the work of Jinapong *et al*. (2008), our results reveal very good flowability and low cohesiveness for powder 1, fair flowability and intermediate cohesiveness for powder 2, and good flowability and intermediate cohesiveness for powder 3. In the studies of Fitzpatrick *et al*. (2004), it is evidenced that the skim milk powder has low cohesiveness when compared to its whole version because of its zero fat content. One of the mechanisms of the formation of particle agglomerates is through solid bridges. These can be built up by chemical reaction, crystallization of dissolved binder substances, hardening binders, solidification of melted components, and by sintering at high temperatures (through the diffusion of molecules from one particle to another at the points of contact). Highly viscous materials and high molecular weight organic liquids can form bonds very similar to those of solid bridges (Pietsch, 1997). We suggest, therefore, that the prebiotics used in our study were responsible for the drop in quality in the flow properties.

The color parameters for the *Bifidobacterium* BB-12 spray-dried powders samples also are shown in Table 3. All the spray-dried powders showed high values for the L^* parameter,

indicating that the samples were white (clearer). Concerning the *a** and *b** parameters, the spray-dried powders showed positive values, indicating a tendency to the color red and the color yellow, respectively. The use of lactose-free milk or lactose-free milk and oligofructose produced spray-dried powders with a more yellowish coloration. We believe that the high contents of proteins and reducing sugars (mainly galactose and glucose) in spray-dried powder 1 favored Maillard reactions during thermal processing. Mensink *et al*. (2015) reported that the hydrolysis of inulin into oligofructose results in a product with high reducing capacity, and therefore, is susceptible to the Maillard reaction. Thus, it was found that in spray-dried powders 1 and 3 there were more reducing sugars, resulting in a more pronounced yellowish color when compared to spray-dried powder 2.

The values of Hue angle (*h*) showed that the spray-dried powder 2 differed significantly from the others, suggesting that this change does not go unnoticed to human eyes. According to Dobrzańska and Cais-Sokolińska (2014), who studied the color measurement systems focused on the protein of milk and whey, a total color difference (ΔE^*) > 2 would already be perceived by an inexperienced observer. Complementing the values found for the Hue angle, this was also observed for the spray-dried powder 2. In the study of Witczak *et al*. (2020), a higher ΔE^* was found for samples containing inulin with a higher degree of polymerization. However, the authors state that the color differences found are not only correlated with DP but also due to the interaction between inulin and other system components, as well as the technologies utilized in the production of this prebiotic. In agreement with the data obtained by Witczak *et al*. (2020), our results suggest that the greater degree of polymerization of inulin contributed to the different values of *h* and ΔE^* for spray-dried powder 2, results that originated from the differences observed ($P < 0.05$) in *a** and *b** parameters.

3.3 Effect of storage on microencapsulated probiotic viability

The viable *Bifidobacterium* BB-12 cell counts throughout the storage time of 120 days at 25 ± 1°C are shown in Table 4. During storage intervals, the spray-dried powders containing microencapsulated bifidobacteria showed a decrease in viable cell count. The protection performance was lower when only lactose-free milk powder was used as the carrier agent, reaching a loss of almost 100% of its viability. Gul (2017) discussed that the viability of

microencapsulated cells during storage depends on various factors such as a high number of irreversible damage cells during spray drying, presence of oxygen, high storage temperature, moisture content, product composition, and exposure to light. According to Liu *et al*. (2016), the maintenance of the moisture content of microcapsules around 4 g 100 g^{-1} is important because as a higher the moisture content, the lower bacterial survival during storage. Thus, the remarkable decrease in viability of the spray-dried powder 1 could be associated with its initial moisture and a_w, which as shown in Table 3, were 7.67 g 100 g^{-1} and 0.396, respectively. Moreover, the decrease in the viable cell count during storage at 25° C may be correlated with the natural mechanism that involves the degradation of life-essential macromolecules. For example, Santivarangkna, Kulozik, and Foerst (2008) affirmed that this viability loss is mainly due to membrane lipid oxidation. The cell counts remained higher than 6 log CFU g^{-1} for spray-dried powder 2 (made with inulin and lactose-free skim milk as carrier agents) for 90 days, while for spray-dried powders 1 and 3 (made with the lactose-free skim milk powder, and lactose-free skim milk powder and oligofructose, respectively) they remained higher than 6 log CFU g^{-1} only for 30 days. This result is following those obtained by Dias *et al*. (2018), who noted that microcapsules with a higher proportion of inulin increased cell viability of *B. animalis* ssp. *lactis* BB-12 compared with samples with no addition of inulin or with a low concentration of this prebiotic, during storage at 25° C. As discussed in the previous item, the use of different carrier agents resulted in spray-dried powders with different physical properties, and according to Verruck *et al*. (2019), this affects the functionality of the microcapsules present. Bedani, Rossi, and Saad (2013) cited that the inulin interacts with available water, forming a gel that consists of a tridimensional network of microcrystals. This structure may involve the bacterial cells, contributing to physical protection and consequent maintenance of viability. Moreover, this fact may be associated with the elevated glass transition temperature inulin presents. It was reported that a carbohydrate-rich formulation with high T_g procured the greater stability for probiotic bacteria (Rokka & Rantamaki, 2010; Nunes *et al*., 2018). Zhang, Lin, and Zhong (2015) observed that the use of trehalose in feed solutions provided better survivability of *Lactobacillus salivarus* NRRL B-30514 (after spray drying) during storage than powders made with only reconstituted skimmed milk. Mouhammad *et al*. (2017) explained that when the carrier agent is at the glassy state (below T_g), its viscosity is higher, which decelerates chemical reactions such as free radical oxidation. Thus, the glassy state could prevent further cellular destruction in spray-dried powders, henceforth supplying additional

safeguards to the cells. Besides, the positive effect of inulin may be attributed to its prebiotic properties, since this carbohydrate is selectively consumed by bifidobacteria.

3.4 Survival of bifidobacteria under *in vitro* simulated gastrointestinal conditions assay

The main objective of this experiment was to evaluate the viability of *Bifidobacterium* BB-12 during their transition through the mouth to the small intestine. The Table 5 and Fig. 2 show, respectively, the survival (%) and the viable cell counts of the free and of the microencapsulated *Bifidobacterium* BB-12 exposed (1, 2, 3 and free cells) and of those not exposed (1C, 2C, 3C and free cells C, i. e., controls) to the *in vitro* simulated gastrointestinal conditions. As the results of the control samples did not change during the assay, it is clear that external factors did not act on the bacteria, and thus, the effectiveness of the *in vitro* simulated gastrointestinal conditions was proven.

The first step was the simulation of mouth conditions. After this step, the free cells showed a decrease ($P < 0.05$) in viable *Bifidobacterium* BB-12 cell count; whereas no differences ($P > 0.05$) in the viable cell count of all the spray-dried powders (1, 2, and 3) were detected after exposure to the same condition. Thus, it was possible to note that the microencapsulation process protected the bifidobacteria in this step. Similar behavior was observed by Verruck *et al*. (2017), who microencapsulated *Bifidobacterium* BB-12 in different matrices, and verified a decrease in the viability of free cells when submitted to simulated mouth conditions. These authors also reported that the maintenance of cell viability in the spray-dried powders after this step is related to the factors such as the buffering capacity of the $NaHCO_3$ solution (used for pH adjustment), and the smaller contact surface between α-amylase and the bifidobacteria.

After exposure to the simulated esophagus-stomach conditions, both the viable cell counts and the survival (%) of *Bifidobacterium* BB-12 decreased ($P < 0.05$) for all samples. When analyzing this step separately, it was verified that there were no significant differences between all spray-dried powders and free cells, which suggests sensitivity towards simulated gastric juice containing HCl and pepsin. Liu *et al*. (2016) noticed that the dissolution of many microcapsules and a loss of protection to the bacteria is strongly correlated to low pH.

The highest protective effect of the spray-dried powders on probiotic survival was observed in the duodenum step of the *in vitro* simulated gastrointestinal conditions. The free cells bacterial viability experienced a dramatic decrease in the duodenal phase when compared to the

microencapsulated forms. As stated by Madureira *et al*. (2011), the amphiphilic nature of bile salts enables its strong inhibitory effect on bacteria, thus heavily constraining bacterial survival throughout the gastrointestinal tract. Thereby, the free cells were apparently in a more fragile state when they reached this step. This occurs due to damages caused by the acidic conditions before exposure to the duodenum conditions, thus recovery of probiotic cells was eventually not possible. Ranadheera *et al*. (2014) also found similar behavior when submitted unencapsulated probiotics to the presence of bile salts during in vitro gastrointestinal tolerance, that is, the cells experienced a significant decrease in their viability. On the other hand, the increase of pH in the duodenum step appeared to be favorable for the survival of probiotic cells in the spray-dried powder 2, since it exhibited higher stability than the other samples. This behavior suggests that lactose-free skim milk in association with inulin may lower toxicity of bile on membrane damage; similar results were reported by Kingwatee *et al*. (2015) and Fritzen-Freire *et al*. (2013).

Remarkably, from the duodenum step to the ileum step, the results of the viable cell count and the survival for the spray-dried powder 2 showed no differences ($P > 0.05$). For the spray-dried powders 1 and 3, as well as for the free cells these parameters increased ($P < 0.05$). Verruck *et al*. (2017) observed the same phenomenon in a microcapsule produced with goat's milk, inulin, and oligofructose, and for free bifidobacteria. Verruck *et al*. (2015) reported that this increase cannot reasonably be attributed to cell multiplication, and probably resulted from a massive release of uninjured bifidobacteria from degraded microcapsules and/or recovery of sublethally injured cells. Besides, Moumita *et al*. (2017) observed that due to acidity shock in the stomach, some of the lineages of lactobacilli entered a dormant state, and regain their growth when the pH in the small intestine reached 6.0. Therefore, at the end of the simulated gastrointestinal conditions, *Bifidobacterium* BB-12 contained in spray-dried powder 3 showed the highest ($P < 0.05$) survival, followed by the spray-dried powders 1 and 2, which did not show differences between them ($P > 0.05$). Free cells exhibited the lowest viability value.

4. Conclusions

While investigating the effect of lactose-free skim milk powder and prebiotics in the spray drying of *Bifidobacterium* BB-12, we observed that all the formulations showed overall positive effects. However, microparticles made from lactose-free skim milk powder and inulin gave a

better result for the survivability of probiotic bacteria during storage. The spray-dried powder produced with lactose-free skim milk powder and oligofructose showed the highest probiotic survival at the end of simulated gastrointestinal conditions. On this approach, we concluded that the spray-dried powders containing prebiotics (2 and 3) were the most appropriate combinations for microencapsulation of *Bifidobacterium* BB-12 and maintenance of cell viability during storage and gastrointestinal system, showing great potential to be used in lactose-free dairy products.

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Conflict of interest

The authors declare that they have no conflict of interest.

Data Availability Statement

Research data are not shared.

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Table 1 Feed solutions composition employed in *Bifidobacterium* BB-12 microencapsulation by spray drying

Formulation	Carrier agent (g L^{-1})			Concentration of culture (mL L^{-1})
	Lactose-free skim milk powder	Inulin	Oligofructose	
Feed solution 1	200	-	-	100
Feed solution 2	100	100	-	100
Feed solution 3	100	-	100	100

Table 2 Viable *Bifidobacterium* BB-12 cells count and encapsulation yields (EY)

Samples	Viable cells (log CFU g^{-1})	EY (%)
Feed solution 1	10.65 ± 0.04^{a}	86.66
Spray-dried powder 1	9.23 ± 0.05^{b}	
Feed solution 2	10.55 ± 0.13^{a}	88.01
Spray-dried powder 2	9.29 ± 0.07^{b}	
Feed solution 3	10.62 ± 0.02^{a}	87.52
Spray-dried powder 3	9.30 ± 0.04^{b}	

Feed solution 1 and Spray-dried powder 1 are the *Bifidobacterium* solution with addition of lactose-free skim milk powder, and its spray-dried powder, respectively. Feed solution 2 and Spray-dried powder 2 are the *Bifidobacterium* solution with addition of lactose-free skim milk powder and inulin, and its spray-dried powder, respectively. Feed solution 3 and Spray-dried powder 3 are the *Bifidobacterium* solution with addition of lactose-free skim milk powder and oligofructose, and its spray-dried powder, respectively. [a-b]Within a column, means ± standard deviations with different superscript lowercase letters denote significant differences ($P < 0.05$) among the samples.

Table 3 Physicochemical properties of probiotic spray-dried powders obtained from lactose-free skim milk and prebiotics

	Spray-dried powders		
	1	2	3
Lactose (%)	nd	nd	nd
Moisture (g 100 g^{-1})	7.67 ± 0.12^{a}	4.45 ± 0.28^{b}	4.54 ± 0.09^{b}
Water activity	0.396 ± 0.004^{a}	0.276 ± 0.006^{b}	0.288 ± 0.009^{b}
Solubility (%)	63.10 ± 0.51^{a}	62.39 ± 1.22^{a}	62.75 ± 1.52^{a}
Loose bulk density (g cm^{-3})	0.32 ± 0.02^{b}	0.32 ± 0.01^{b}	0.44 ± 0.05^{a}
Tapped bulk density (g cm^{-3})	0.37 ± 0.03^{b}	0.42 ± 0.02^{b}	0.53 ± 0.06^{a}
Interstitial air (IA) (cm^{-3}100 g)	42.21 ± 4.54^{b}	61.18 ± 2.36^{a}	42.61 ± 1.91^{b}
Flowability (Carr's index) (%)	12.68 ± 2.58^{b}	23.43 ± 5.99^{a}	17.65 ± 0.36^{ab}
Cohesiveness (Hausner ratio)	1.15 ± 0.03^{b}	1.31 ± 0.11^{a}	1.21 ± 0.01^{ab}
*L**	95.58 ± 0.32^{a}	93.90 ± 1.81^{a}	93.93 ± 0.35^{a}
*a**	0.26 ± 0.04^{b}	0.04 ± 0.01^{c}	0.37 ± 0.06^{a}
*b**	8.87 ± 0.42^{a}	6.68 ± 0.06^{b}	9.37 ± 0.04^{a}
h	88.31 ± 0.19^{b}	89.69 ± 0.05^{a}	87.74 ± 0.36^{b}
ΔE*	-	2.76	1.72

[a–c]Within a line, means ± standard deviations with different superscript lowercase letters denote significant differences ($P < 0.05$) between the samples.

nd: not detected, (1): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced only with lactose-free skim milk powder, (2): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced with lactose-free skim milk powder and inulin, (3): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced with lactose-free skim milk powder and oligofructose.

Table 4 Viable *Bifidobacterium* BB-12 cells count from spray-dried powders during 120 days of storage at 25 ± 1 °C

	Viable cell count of the spray-dried powders (log CFU g^{-1})		
Storage (days)	1	2	3
0	9.23 ± 0.05^{a}	9.29 ± 0.07^{a}	9.30 ± 0.04^{a}
30	8.18 ± 0.16^{b}	9.24 ± 0.01^{a}	9.13 ± 0.08^{a}
60	1.95 ± 0.07^{c}	7.81 ± 0.02^{b}	5.88 ± 0.13^{b}
90	1.78 ± 0.25^{c}	6.50 ± 0.43^{c}	4.08 ± 0.18^{c}
120	0.17 ± 0.01^{d}	4.49 ± 0.50^{d}	2.23 ± 0.23^{d}

1 is the spray-dried powder that was prepared employing the Feed solution 1, with 200 g of lactose-free skim milk powder. 2 is the spray-dried powder that was prepared using the Feed solution 2, with 100 g of inulin and 100 g of lactose-free skim milk powder. 3 is the spray-dried powder prepared using the Feed solution 3, with 100 g of oligofructose and 100 g of lactose-free skim milk powder. $^{a-d}$Within a column and for the same temperature, different superscript lowercase letters denote significant differences ($P < 0.05$) among the samples at different storage day.

Table 5 Survival (%) of *Bifidobacterium* BB-12 free and microencapsulated after each step of the under simulated gastrointestinal conditions

Spray-dried powder	Mouth	Esophagus-Stomach	Duodenum	Ileum
1	100.68 ± 1.02^{aA}	78.07 ± 5.34^{bcB}	70.52 ± 1.82^{cC}	80.01 ± 1.08^{bC}
1C	100.85 ± 0.82^{aA}	99.75 ± 0.55^{aA}	99.58 ± 0.39^{aA}	99.50 ± 0.91^{aA}
2	100.40 ± 0.16^{aA}	80.80 ± 1.61^{bB}	78.19 ± 1.80^{bB}	81.29 ± 0.76^{bC}
2C	99.44 ± 1.95^{aA}	97.72 ± 0.48^{aA}	97.75 ± 0.44^{aA}	98.74 ± 0.64^{aA}
3	100.25 ± 0.31^{aA}	83.72 ± 0.42^{bB}	67.36 ± 2.95^{cC}	87.59 ± 0.02^{bB}
3C	100.04 ± 0.72^{aA}	100.39 ± 3.36^{aA}	99.28 ± 1.80^{aA}	101.66 ± 1.57^{aA}
Free cells	90.10 ± 0.37^{aB}	81.34 ± 6.97^{abB}	44.68 ± 3.73^{cD}	75.53 ± 0.85^{bD}
Free cells C	100.15 ± 0.19^{aA}	99.10 ± 0.46^{aA}	100.41 ± 0.18^{aA}	99.35 ± 1.32^{aA}

$^{a–c}$Within a line, means ± standard deviations with different superscript lowercase letters denote significant differences ($P < 0.05$) among different steps of the simulated gastrointestinal conditions for each sample.

$^{A–D}$Within a column, means ± standard deviation with different superscript uppercase letters in the same column indicate significant differences ($P < 0.05$) among the same step of the simulated gastrointestinal conditions for all samples.

(1): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced only with lactose-free skim milk powder, (2): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced with lactose-free skim milk powder and inulin, (3): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced with lactose-free skim milk powder and oligofructose. The letter C after each respective identification represent the samples not exposed to the simulated gastrointestinal conditions, i.e., used only as control.

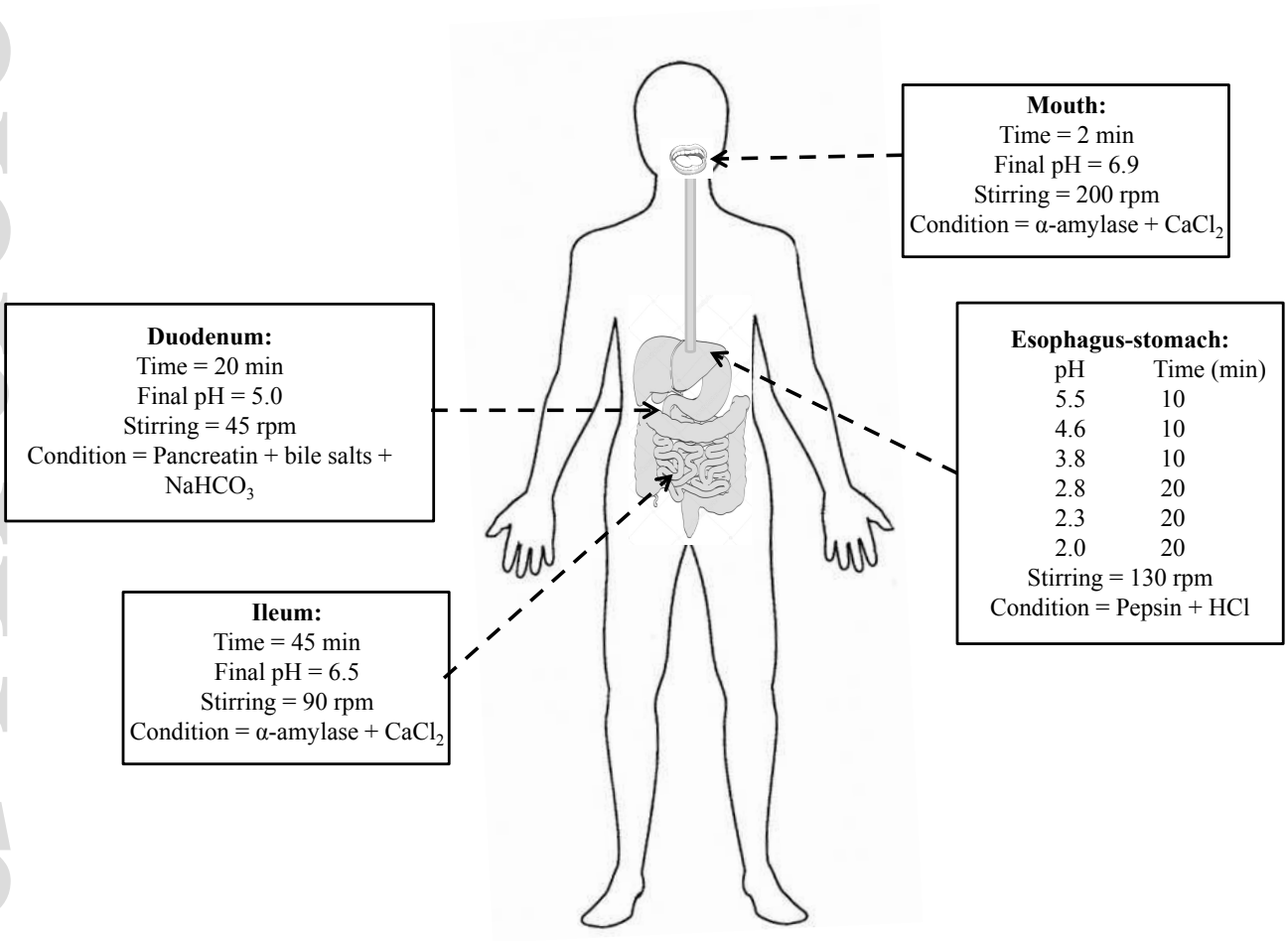

Figure 1: Protocol of under *in vitro* simulated gastrointestinal conditions steps according to Verruck *et al*. (2017). All enzymes and bovine bile salts were purchased from Sigma Aldrich (St. Louis, USA), while all others reagents were of analytical grade.

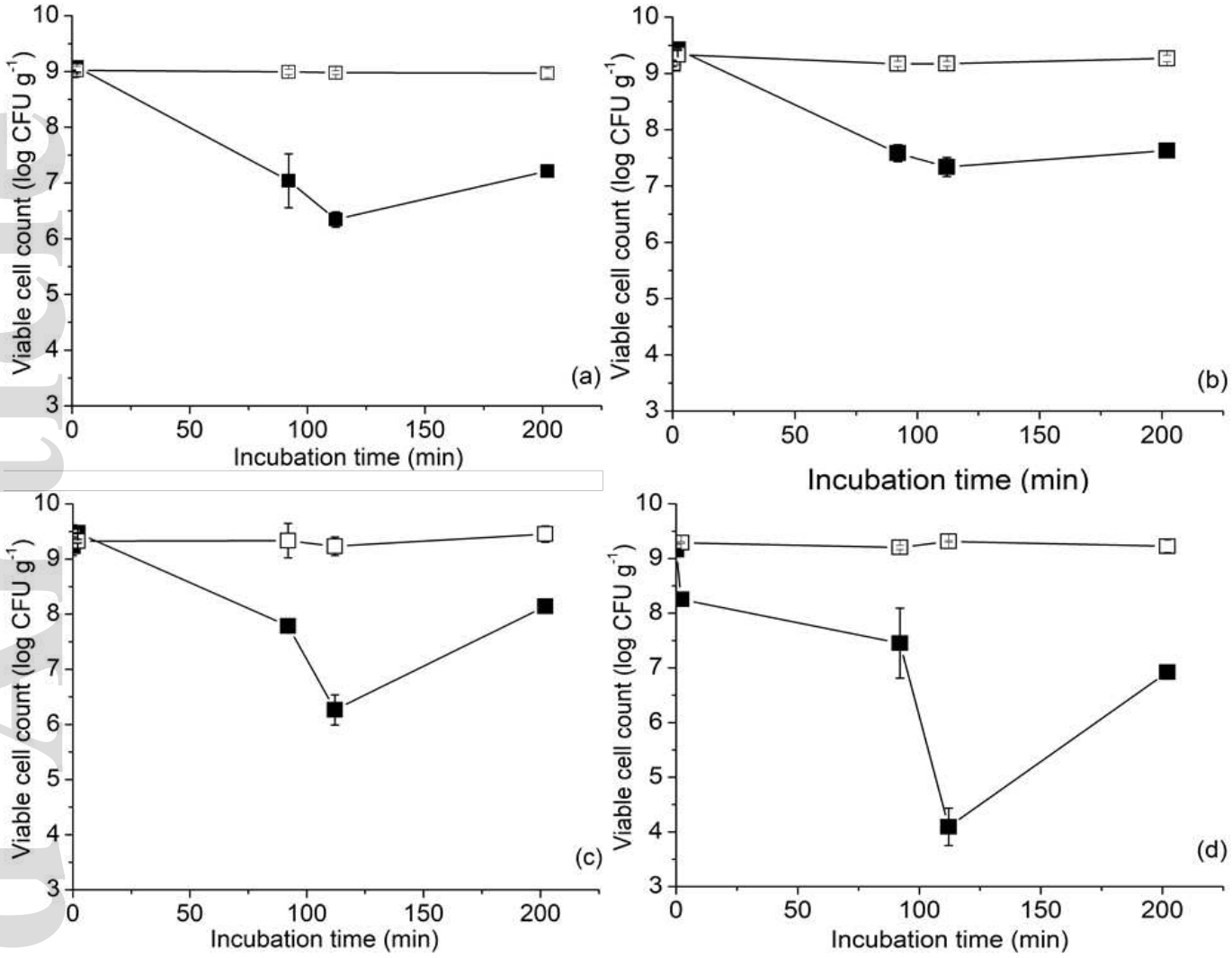

Figure 2: Survival of free and microencapsulated *Bifidobacterium* BB-12 after each step of the simulated gastrointestinal conditions. (a) 1 (■) and 1C (□); (b) 2 (■) and 2C (□); (c) 3 (■) and 3C (□); (d) Free cells (■) and free cells C (□). (1): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced only with lactose-free skim milk powder, (2): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced with lactose-free milk powder and inulin, (3): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced with lactose-free skim milk powder and oligofructose. The letter C after each respective identification represent the samples not exposed to the simulated gastrointestinal conditions, i.e., used only as control. The error bars represent standard deviation of mean.