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STABILITY OF *Lactobacillus plantarum, L. casei* AND *L. rhamnosus* IN TWO TYPES OF MICROCAPSULES

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Abstract

Probiotics require proper encapsulation in order to allow development of useful products with biological activity. Survival of encapsulated probiotics was monitored as a function of the carbohydrate source and of time, over a period of 45 days. The starting material was a mixed culture of Lactobacillus casei, Lactobacillus rhamnosus and Lactobacillus plantarum with a concentration of 10^{11} colony-forming unit/ml (CFU/ml). Standard culture medium with glucose allowed higher initial concentration of probiotics compared with oligofructose medium. However, reduction of viable probiotics in oligofructose supplemented microcapsules was of only 4 log CFU/g, while in glucose supplemented microcapsules was of 8 log CFU/g. Specifically, numbers of probiotics varied from 10^{10} CFU/g immediately after encapsulation to 10^{2} CFU/g, after 30 days of storage for glucose supplemented microcapsules, while, for oligofructose supplemented microcapsules, numbers were 10^{1} CFU/g initially and 10^{6} CFU/g after 30 days. Our results indicates that oligofructose is a more effective prebiotic than glucose, allowing higher survivability of probiotics.

Key words*: Lactobacillus, glucose, oligofructose, prebiotics, probiotics.*

INTRODUCTION

As has been known for more than a century, the Nobel Prize laureate, Russian researcher Elie Metchnikoff is the one who brought the new concept about the existence of "pro-life" bacteria. But what led to this concept was the hypothesis that the long and healthy life of Bulgarian peasants was due to the consumption of dairy products containing lactic bacteria. Probiotics have been defined by the FAO/WHO Expert Committee in 2001, as being "living microorganisms that, when administered in adequate quantities, provide health benefits to the host" (De Prisco et al., 2016). Taking into account all the definitions issued so far and accepted, it has been found that more than 30 species and genera of bacteria are accepted as probiotics. (Fijałkowski et al., 2016).

The probiotic agents most frequently used are those microorganisms which produce lactic-acid, including in particular *Lactobacillus* species (Serna-Cock et al., 2016). The most important benefits of these microorganisms are found especially in the intestinal level, where they are particularly concerned with the maintenance of the microbiota ecosystem (Ozyurt & Otles, 2014).

In the present study were analyzed three species from the group of lactobacilli and they are: *Lactobacillus plantarum, Lactobacillus casei* and *Lactobacillus rhamnosus*, about which the specialized literature states that they are effective in many treatments. For example, in the treatment of patients who had chronic fatigue syndrome it was used the *Lactobacillus casei* strain *Shirota* and it was observed a significant reduction on the Beck anxiety (Rao et al., 2009); Other authors have argued that the *L. plantarum* is responsible for the decrease and even treating the symptoms of colitis and irritable bowel Schultz et al. (2002); Regarding *L. rhamnosus* and other probiotics, it was found to be responsible for the anti-inflammatory induction of cytokines, interleukin-10 (Tenea et al., 2018). It is also known that there is an interaction between the innate immune system, the adaptive immune system and probiotics contributing to intestinal homeostasis. (Latvala et al., 2011).

In order for a probiotic to be used, it must follow basic principles such as: it must survive so that the concentrations of living microorganisms at the time of food consumption are above 10-10^{7} CFU g^{-1} or ml^{-1} (FAO/WHO, 2001). For more than 10 years, in order to cope with the

disruptive factors, different physical barriers to the protection of these microorganisms are analyzed. (Bernucci et al., 2017, Burgain et al., 2011). The most studied method of protection is microencapsulation, which involves retaining the culture of microorganisms in a capsule made of materials that must be generally recognized as safe (GRAS), be able to ensure the integrity of probiotic cells and be soluble in their place of action (Valero-Cases, 2015). Starch, chitosan, alginate, xanthan gum, cyclodextrins, whey proteins etc. are among the most analyzed biopolymers and used as a material for the capsule protection wall (Ashwar et al., 2018). In order to ensure a higher survival rate throughout the manufacturing process and up to the place of action of probiotics in the encapsulation matrix, a nutritious substrate such as prebiotics was incorporated. These prebiotics were defined by International Scientific Association for Probiotics and Prebiotics (ISAPP) as being "a substrate selectively used by host microorganisms that confer health benefits". From the combination of a probiotic and a prebiotic, results a symbiotic product that can increase the probiotic's survival rate during products and storage but at the same time enhances the effects on the intestine. (Raddatz et al., 2019; De Prisco et al., 2016). As far as we know, there are no studies investigating the survival of the three strains mentioned above in mixed culture. Therefore, this study aimed to analyze the viability of the three strains of *Lactobacillus* in two different capsule types for 45 days of storage at 4°C. The capsules contain different sources of carbon, one source is glucose (GLU) and the other is a prebiotic, oligofructose (OLI).

MATERIALS AND METHODS

Chemicals and reagents

De Man-Rogosa-Sharpe agar was purchased from VWR International bvba/sprl; MRS broth, manganese (II) sulfate hydrate, potassium chloride, sodium chloride sodium citrate, peptone from casein, meat extract, yeast extract, magnesium sulfate hepta-hydrate glucose, oligofructose were purchased from Sigma-Aldrich, Germania; Alginic acid Sodium salt from AppliChem GmbH. Calcium chloride hexa-hydrate from Lach - Ner Company - Czech Republic; acetic acid-ammonium salt, sodium salt tri-hydrate and sodium phosphate, acetic acid, and sodium phosphate dibasic hydrate from Across organics-Spain.

Microorganism and culture preparation

In order to determine the ability of *Lactobacillus* cultures to use oligofructose to have long-term viability, a De Man-Rogosa-Sharpe broth (MRS broth) was used as a basal medium as described by De Man et al., 1960 and a modified medium where the initial source of carbohydrate with oligofructose (OLI) was replaced. In brief, the medium containes the following components (g/l): peptone (10.0), meat extract (10.0), yeast extract (4.0), Na × $3H_2O$ acetate (5.0), K_2HPO_4 × $3H_2O$ (2.0), (NH_4) $3C_6H_5O_7$ × $2H_2O$ (2.0), $MgSO_4$× $7H_2O$ (0.2), $MnSO_4^-$ × $4H_2O$ (0.05), Tween 80 (1 ml) and glucose (20.0). For the homogenization of the two types of culture media, a homogenizing vortex (Vortexer, Heathrow Scientific ® LLC) was used for 15 min at 1400 rpm. Before being sterilized in the autoclave at 121°C for 15 min, the pH was adjusted to 6.2.

Probiotic strain and culture condition

In this study were used three strains of lactobacillus. *L. casei 431* is a registered trademark, and it was received from Christian Hansen. From this strain, pure cultures were obtained after three reactivations in MRS broth and stored at -20°C in glycerol. *Lactobacillus plantarum* and *L. rhamnosus* (BIOPROX RP 80) were purchased from Bioprox Noyant, France. Prior to use, each culture was reactivated in MRS broth at 37°C for 24 h. Then another 24 h reactivation took place at 37°C, to obtain a densitometry of 4 McFarland units for *L. casei* and 8 McFarland units for the other two strains (*L. plantarum* and *L. rhamnosus*) grown together. These values were determined on a McFarland densitometer with McFarland measuring range 0.3-15.0 at wavelength $\lambda = 565 \pm 15$ nm. After this, the cultures, also separately, were inoculated in modified MRS broth at 37°C for another 24 h. The three strains were then put together in equal proportions and incubated in modified MRS broth for 24 h at 37°C. and then for another 16 h in order to obtain the final culture for this study. The cell suspensions were subsequently subjected to micro-encapsulation as described later.

Microencapsulation procedure

For the preparation of all solutions including culture media, distilled water (Aquatron A4000D, Cole-Parmer Ltd) was used, and to avoid any contamination, sterilized reagents and glassware were used. The encapsulation technique used was extrusion, a technique described by Darjani et al. (2016), Krasaekoopt et al. (2004) and Peredo et al. (2016), and then adapted and modified to the needs of the present study. The materials used to form the encapsulation matrix were: 1.75% sodium alginate, 1% carbon source, 10% vegetable oil, 85% distilled water. All the materials were mixed with the help of a stirrer at 10,000 U/ min. The pellet was added and the mixing was continued using a magnetic stirrer (15 min/600 rpm). This pellet was obtained by centrifuging the culture for 16 h for 10 min at 2500 g at 4°C.

After this homogenization, a peristaltic pump, a 3 mm diameter hose and a 0.4 mm diameter needle were used to pass the prepared emulsion. The resulting drops reached into aliquots of 2% sterile calcium chloride at a distance of about 10-15 cm with a power of 3.2 rpm/ml/min. Once the drops arrived in the calcium chloride solution, they immediately formed micrometer-sized gel spheres. The microcapsules were allowed to stand for 30 min to harden, then harvested using vacuum pump (EZ-Stream® vacuum filtration pump). The whole process was performed by autoclaving all the solutions involved in the process (121°C, 15 min) and under sterile conditions in a horizontal laminar air-flow cabinet.

Characterization of capsules

Considering the dimensions of the micrometers, the morphological characterization and the dimensions of the microcapsules were performed using microscopy. An optical microscope and an electronic microscope were used. The morphology was performed by scanning electron microscope-SEM.

Viable cell count

For the determination of the number of living cells from the emulsion, serial dilutions were performed in distilled water. 1 ml of the final dilution was inoculated in triplicate in MRS agar plates and incubated at 37°C for 48-72 h under anaerobic conditions in anaerobic jars. The results were reported in CFU/ml.

Encapsulation yield

Regarding the determination of the number of viable cells in the fresh microcapsules, it was necessary to dissolve the capsule and release the cells. This protocol was performed by dissolving 0.1 g of fresh microcapsules in 9.9 ml of 1% sterile sodium citrate solution with pH 6 and slightly stirred at room temperature for approximately 12 min, after which serial dilutions were performed as mentioned above. From the last dilution 1 ml was inoculated into small plates with MRS agar and incubated for 48-72 h at 37°C in anaerobic jars with anaerobic generator. All experiments were performed in triplicate and the results were reported as CFU/g of microcapsules. The encapsulation yield (EY) was calculated according to the formula used by Chávarri et al., 2010; Picot & Lacroix, 2004; Rather et al., 2017:

$$\mathrm{EY} = \frac{log_{10} N_1}{log_{10} N_0} ,$$

where $log_{10}N_1$ represents the number of viable cells trapped in the capsule and $log_{10}N_0$ represents the amount of free viable cells added to the emulsion during the encapsulation process and the result of the equation is expressed as number of CFU/ml. This formula, EY, represents a criterion for measuring how the encapsulation process influences the number of viable cells (Picot & Lacroix, 2004).

Viability of encapsulated bacteria during storage

To determine the number of viable cells encapsulated during storage at 4°C for 45 days, the microcapsules were stored in sterile and sealed petri dishes. The cell viability was analyzed both from the microcapsules containing glucose in their matrix as carbon source, as well as from microcapsules with prebiotic, oligofructose.

The testing was performed on microcapsules collected from day 0, 7, 14, 28 and 45. The same protocol described above was used. After dissolution of the capsule, serial dilutions were made and then pour plated in MRS agar. Incubation was carried out under the same conditions as above. An average of three

replications were made, and was expressed as log CFU/g of microcapsules.

RESULTS AND DISCUSSIONS

Microscopic examination of alginate beads/Morphological characterization of micro-particles

One of the objectives of this study was to evaluate the effect of prebiotic on morphology and on the size of the microparticles. For this purpose, optical microscopy and electron microscopy were used. Thirty-five microcapsules were randomly selected and analyzed under the optical microscope which was equipped with a digital component (Table 1). Optical microphotographs of microcapsules showed different types of beads with a defined limit, but spherical shape was more abundant and particles were isolated without adherence to each other. This aspect leads us to the idea that this technique is improved because it produces capsules of micron size, much better than those of millimetric dimensions produced by other researchers (Hyndman et al., 1993; Arnaud et al., 1992) who have used a similar technique, and will offer a smooth texture when incorporated into products. Some researchers (Mokarram el al., 2009; Hansen et al., 2002) claim that large alginate capsules (> 1 mm), cause the coarse texture of foods that are supplemented with probiotic cultures.

Therefore, two samples of fresh microcapsules were subjected to electron microscopy (SEM) scanning: microcapsules with prebiotic and probiotic bacteria - oligofructose and microcapsules with probiotic bacteria and without prebiotic (Figure 1). It was found, according to Table 1, that there are no significant differences in morphology and size of beads. Following the SEM analysis, it was found that: microcapsules varied in size (which was also observed in optic microscopy), were compact and continuous, spherical but with irregular surface. As an explanation for the uneven surfaces, there may be a greater concentration of polymer there (Fareez et al., 2015). Also, the absence of free *Lactobacillus* cells on the surface of the capsules was noted, and as mentioned above the encapsulation process means that it is efficient from this point of view.

Evaluation of the mean diameter and distribution of microparticles size

Below, in Table 1 are given the averages of the diameters of the microcapsules that were analyzed. It is considered that the distribution and size of the capsules may be influenced by several factors such as: firstly, the stirring speed, then the ratio between water and oil (v/v) or surfactant concentration.

Table 1. Beads size expressed as mean of 35 microcapsules ± standard deviation

Parameter	Size (μm)	
	Oligofructose	Glucose
Mean	0.478 ±0.13 [a]	0.42 ±0.14 [a]
Median	0.478	0.42
Minimum	0.20	0.21
Maximum	0.65	0.62

Means in the same row with superscripts (a) not differ significantly: *P > 0.05.

Some authors believe that with the introduction of probiotic cells into the emulsion and then injected with needle into calcium chloride, the size of the microparticles (Sousa et al., 2015; Martin et al., 2013) is reduced and the capsules are more compact. This happens due to the presence of probiotics cells, but also due to the gel layer that forms which compresses the capsule matrix and thus expels some of the inner water (Sánchez et al., 2017). On the other hand, another explanation regarding the aforementioned might be that probiotic cells would replace Ca^{2+} ions which leads to a change in Ca^{2+} concentration which will lead to syneresis. At the same time, the size of the microcapsules can be influenced by other factors different from those mentioned, such as: alginate concentration, calcium chloride concentration, needle diameter, pump pressure, distance between needle and calcium chloride solution. In previous studies with microencapsulation by extrusion, Lenton et al., (2012) obtained an average size for microcapsules of 2.9 mm; Muthukumarasamy et al. (2006) obtained an average size of 2.37 mm with a similar needle (G21) with the one used in the study performed by Valero-Cases et al., 2015, for microencapsulation by extrusion, which obtained an average of 1.86 mm, which was larger than the average size obtained in this study for all the beads (0.48 mm). In other previous studies with internal emulsion microencapsulation, Cai et al. (2014) obtained

average microcapsule dimensions of 343 µm, which were similar to those of the present work obtained using the method of extrusion encapsulation.

Viable cell count and Microencapsulation efficiency and yield

The presence of prebiotic in the culture medium significantly influenced the growth of lactobacilli in final cultures of 16 h thus, the growth of lactobacilli in the culture medium with oligofructose was 11.08 ± 14.11 log CFU/ml and 12.08 ± 7.06 log CFU/ml for glucose.

This is explained by the fact that lactobacilli metabolize glucose much faster as a carbon source compared to oligofructose (Luca and Oroian, 2019).

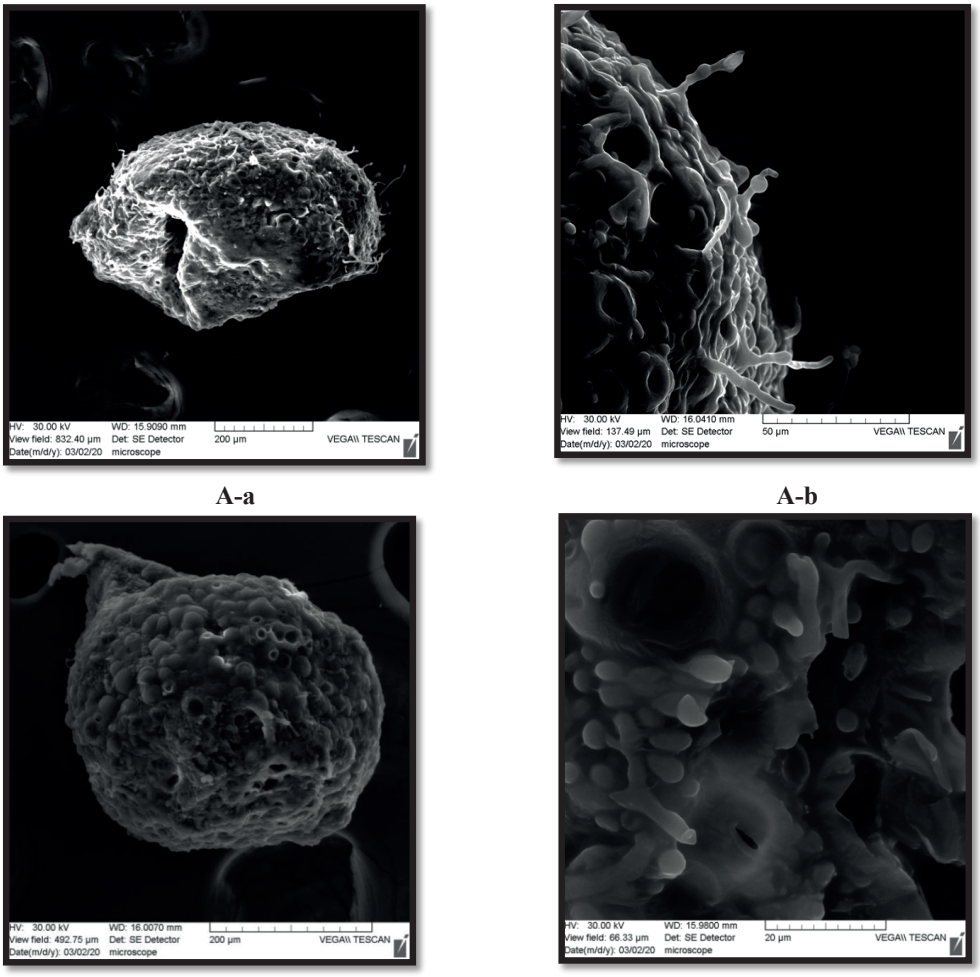

Figure 1. Scanning electron microscope photographs of fresh beads: (A-a) bead whit prebiotic; (A-b) surface of a bead with oligofructose; (B-a) bead whit glucose; (B-b) surface of a bead with glucose

Another objective of this study was to identify the most efficient encapsulation matrix of each type of microcapsule for *Lactobacillus* strains. For this it was analyzed how the *Lactobacillus* cells were protected during the encapsulation process - the of microcapsulation but also EY.

Table 2 shows the results obtained for glucose microcapsules efficiency and also for oligofructose microcapsule. It can be seen that both types have formulated an optimal encapsulation efficiency of 100% but with a significant difference between them ($p > 0.05$).

The encapsulation efficiency of the employed process was very high and is not a critical parameter that needs to be adjusted. The results showed an encapsulation efficiency of over 100%, given the fact that the number of CFU/g of capsules is almost 1 log higher than that of CFU/ml of microorganism containing emulsion. This effect appeared due to the capsules being a proper medium for encapsulated bacteria growth, as it contains prebiotics and of the fact that enumeration of encapsulated bacteria was performed approximately 6 h after the actual encapsulation. The growth of probiotics in prebiotic supplemented media was already shown to occur rather rapidly. For instance, *Lactobacillus casei* has a rate of approximately 1 log of growth over 6 h in 4 different prebiotic supplemented media (Luca and Oroian, 2019), while *Lactobacillus plantarum* and *L. rhamnosus* displayed a similar behavior with the same prebiotics (Luca et al., 2019). A probiotic encapsulation report by Sánchez et al in 2017 showed similar results but they did not use prebiotics. Other authors such as Raddatz et al., 2020, found that their capsules obtained by internal emulsification/gelation, had an EY% between 82.65% and 91.24%; capsules obtained by Jantarathin et al. (2017) using extrusion technique with *L. acidophilus*, sodium alginate and inulin had an EY % = 88.19%; Zou et al., 2011, using the internal gelling technique, produced alginate microspheres mixed or coated with other polymers containing *Bifidobacterium bifidum F-35*, and the average EY% ranged from 43% to 50%. As noted above, it is observed that maintaining cell viability is a very important factor, regardless of the type of encapsulation.

Viability of encapsulated bacteria during storage

The cellular viability of glucose and oligofructose microcapsules stored in the refrigerator for 45 days at 4°C is shown in Figure 2. After 45 days, the survival rate of *Lactobacillus* cells in glucose microcapsules indicates a decrease greater than 7.60 CFU log/g. In terms of cell survival in prebiotic microcapsules, it decreased from 2.96 x 10^{10} ± 20.3 CFU/g to 6.93 x 10^{5} ± 1.33 CFU/g, so with a loss of viability of 4, 63 CFU log/g after this period of 45 days. The survival of cells in oligofructose beads was significantly ($p < 0.05$) better than that of glucose beads.

It was statistically analyzed both the cell viability at the same time between the two types of microcapsules, but also between the obtained values of the same type of microcapsules at the same moment. It was found that there are significant differences ($p < 0.05$) in both cases at all times. These differences were maintained during the 45 days.

In the first 7 days after encapsulation, a reduction in the viability of probiotics was observed in both prebiotic and non-prebiotic microcapsules. In the first case the reduction was 1.899 log CFU/g, and in the second case the reduction was 2.90 log CFU/g. Therefore, the viability decreased during the first 7 days by 1.26% for probiotics encapsulated with oligofructose and by 1.25% for probiotics encapsulated with glucose. If in the next 7 days the decrease in cell viability of oligofructose microcapsules was insignificant of 0.073 log CFU/g, in the case of the other type of microcapsule, the cell viability decreased by approximately 2 log CFU/g. After 28 days of storage, it was observed a decrease in cell viability of 3.169 log CFU/g in prebiotic microcapsules compared to day 14 and a decrease of 5.14 log CFU/g compared to the time of encapsulation, but this decrease will not be the same after 45 days when the reduction was only 0.49 log CFU/g. A decrease in cell viability also occurs in the case of glucose microcapsules, but it was 2.53 log CFU/g less than day 14 and much higher (7.41 log CFU/g) compared to day 0. Comparing the last 2 measurement points between the cell viability of the capsules with and without prebiotic, day 28 and day 45, it was found a greater decrease of cell viability in microcapsules without prebiotic, of 1.2 log CFU/g. According to the above it can be stated that these microcapsules which have a source of carbon in their matrix the microorganisms are metabolically active in capsules at 4°C. Whether prebiotics, or glucose was used as a substrate in the capsule matrix, the encapsulation is not responsible for decreasing cell viability. In our opinion, some of the main causes would be the passage of time, the consumption of the nutritious substrate, the presence of compounds resulting from the metabolism process such as metabolic acids and

bacteriocins. Other explanations for this would be the presence of residual water in microcapsules and the fact that they were stored in petri dishes during the storage test. Each sample tested was taken from these plates, where the humidity in the atmosphere could have entered which could have led to increased water activity. (Sánchez et al., 2017). Also, it is well known that humidity has a negative effect on cell viability (Heidebach et al., 2010). Using extrusion as the encapsulation method for *Lactobacillus gasseri* and *Bifidobacterium bifidum*, Chavarri et al. (2010) reported that they observed a decrease in viability in the first 11 days of 3.34 log CFU/ml and 4.11 log CFU/ml; respectively after 14 days they did not observe any survival.

As shown in Figure 2, at the end of the storage period, the number of microorganisms in the encapsulated probiotic with oligofructose was higher than the probiotic encapsulated with glucose.

Table 2. Enumeration of probiotic cells and the encapsulation yield

	Oligofructose	Glucose
Culture of 16 h	1.39E+11±14.11	1.23E+12±7.06
Probiotic population before encapsulation (CFU/ml)	8.16E+10^{a}±23.9	1.12E+11^{a} ±8.00
Encapsulated probiotic population (cfu/g)	2.96E+11^{b} ±2.01	1.87E+11^{b} ±2.67

All values are mean ± standard deviation of three replicates

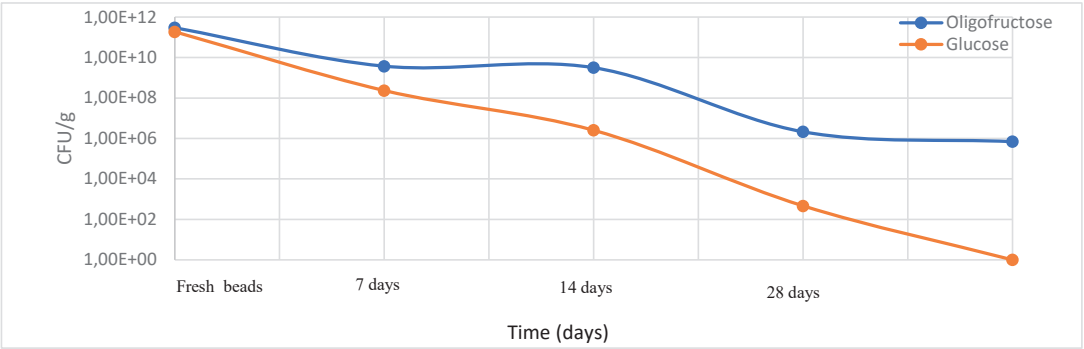

Figure 2. Stability of oligofructose and glucose microparticles of *Lactobacillus* during 45 days at 4ºC. Means (n = 3) ± SD (P < 0.05 between oligofructose and glucose)

CONCLUSIONS

In this study, two types of microcapsules obtained by the extrusion encapsulation method were analyzed. They had different carbon sources in their matrix. Checking the cell viability after encapsulation showed that the encapsulation process chosen did not reduce the cell number. The results also showed that the presence of a carbon source such as oligofructose is much more efficient than glucose in terms of cell survival, having a much greater protective role against environmental conditions. *Lactobacillus* strains had a much higher survival rate in oligofructose capsules than glucose capsules during 45 days of storage. At the same time, the results showed that regardless of the type of emulsion used for encapsulation in this case, the size and shape of the beads were similar without statistically significant differences, and this behavior could be attributed to prebiotics ability to improve capture efficiency and accumulation capacity over time of the encapsulation process.

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