Optimization of the encapsulation of *Lactobacillus rhamnosus* ATCC 7469 by extrusion and subsequent lyophilization on *Passion fruit* from caatinga
ABSTRACT

Aim: The encapsulation of *Lactobacillus rhamnosus* ATCC 7469 by extrusion and subsequent lyophilization for addition on passion fruit from Caatinga (*Passiflora cincinnata* Mast.) juice was evaluated. Methods: A central composite rotatable design was employed to investigate the effects of pectin and CaCl$_2$ concentrations on the encapsulation efficiency (EE). Wet beads were lyophilized with or without sucrose as cryoprotectant. Free and encapsulated cells were added on juice to evaluate cell viability during the refrigerated storage. Results: EE achieved 90.56 ± 0.48 % after extrusion method, and values between 70.36 ± 0.35 and 87.29 ± 0.24 % were observed after lyophilization. Sucrose did not influence the cell viability in lyophilized beads. Higher viabilities were found in free and encapsulated cells without lyophilization (8.2 and 7.28 log CFU/mL, respectively) at 120 days of storage. Conclusion: Passion fruit from Caatinga proved to be an effective vehicle for the incorporation of probiotics.

Keywords: Encapsulation; Lyophilization, Passion Fruit From Caatinga, Probiotic, Storage.
INTRODUCTION

During the design of new probiotic products, the main technological requirement is microbial stability during the processes and the shelf life period. This involves the required number of viable and active microorganisms in the final formula (Tomás et al., 2015). It has been suggested that probiotic-based products should contain at least 6 log CFU/g of viable cells at the time of consumption to provide probiotic benefits (Food and Agriculture Organization & World Health Organization, 2001).

Fruit juices have been reported as novel and appropriate media for probiotic beverage production as they contain essential nutrients and are generally accepted by consumers regardless of age, gender, or geographic region (Mantzourani et al., 2018). A variety of fruits are used in the production of commercial beverages. Passion fruit from Caatinga (*Passiflora cincinnata* Mast.) has been highlighted by Brazilian researchers as a good food matrix for the production of functional juice (Farias et al., 2016; Santos et al., 2017).

An important criterion should be considered when marketing fruit juices containing probiotics: viability until the end of shelf life (Shori et al., 2016). Many methods have been studied to increase the viability of food probiotics, such as cell entrapment or immobilization on various food-grade carriers (Mantzourani et al., 2018). Lyophilization and encapsulation processes via extrusion are methods widely applied to preserve and protect microbial viability (Albadran et al., 2015; Etchepare et al., 2016; Li et al., 2016; Halim et al., 2017).

The entrapment of probiotic cells by encapsulation provides a physical barrier against environmental stressors (Burgain et al., 2011). The selection of a wall material and the encapsulation process must be compatible with the probiotics. Pectin has been widely used as a microencapsulation material because it is inexpensive and nontoxic, having been evaluated and declared toxicologically harmless by Joint FAO/WHO (Food and Agriculture Organization & World Health Organization, 2001).

Drying is a crucial step in food production processes and aims to lower the moisture content of the product to increase shelf life (Broecky et al., 2016). Despite its potential, a limited number of studies have reported the application of lyophilized beads in foods (Ribeiro et al., 2014; Moumita et al., 2016). As well, the viability of probiotic strains during encapsulation, lyophilization and subsequent storage in fruit juice has not yet been reported.

The main goal of this work was to encapsulate *L. rhamnosus* ATCC 7469 using pectin as the encapsulating matrix by extrusion method. The effects of lyophilization and subsequent storage on the viability of *L. rhamnosus* ATCC 7469 were studied. Finally, beads with and without lyophilization were applied to passion fruit from Caatinga juice.
MATERIALS AND METHODS

Collection of passion fruit pulp, Microorganism, preservation and inoculums

The passion fruit came from Tapiramutá (Bahia, Brazil), located at latitude 11°50'50", longitude 40°47'29" and altitude 820 meters. The passion fruit was cut manually and the pulp was stored in a freezer (-20 °C) in sterile glass vials for use in the next steps of the work. Lactobacillus rhamnosus ATCC 7469 strain was used. A commercial culture, freeze-dried, was purchased from the American Type Culture Collection (ATCC, USA). The culture was rehydrated in 100 mL of MRS broth (MERCK, KgaA Germany) and incubated for 24 h at 37°C. The cellular suspension was inoculated in sloped glass tubes with MRS-agar (MERCK, KgaA Germany) and re-incubated at 37°C for 24 h. Afterwards, the cell culture was resuspended in glycerol (10 % V/V), distributed (1 mL) in Eppendorf tubes and kept in a freezer at -20 °C. For the preparation of the inoculums, the cells maintained in glycerol were cultured in 50 mL of MRS broth at 37°C for 24 h, then the cells were harvested by centrifuge at 10.000 rpm (3.468 g) for 10 min (THERMO, Electron Corporation) and 4 ºC. The cell pellets were washed and suspended in a sterile saline solution (0.9 % W/V) for further addition in the pectin suspension.

Preparation of beads

Preparation of beads by extrusion method

The beads were prepared by the extrusion method according to the methodology of Nualkaekul et al. (2013). Briefly, a proportion of 1 mL of cell suspension, produced as described in Section 2.2, was mixed with 9 mL of low-methoxyl pectin suspension (GENU® Pectin Type LM, with metoxilation lower than 50%). Before use, the pectin suspensions were heated at 72 °C for 30 min in a thermostatic bath and immediately put on ice to cool down. The cell/pectin mixture (600 mL) was pumped (Gilson®) at a flow rate of 344.5 ml/min and extruded through four sterile needles (25 × 0.70 mm diameter) into sterile 0.15 M CaCl$_2$ (Vetec®, 100 mL) under constant stirring. The beads were allowed to harden for 30 min and were then harvested using a sieve.

A central composite rotatable design (DCCR) $2^2$ was applied with four axial points and two central points, totalizing 10 trials, to investigate the influence of the pectin and CaCl$_2$ concentrations on the encapsulation efficiency. The central point was selected at 4% (w/v) pectin, since this concentration has been used in the beads formulations with high efficiency of encapsulation ( Nualkaekul et al., 2013). Based on the work of Nualkaekul et al. (2013), the concentration of 0.15 M was used as the central point of CaCl$_2$. Experiments were performed using a random run order to minimize the effects of unexpected variations in the observed
responses due to extraneous factors. The independent variables were $x_1$ for the pectin concentration and $x_2$ for the CaCl$_2$ concentration (Table 1).

<table>
<thead>
<tr>
<th>Factors</th>
<th>Levels</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>-1.41</td>
</tr>
<tr>
<td></td>
<td>-1</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+1</td>
</tr>
<tr>
<td></td>
<td>+1.41</td>
</tr>
<tr>
<td>Pectin (% w/V)</td>
<td>3.29</td>
</tr>
<tr>
<td></td>
<td>3.50</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td>4.50</td>
</tr>
<tr>
<td></td>
<td>4.70</td>
</tr>
<tr>
<td>CaCl$_2$ (M)</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>0.22</td>
</tr>
</tbody>
</table>

Table 1. Variables and levels of central composite rotatable design.

The dependent variable was the efficiency of the encapsulation (EE %), determined in section 2.4. Response surface was drawn by using the analysis design procedure of Statistic (7.0 version) for Windows software. The polynomial model generated was adjusted according to Equation 1.

$$\hat{y}_i = b_0 + b_1 x_1 + b_2 x_2 + b_{11} x_1^2 + b_{22} x_2^2 + b_{12} x_1 x_2$$  

Eq. (1)

Where $\hat{y}_i$ is the efficiency of the encapsulation. The validation of the model was performed through the analysis of EE% with the best condition proposed by the generated polynomial model and the tests performed in triplicate.

**Preparation of beads by lyophilization**

Beads were prepared using the selected encapsulation conditions from the experimental design and were subsequently lyophilized. Wet beads were frozen in a freezer at -80°C for 12 hours and then lyophilized at -50°C for 24 hours under vacuum (1.720 mT) in a lyophilizer (VirTis SP scientific, Sentry 2.0). Four types of lyophilized beads were analyzed (L1, L2, L3 and L4), varying in the presence of cryoprotectants (L3 and L4) and passion fruit juice (L2). Control beads (L1) were prepared without cryoprotectant and without juice.

The passion fruit juice used in L2 beads lyophilization was composed of 20 % (V/V) pulp and 10 % (w/V) sucrose. These amounts were chosen in order to have a dehydrated functional product containing *L. rhamnosus* and passion fruit juice. In L3 and L4 lyophilized beads, the cryoprotectant used was sucrose (10 % w/V), which differed in relation to the time of sucrose addition. Under the L3 condition, the wet beads were resuspended in a sucrose solution and then lyophilized. Under the L4 condition, beads were prepared by the addition of sucrose to a pectin solution, still in the extrusion step, and subsequently lyophilized. The flowchart of the four conditions is shown in Figure 1.

After lyophilization, the beads (L1, L2, L3 and L4) were stored at room temperature and the cell viability was analyzed at 0, 30 and 60 days. The efficiency of lyophilization and viability were determined in sections 2.4 and 2.5, respectively.
Figure 1. Scheme summarizing the preparation of encapsulated *L. rhamnosus* ATCC 7469 with and without lyophilization. Storage conditions: room temperature and 4 °C, respectively.

Efficiency of encapsulation

Non-lyophilized and lyophilized beads were disintegrated to calculate the encapsulation efficiency by extrusion and the lyophilization efficiency. 1.0 g and 0.1 g of non-lyophilized and lyophilized beads, respectively, were disintegrated in 10 ml of sodium citrate buffer (0.1 M) at 37 °C and stirred for 5 min. An aliquot of the suspension (1 mL) was used for the plating, as described in section 2.5. The efficiencies of both extrusion encapsulation (EE)
and lyophilization (EL) were calculated according to Equations 2 and 3, respectively. The efficiency tests were performed in duplicate.

\[
EE = \frac{(CV/CL) \times 100}{\text{Eq. (2)}}
\]

\[
EL = \frac{(CS/CV_0) \times 100}{\text{Eq. (3)}}
\]

CV is the number of viable cells in Log, released from the non-lyophilized beads, and CL is the number of free cells in Log added to pectin during bead production by extrusion. CS is the number of viable cells in Log, released from the dry beads after lyophilization, and CV₀ is the number of viable cells in Log, contained in the wet beads before to lyophilization.

**Viability of *L. rhamnosus* ATCC 7469**

Quantification of cell viability was performed by the spread-plate technique. One mL of samples containing the cells was serially diluted in 9 mL of saline solution (0.9 % w/V) and aliquots were seeded in Petri with MRS agar. The plates were incubated at 37 °C for 48 hours.

**Bead characterization**

The moisture content of non-lyophilized and lyophilized beads was carried out using a drying oven (BUNKER) at 80 °C for 24 hours until a constant weight was reached. The tests were performed in triplicate. Size measurements and the direct observation of non-lyophilized beads were carried out as follows: thirty randomly selected beads were placed on dark paper and photographed with a digital photo camera (Canon EOS 70D) with a 100 mm macro lens. A scale bar was added from a calibrated digital image using the software Image J 1.47v and the bead size was determined (Lopes et al., 2017).

For micromorphological analysis, non-lyophilized beads were stained by 1 ml of concentrated Giemsa (Sigma-Aldrich, St. Louis, MO) for 1 hour and rinsed with distilled water for 5 minutes. The stained beads were placed on glass slides, covered with a coverslip and photographed using a digital camera (Axiocam ERc5s, Zeiss) coupled to an optical microscope (Primostar, Zeiss) immersed in oil at x1000 magnification. The microscopic study of the lyophilized beads was performed on a scanning electron microscope (Hitachi TM3030), operated at 15 kV, equipped with a Quantax70 X-ray dispersive energy system for microanalysis of the constituent elements. Samples were added under a carbon tape and evaluated later.
Application of non-lyophilized and lyophilized *L. rhamnosus* beads in passion fruit from Caatinga juice

To investigate the influence of encapsulation and lyophilization on the viability of the *L. rhamnosus* ATCC 7469, the non-lyophilized and lyophilized beads were stored for 120 days at 4 °C. The lyophilized beads L1, L3 and L4 (0.1 g) were added at 10 mL of passion fruit from Caatinga juice (20 % V/V pulp and 10 % w/V sucrose) and named L1*, L3* and L4*, respectively. The L2 condition corresponded to an instant functional juice powder and was not stored under refrigeration. The purpose of this evaluation was to elaborate a product ready for immediate consumption. To compare with the lyophilized beads, 1.0 g of non-lyophilized beads (NL) was placed in 10 mL of passion fruit juice (20% V/V of pulp and 10% w/V of sucrose). The control condition in relation to the encapsulation was passion fruit juice containing the free cells (FC). Cell viability, lactic acid concentration and pH were determined during storage. A scheme of storage conditions is shown in Figure 1.

**pH measurement and determination of lactic acid concentration**

pH variation was analyzed using a digital pH meter (3510, Jenway). The lactic acid concentrations were determined by high performance liquid chromatography (M20A, Shimadzu) with the following conditions: ionic exchange column (Aminex® HPX-87H, Bio-Rad, USA), sulfuric acid (5 mM) as the mobile phase, detection at 210 nm, flow rate of 0.6 mL/min and 28 °C (Farias *et al*, 2017).

**Statistical analysis**

The results were evaluated according to the Analysis of Variance (ANOVA), considering the significance level of 5 % (p < 0.05), using Statistica 7 software and the means were compared by the Tukey test, using Past®.

**RESULTS**

**Application of a central composite rotatable design for optimization of the encapsulation**

A Central Composite Rotatable Design (CCRD) was applied to investigate the efficiency of the encapsulation of *Lactobacillus rhamnosus* ATCC 7469. Assays were carried out as reported on Table 2.
Table 2. Conditions of assays of the central composite rotatable design and response.

<table>
<thead>
<tr>
<th>Assays</th>
<th>Pectin (% w/V)</th>
<th>CaCl₂ (mol/L)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.5</td>
<td>0.10</td>
<td>81.29</td>
</tr>
<tr>
<td>2</td>
<td>3.5</td>
<td>0.20</td>
<td>81.28</td>
</tr>
<tr>
<td>3</td>
<td>4.5</td>
<td>0.10</td>
<td>77.07</td>
</tr>
<tr>
<td>4</td>
<td>4.5</td>
<td>0.20</td>
<td>75.09</td>
</tr>
<tr>
<td>5</td>
<td>3.2</td>
<td>0.15</td>
<td>86.74</td>
</tr>
<tr>
<td>6</td>
<td>4.7</td>
<td>0.15</td>
<td>80.90</td>
</tr>
<tr>
<td>7</td>
<td>4.0</td>
<td>0.08</td>
<td>75.75</td>
</tr>
<tr>
<td>8</td>
<td>4.0</td>
<td>0.22</td>
<td>81.74</td>
</tr>
<tr>
<td>9</td>
<td>4.0</td>
<td>0.15</td>
<td>92.03</td>
</tr>
<tr>
<td>10</td>
<td>4.0</td>
<td>0.15</td>
<td>94.26</td>
</tr>
</tbody>
</table>

The analysis of part of the factorial planning (runs 1, 2, 3 and 4) indicated that when the CaCl₂ concentration was increased (from 0.10 to 0.20 mol/L) and the pectin concentration was 3.5 % w/V (runs 1 and 2), there was no variation in encapsulation efficiency. However, an increase in pectin concentration to 4.5 % w/V and variations in chloride concentration (from 0.10 to 0.20 mol/L) led to a decrease in encapsulation efficiency (runs 3 and 4). In general, these assays (runs 1, 2, 3 and 4) indicated that the variation in the encapsulation efficiency was independent of the CaCl₂ concentration. In the axial part of the planning, the encapsulation efficiency decreased when the pectin was increased from 3.2 to 4.7 % w/V (runs 5 and 6). On the other hand, encapsulation efficiency increased when the CaCl₂ concentration was increased from 0.08 to 0.22 mol/L (runs 7 and 8). The highest efficiency was found in the center point (runs 9 and 10), with pectin at 4 % w/V and a CaCl₂ concentration of 0.15 mol/L, as shown in the response surface for encapsulation efficiency as a function of independent variables (Figure 2). Equation (4) shows the polynomial quadratic model.

\[ \hat{y}_1 = 93.14 - 5.31x_1^2 - 7.85x_2^2 \]  
Eq. (4)

Table 3. shows the analysis of variance (ANOVA) for the polynomial quadratic model. The statistical significance of the model was evaluated by the F-test, showing a statistically significant regression. The ratio between the mean square (MS) of regression and MS residual was higher \( (F_{\text{calc}} = 17.23) \) than the F-test, tabulated at 95 % confidence \( (F_{\text{tab}} = 5.05) \). The ratio between the MS of lack of fit and MS of pure error, however, was lower \( (F_{\text{calc}} = 2.88) \) than the F-test tabulated at 95 % confidence \( (F_{\text{tab}} = 224.6) \). MS were calculated by the ratio between Sum of Squares (SS) and Degrees of Freedom (DF). The coefficient of determination \( (R^2) \) of the model was 0.9323, which further indicated that the model adequately represented the real relation among the selected variables.
The concentrations of the investigated variables that allowed the highest efficiency of the encapsulation (EE%) were determined with Statistica software. These concentrations were 3.88 % w/V and 0.15 M of pectin and CaCl$_2$, respectively. To validate the quadratic model, beads were prepared under the optimized conditions and the efficiency was 90.2 %.

**Efficiency of the lyophilization**

Beads obtained under the optimized condition of encapsulation were dried by lyophilization, with and without the addition of sucrose and passion fruit juice. All relative standard deviations were less than 0.38 % (Table 4).

<table>
<thead>
<tr>
<th>Condition of lyophilization</th>
<th>Sucrose (% w/V)</th>
<th>Pulp (% w/V)</th>
<th>EL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>-</td>
<td>-</td>
<td>83.36</td>
</tr>
<tr>
<td>L2</td>
<td>10</td>
<td></td>
<td>87.51</td>
</tr>
<tr>
<td>L3</td>
<td>10</td>
<td></td>
<td>70.45</td>
</tr>
</tbody>
</table>

All conditions of lyophilization resulted in efficiencies higher than 70 %. Higher efficiency was obtained for L3 (beads added to sucrose solution before lyophilization). The efficiency
of lyophilization without sucrose or pulp (L1) was 13 % higher than when the beads were added to the juice (20 % V/V pulp) containing sucrose (10 % V/V) before lyophilization (L2).

When sucrose was added to a pectin solution (L4), the encapsulation efficiency was similar to the L2 condition. The presence of the pulp (L2) or sucrose (L4) when added to pectin solution decreased efficiency; however, efficiency remained above 70% under these conditions. There was no significant difference between the efficiencies obtained under conditions L2 and L4 at a 95% confidence level. The difference between the L3 and L4 conditions was the sucrose addition. This difference resulted in a 17.14 % higher level for the L3 condition.

Cell viabilities of the lyophilized beads (L1, L2, L3 and L4) were measured at 0, 30, and 60 days of storage at 25 ºC to investigate the survival of *L. rhamnosus*. This evaluation aimed to investigate if lyophilized beads could be used as vehicles in some foods, with exception of the material obtained under the L2 condition. L2 was a product ready for consumption after resuspension in water, since the beads had been added to juice containing sucrose before lyophilization.

Figure 3. shows viability and survival of *L. rhamnosus* ATCC 7469 in lyophilized beads, stored at room temperature after 30 days. There was no significant difference in relation to viability or survival at a level of 95 % confidence. After 60 days, the cells were not viable under any of the four lyophilized conditions.

In the presence of sucrose and pulp (L2), or sucrose added to obtain the beads (L4), bead survivals were higher than in L1 (without pulp and without sucrose) and L3 (without pulp and with sucrose) (Fig. 3). This was interesting because this was the opposite of what had occurred with the lyophilization efficiency (Table 4). This fact is an indication that the efficiency of encapsulation should not be evaluated separately from assessment of survival during storage.
Influence of cell conditions on maintenance of probiotic juices during refrigerated storage

The lyophilized beads under conditions L1, L3 and L4 were added to the passion fruit juice and the cell viability was determined during 120 days under refrigerated storage (4 °C) for evaluation of the preservation of the probiotic juices (Figure 4). The L1* juice (cells lyophilized without pulp or sucrose, L1 condition) was more viable than the other two juices containing the lyophilized beads under conditions L3 and L4 (Figure 4). Cells obtained under the L1 condition remained viable until about 100 days of refrigerated storage in the juice (L1*). However, cells L3 and L4 remained viable only for 30 days (L3* and L4* juices). This result was similar to that observed when the lyophilized beads remained dried and without refrigeration. The difference between the beads L1, L3 and L4 was the presence of sucrose, which had been added after obtaining the beads by extrusion (L3) or in the pectin solution (L4).

Cell viability in the non-lyophilized (NL) beads remained practically constant throughout the storage period (120 days), ending at 7.28 Log CFU/mL. Free cells (FC) decreased by 2.47 Log in the first 15 days of storage, and after this period the cell viability remained constant and above 8.2 Log CFU/mL.

Figure 4. Viability of L. rhamnosus ATCC 7469 free and encapsulated, both maintained in the juice during refrigerated storage. NL – non-lyophilized beads; FC – Free cells; L1*, L3* and L4* – L1, L3 and L4 beads added to the juice, respectively.

The survival of L. rhamnosus ATCC 7469 free and encapsulated in passion fruit juice is shown in Figure 5. In the condition L1*, cell survival was 40% after 90 days of refrigerated storage. For juices containing the lyophilized L3 and L4 beads (L3* and L4*), there was no viability after 30 days. The survival of the probiotic was 92.9 % and 76.7 % for the non-lyophilized beads and free cells after 120 days, respectively (Figure 5). There was an increase of almost 20 % in the survival of free cells in relation to those encapsulated without lyophilization.
Figure 5. Survival of *L. rhamnosus* ATCC 7469 free and encapsulated, both maintained in the juice during refrigerated storage. NL – non-lyophilized beads; FC – Free cells; L1*, L3* and L4* – L1, L3 and L4 beads added to the juice, respectively.

It was observed that the storage of the lyophilized beads in the juice did not provide increase cell survival, regardless of the lyophilization conditions. In beads stored for 30 days and maintained at room temperature (L1, L2, L3 and L4), however, viability remained above 5 Log CFU/mL (survival above 70%). This is an important result, since in this period the lyophilized beads can be added to some food vehicles. To confirm this hypothesis, however, the lyophilized beads should be added to the juice up to 30 days of storage at room temperature, for further evaluation of cell viability in the refrigerated juice.

The pH decreased during the storage of free or encapsulated cells, with and without lyophilization (Table 5). The lowest decrease was observed for the conditions L3* and L4*, which also presented the lowest productivity of lactic acid. Higher productivity was obtained for free cells.

Table 5. Productivity and decrease pH during refrigerated storage of Caatinga passion fruit juice containing free cells, encapsulated with and without lyophilization.

<table>
<thead>
<tr>
<th>Passion fruit juice</th>
<th>Productivity (mg/L.d)</th>
<th>Decrease pH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC</td>
<td>10.2</td>
<td>10.0</td>
</tr>
<tr>
<td>NL</td>
<td>6.2</td>
<td>7.4</td>
</tr>
<tr>
<td>L1*</td>
<td>6.4</td>
<td>4.2</td>
</tr>
<tr>
<td>L2*</td>
<td>2.1</td>
<td>1.0</td>
</tr>
<tr>
<td>L3*</td>
<td>2.4</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Bead characterization

Non-lyophilized and lyophilized beads were characterized according to moisture, size and morphology. The non-lyophilized beads had a spherical shape, opaque in color and ranged in size from 2.72 to 3.02 mm (Figure 6a). A cross-section of a bead (x1000 magnification)
shows the integrity of *L. rhamnosus* cells entrapped within the capsule (Figure 6b). The lyophilized beads formed agglomerates after the drying process, which made it difficult to measure their size. Beads presented porous aspect and without defined format (Figure 6c).

Scanning electron microscopy images for lyophilized beads show dry beads of irregular shapes, presence of multi-cavities and wrinkled surfaces. There was no morphological variation among the four conditions of lyophilized beads (Figure 7). The moisture of the non-lyophilized beads was 97.1 % ± 0.70. For the lyophilized beads, the moisture content was 6.38 % ± 0.46, 8.38 % ± 0.62, 8.46 % ± 0.54 and 8.65 % ± 0.35 for L1, L2, L3 and L4, respectively.

**Figure 6.** Photographic images of pectin beads. (a) Direct observation; (b) Cross section of bead (x1000 magnification); (c) Lyophilized beads.
Figure 7. SEM pictures of lyophilized beads. (a) Whole capsule. Magnification 40x. (b) Cross section of bead. Magnification 100x. L1 – lyophilized beads without juice or sucrose; L2 – beads resuspended in the passion fruit juice (20 % w/V pulp ad 10 %w/v sucrose) before lyophilization; L3 – beads resuspended in sucrose (10 % w/V) solution before lyophilization; L4 – lyophilized beads contained sucrose in the pectin solution.

DISCUSSION

Comparison between encapsulation processes becomes complicated, since parameters such as diversity of microorganisms and the type of material used are too diverse to achieve good encapsulation efficiency. Eckert et al. (2018) and Vaziri et al. (2018) found efficiencies above 84 % when encapsulating Lactobacillus spp. by the extrusion method and using a
concentration of 2 % w/v pectin with coating materials. In the present work, it was possible to obtain efficiencies greater than 85 % without the need to add other coating polymers.

Fraeye et al. (2010) and Brinques et al. (2011) reported that higher pectin concentrations lead to the greater cross-linking of the pectin molecule. In the present study, we used a LM pectin and Ca\(^{2+}\) for encapsulation of *Lb rhamnosus* ATCC 7469, and greater encapsulation efficiencies were observed at intermediate values in the tested concentration range (4 % w/V pectin and 0.15 mol/L CaCl\(_2\) concentration). For lower or higher values, the encapsulation efficiency has been decreased. Ngouemazong et al. (2012) reports that for low Ca\(^{2+}\) concentrations occurs a pectin chain entanglements, while at higher Ca\(^{2+}\) concentrations occurs the formation of a cross-linked network, following an egg-box model. The according to the results of the present study, the variation in the encapsulation efficiency was independent of the CaCl\(_2\) concentration.

Freeze drying process can destroy membrane structure, as recently reported by Li and coworkers (Li et al., 2016) when studied the effects of cryoprotectants on viability of *Lactobacillus reuteri*. Sucrose is a disaccharide used as cryoprotectant agent and is related to prevent membrane damage and maintain the structure of proteins and biomolecules during the freezing stage (Leslie et al., 1995). However, in our study, sucrose did not exert the desired protective effect. The requirements for an effective cryopreservation are specific to each system, including the strain and the complexity of medium. Use of a combination of cryoprotectants agents is related as an alternative to enhance the cells survival after the freeze drying process.

Shoji et al. (2013) evaluated the survival, in yogurt of *L. acidophilus* encapsulated by coacervation, using pectin and casein, with subsequent lyophilization without cryoprotectant. The authors reported a survival of 84% after 28 days of refrigerated storage. Ribeiro et al. (2014) analyzed the survival of free and encapsulated *L. acidophilus* by extrusion (with and without lyophilization), using pectin and whey as a coating, in yogurt for 35 days. This condition was similar to L1*, but without use of a coating. The authors reported 89, 97 and 97 % survival for free and encapsulated cells with and without lyophilization, respectively. Moumita et al. (2016) analyzed the viability of *L. plantarum* and *L. bulgaricus* encapsulated by extrusion, using alginate and subsequent lyophilization and addition in two dry products. Microorganisms remained viable for only 15 days, in both dry food matrices, with survivals below 40% during this period. In this present work, the survival of *L. rhamnosus* ATCC 7469 under the L1* condition was 69.5 % for 30 days and about 40% after almost 100 days.

Nualkaekul et al. (2013) produced pectin beads (4% w/V) by extrusion method containing *L. plantarum* and reported 60 and 90% survival for free and encapsulated cells, respectively, in pomegranate juice for 30 days. Gandomi et al. (2016) encapsulated *L. rhamnosus* GG with
alginate and chitosan and obtained 15 and 85% survival for free and encapsulated cells, respectively, in apple juice after 90 days. Survival for *L. rhamnosus* ATCC 7469 was higher in non-lyophilized (99 %) beads followed by free cells (84 %) and finally in lyophilized beads (40 %) with about 100 days of refrigerated storage. The moisture values found in this work for the four lyophilized beads are within the limit defined by Albadran *et al.* (2015) for beads containing lyophilized probiotic cells, which is below 10%.

**CONCLUSION**

In this present work, it was possible to optimize the encapsulation of *L. rhamnosus* ATCC 7469 using pectin and CaCl$_2$. The lyophilization was evaluated in relation to multiple factors, such as efficiency and viability/survival during the storage. Five probiotic juices containing free, encapsulated and encapsulated/lyophilized cells were formulated based on the passion fruit from Caatinga juice. The presence of sucrose and/or pulp in the lyophilized beads of the passion fruit decreased the preservation of the probiotics juices. A better study on the influence of the cryoprotectant should be made for this strain. All probiotic juices presented stability in pH and the production of lactic acid. Products containing free and encapsulated cells could be recommended for 120 days of refrigerated storage.

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