Polímeros
ISSN 1678-5169 (Online)

https://doi.org/10.1590/0104-1428.03020

ORIGINAL ARTICLE

Investigation of *Lactobacillus paracasei* encapsulation in electrospun fibers of Eudragit® L100

Juliana Mikaelly Dias Soares[1,2], Ruan Emmanuell Franco Abreu[2], Mateus Matiuzzi da Costa[1,2], Natoniel Franklin de Melo[3] and Helinando Pequeno de Oliveira[1,2]*

[1]Rede Nordeste de Biotecnologia – RENORBIO, Universidade Federal Rural de Pernambuco – UFRPE, Recife, PE, Brasil
[2]Universidade Federal do Vale do São Francisco – UNIVASF, Petrolina, PE, Brasil
[3]Embrapa Semiárido, Petrolina, PE, Brasil
**helinando.oliveira@univasf.edu.br*

Abstract

Some species of *Lactobacillus* have demonstrated beneficial health effects being applied in the production of food supplements. Thus, the incorporation of viable cells as encapsulated probiotics represents an essential condition to be considered in new strategies for the controlled release of microorganisms. Herein, the massive encapsulation of *Lactobacillus paracasei* is provided by the use of alternative electrospinning technique. Is spite of the high voltage required for the production of fibers, a high density of viable cells is observed into the polymeric electrospun web, allowing the controlled release at targeted pH (characteristic of Eudragit® L100 polymer support). The reported procedure circumvents typical drawbacks of degradation of microorganisms under adverse conditions (storage, package and low pH) and preserves its biologic action after complete release from polymer fibers.

Keywords: *probiotics, encapsulation, electrospinning, food, Lactobacillus.*

How to cite: Soares, J. M. D., Abreu, R. E. F., Costa, M. M., Melo, N. F., & Oliveira, H. P. (2020). Investigation of *Lactobacillus paracasei* encapsulation in electrospun fibers of Eudragit® L100. *Polímeros: Ciência e Tecnologia*, *30*(3), e2020025. https://doi.org/10.1590/0104-1428.03020

1. Introduction

Some probiotic bacteria have positive physiological effects and have been considered as important components for the production of foods supplements[1,2]. In particular, *Lactobacillus* spp. has been considered as a promising probiotic that confers health benefits to the host. Recent studies considered the use of *Lactobacillus* spp. in the prevention and treatment of inflammatory bowel disease[3,4], food hypersensitivity[5], cardiometabolic disorders[6] and anti-tumor activity[7].

However, it has also been reported that bioactive living cells, such as the *Lactobacillus* spp. present low bioavailability/ biofunctionality as a consequence of transport through the gastrointestinal tract, in the processing and/or prolonged storage[8,9]. The acidic medium can induce changes on bacterial membrane components, modifying and disturbing the peptidoglycan components, lipids, proteins and DNA in Gram-positive bacteria[10].

As a consequence, the survivability and colonization in the digestive tract are considered critical to ensure optimal functionality of *Lactobacillus* species[11]. The poor survival rate of bioactive cells can be attributed to environmental conditions such as acidic medium, the toxicity of oxygen and UV light[12-16]. Healthy-promoting effects of probiotics are extremely dependent on cell viability degree and the concentration of living cells as high as 10^9 CFU/ day for administration[17]. To circumvent the drawbacks related to low shelf-life of food products and adverse conditions at the acidic environment (stomach/ bile salts) the creation of an anaerobic environment for probiotics growth received increased attention in the literature with promising strategies to maintain the viability of cells until to reach the colon lumen. These encapsulation strategies are based on the production of fruit bubles[18], nanoencapsulation by electrospinning[17,19] and by the production of microcapsules[20,21].

The electrospinning technique has been drawing attention in the encapsulation of *Lactobacillus* spp.[22-24]. Despite the adverse conditions from experimental setup (high voltage and the nature of organic solvents) – that could be harmful to remaining viable cells in culture, studies are reporting that *Lactobacillus*-loaded electrospun fibers may preserve metabolic activity, with increased stability and protection[25-28].

The basic experimental setup for electrospinning production requires the dispersion of additives (molecules of interest) in a polymeric solution to be incorporated in a compartment (a syringe) kept at a fixed pressure. The needle in the syringe is connected to a high voltage source and depending on a series of factors (such as the distance of dip of the syringe and the grounded target, density of the solution, the intensity of the electric field, local humidity and infusion rate) the production of the fibers takes place[29,30]. Under an adequate

combination of parameters, the atomization of a polymeric solution results in fibers with the diameter ranging from micrometer scale to nanometer scale[31,32].

The nature of the polymeric support is a critical parameter applied in the determination of the density and physical-chemical properties of the resulting fibers. The family Eudragit® is a commercial group of pH-dependent block polymer materials that presents important properties for encapsulation and release of active molecules at targeted pH, avoiding side effects for adsorption of drugs at low pH conditions in the organism. In particular, the Eudragit® L100 is an anionic methacrylic acid and methyl methacrylate copolymer which presents dependent solubility, with rapid dissolution in the upper intestine (pH ≥ 6)[33], that protects the encapsulated species from degradation in the stomach.

The fast dissolution of the polymeric matrix at high pH can be explored as an alternative strategy to protect probiotics against adverse conditions in the stomach. Despite the conventional use of enteric polymers of this family in the form of microparticles for encapsulation, it is observed an important possibility of use of the matrix as support for electrospinning – as a consequence, the massive production of matrix encapsulating species can be reached.

Thus, the aim of this work was to developing electrospun fibers of enteric polymers for the encapsulation of *L. paracasei*. Several studies have already demonstrated efficient encapsulation of *Lactobacillus*-loaded electrospun fibers[22,26,28,34,35]. However, we have explored a simple procedure for encapsulation of probiotics in electrospun fibers from Eudragit® L100 polymer solution in alcohol. The successful encapsulation of microorganisms into this matrix represents a step forward in the direction of the massive production of encapsulated probiotics with well-defined targeted pH for release.

2. Materials and Methods

2.1 Materials

The *Lactobacillus paracasei* probiotic strain was isolated from silage composed of elephant grass (*Pennisetum purpureum* cv. *Cameroon*) plus grape residue and had its identification confirmed by previous 16S rDNA sequencing. The microorganisms were kept in culture medium plates containing Rogosa and Sharpe Agar in anaerobic conditions for 48 hours at 37 °C[36]. The strain was phenotypically and genotypically characterized[37] by sequencing the 16S rRNA gene[38]. Eudragit® L100 was donated from Evonik, alcohol and sodium alginate were purchased from Sigma-Aldrich and MRS broth and agar from Neogen. Potassium monobasic phosphate and sodium hydroxide were purchased from *Vetec* Quimica Fina Ltda. Ultrapure water was obtained by the Milli-Q® equipment.

2.2 Microorganisms growth conditions

The cultures of *L. paracasei* were prepared by transfer into MRS broth cultures and then incubated anaerobically at 37 °C for 48 h, as reported in the literature with some modifications[39]. Following the incubation step, the media-containing cells were centrifuged at 5,000 rpm for 10 min at 10 °C, after that the supernatant was removed and the cells were further washed twice in sterile simulated intestinal fluid solution, with centrifugation after each step. The washed cells were suspended in sterile simulated intestinal fluid solution and stored for later use.

2.3 Preparation of electrospun fibers

Polymeric solutions were prepared from dispersion of 0.4 g of Eudragit® L100 and 2% of sodium alginate (w/v) in 2 mL of alcohol. After that, 500 µL of suspended *L. paracasei* in sterile simulated intestinal solution was added in the previous solution. Solutions without and with *L. paracasei* were loaded into 5 mL syringes fitted with a capillary (metal needle), which was mounted horizontally on a syringe pump.

The electrode at high-voltage power supply was clamped to the capillary and an aluminum plate was used as a collector was grounded. The voltage of 15 kV was established with the nozzle-to-collector distance of 20 cm and the flow rate of 1.0 mL/h. The resulting samples were: electrospun fibers of Eudragit® L100 (EDGT) and electrospun fibers with *L. paracasei (*EDGT-*L.paracasei).*

2.4 Characterization of electrospun fibers morphology

Scanning Electron Microscopy (SEM) was performed in an SEM Vega 3XM Tescan. The electrospun fibers of EDGT and EDGT-*L.paracasei* were examined from SEM and the mean diameter was measured using the ImageJ from 25 electrospun fibers randomly selected.

2.5 Fourier transform infrared spectroscopy

Fourier Transform Infra-Red (FTIR) analysis was performed using an IR Prestige-21 FTIR Shimadzu by KBr method in the range of 4000 cm^{-1} - 500 cm^{-1}. The FTIR spectra were used to identify the influence of *L. paracasei* in the overall structure of electrospun fibers.

2.6 Viability of L. paracasei in Eudragit® L100 electrospun fibers

The viabilities of the *L. paracasei* cells in electrospun fibers were determined from the dissolution of the fibers into sterile simulated intestinal fluid and then plating in MRS broth and agar. The sterile simulated intestinal fluid solution was prepared with potassium monobasic phosphate, sodium hydroxide and ultrapure water with pH adjustment to 6.8. All assays were performed in duplicate.

2.7 Acridine Orange/DAPI staining and data analysis

The cells of *L. paracasei* encapsulated in electrospun fibers were stained with Acridine Orange - AO (1 mg/mL) for 20 min, which was mounted in a glass slide for posterior analysis under a fluorescence microscope. The material (cells and/or electrospun fibers) were analyzed using a Leica DM2000 epifluorescence microscope with a set of four filter cubes (A, L5, N3 and E4) and the images were captured with a Leica FX-350 camera using Leica QFish software.

3. Results and Discussions

The morphology of *L. paracasei*, electrospun fibers of Eudragit® L100 (EDGT) and electrospun fibers with *L. paracasei (*EDGT-*L.paracasei)* were compared from SEM images, as shown in Figure 1 – the diameter was calculated from 25 different fibers per image. The EDGT fibers prepared in the absence of *L. paracasei* were uniform (with no imperfection) and presented a mean diameter of (2.134 ± 0.4127 µm) (Figure 1b). The morphology of pristine (non-encapsulated *L. paracasei*) is characterized by cells with an average length of (1.536 ± 0.370) µm and an average width of (0.526 ± 0.068) µm (Figure 1a). The incorporation of *L. paracasei* into electrospun fibers is followed by the formation of imperfections localized along with the structure. As can be seen in Figure 1c, it is possible to identify aggregates of cells along with the polymeric structure.

The EDGT-*L.paracasei* presented an average diameter of 1.508 ± 0.477 µm (Figure 1a). The EDGT-*L. paracasei*-loaded presented a reduction in diameter size (Figure 1d) and that the aggregates correspond to several *L. paracasei* cells, which individually evaluated show the same average diameter of *L. paracasei* (0.51 to 0.77 µm) (Figure 1d).

The decrease in the average diameter of EDGT-*L. paracasei* samples may be due to the viscosity modification that results in reduced finer fiber size. Fung et al.[23] reported that PVA-based electrospun fibers with agrowaste containing

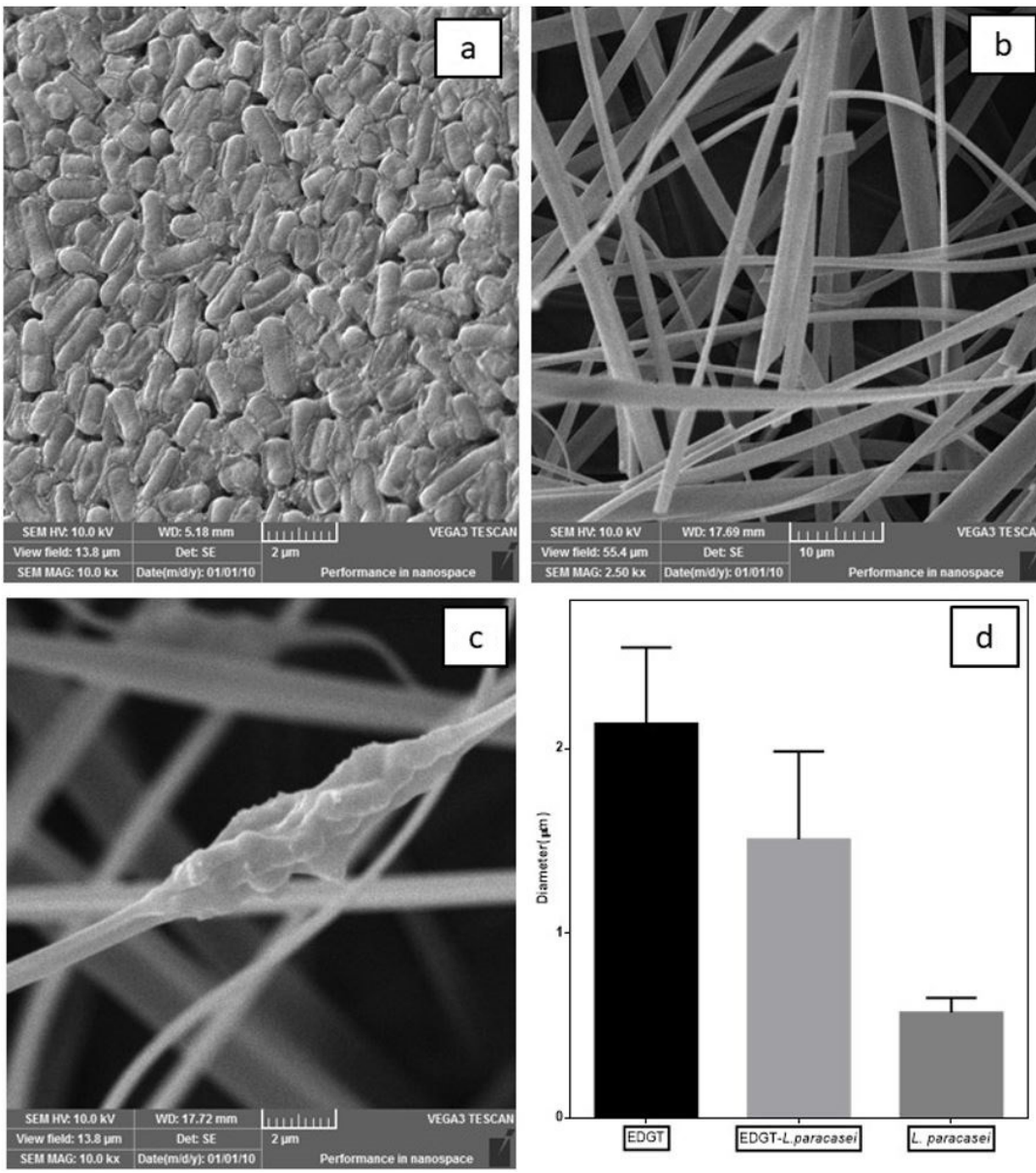

Figure 1. Scanning electron microscopy images are shown for (a), *L. paracasei* (L) (b), EDGT (c) EDGT-*L.paracasei* and (d) average diameter EDGT, EDGT-*L.paracasei* and *L. paracasei* – *calculated from sets of 25 different fibers per SEM image.*

L. acidophilus have decreased in mean diameter size by comparison with electrospun neat fibers due to the higher viscosity of pristine solution.

An important aspect to be reported from these images is that there are no *L. paracasei* cells on the surface of electrospun fibers, in agreement with reported in the literature[27,28,40]. According to Heunis et al.[26], the high voltage applied during the electrospinning process inhibits bioactive cells not included in the electrospun fibers (non-protected species) that are prone to destruction in consequence of applied high voltage. In Figure 1c it is possible to observe that the *L. paracasei* cells were concentrated into the electrospun fibers as also randomly oriented along the electrospun fibers, such as reported in the literature[22,28].

In terms of the FTIR responses sample, it is possible to observe the presence of the peaks at 3507 cm^{-1} as a response of free carboxylic acid form and 3000 cm^{-1} and 2957 cm^{-1} assigned to vibrations CH_x, 1723 cm^{-1} for esterified carboxylic groups and 1162 cm^{-1} given by carboxylic acid ester bonds stretching vibrations[41-43], see Figure 2.

The spectrum of *L. paracasei* shows FTIR at positions that are in agreement with reported in the literature with the fingerprint of *Lactobacillus* spp. for peaks between 1300 and 900 cm^{-1} that indicate specific vibrational features of nucleic acids and bacterial proteins[40,44,45].

For samples EDGT-*L. paracasei*, the FTIR spectrum is characterized by peaks of the Eudragit® L100 polymer that stands out above the *L. paracasei* peaks. Ceylan et al. [24] reported similar results in which the peak assignments of each electrospun fiber component (pristine electrospun fibers and *Lactobacillus* loaded electrospun fibers) were highly similar to the individual components. Other studies have also reported the hardness visualization of *Lactobacillus* spp. peaks in electrospun fibers due to the complex overlap of polymer peaks and/or additives, showing that the evidence of encapsulation of *Lactobacillus* spp. was supported by the SEM images and fluorescence microscopies[28,40].

The staining procedure for the detection of cells (viable and killed organisms) is based on the interaction of fluorochromes and cells for the following identification of fluorescence levels in microscopy images[46]. AO and 4"

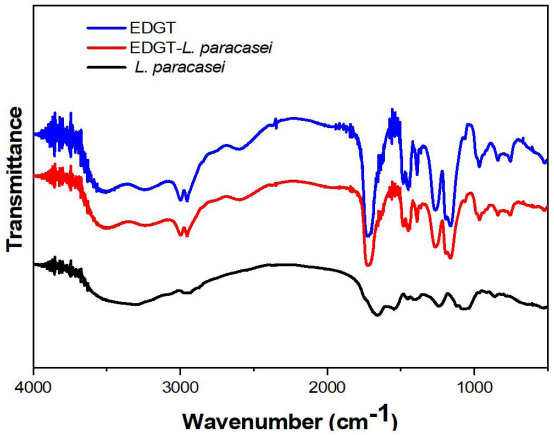

Figure 2. FTIR of EDGT, EDGT-*L.paracasei* and pristine *L. paracasei*.

6-diamidino-2-phenylindole (DAPI) are two of the most used fluorochromes in microbiology[47]. Particularly, the AO at low concentration binds with RNA and allows that microorganisms at high growth rates present high fluorescence at the red-orange region. On the contrary, for death cells, abundant DNA binds with AO and shifts the emission to the green region[48]. Based on these properties, it is possible to differentiate active cells´ signature (RNA fluorescence) from dead cells signature (prevailing DNA fluorescence)[49,50].

Assays of fluorescence microscopy were performed using AO and are summarized in Figure 3. The presence of free *L. paracasei* (viable cells) (from the fluorescence of AO at red region) can be visualized in Figure 3a. As shown, strong fluorescence reveals the viable character of cells before incorporation into the polymer solution. After encapsulation of microorganisms into electrospun fibers, it is possible to identify the presence of viable cells in fibers from strong red fluorescent dots (shown in Figures 3b, 3c and 3d) characterizing by the interaction of prevailing RNA of the viable cells and AO[50].

The fluorescence images are in agreement with previous SEM images that shown organisms dispersed as aggregates at specific sites of the fibers. The strong fluorescence reached form interaction with fluorochrome confirms the presence of viable cells encapsulated in electrospun fibers. It can be attributed to a covering layer of polymer that protects cells against the effects of high voltage and allows that a reasonable number of cells remain active after encapsulation.

The confirmation of the presence of viable *L. paracasei* in the Eudragit® L100 electrospun fibers is already considered a notable result because to the best of our knowledge, there is no literature reporting the encapsulating of probiotics in electrospun fibers derived from Eudragit® L100 polymer solution in alcohol. Therefore, it is essential to test whether encapsulated *L. paracasei* are viable after fiber production and collection.

For this, the *L. paracasei*-loaded Eudragit® L100 electrospun fibers were dissolved in the intestinal fluid solution for later plating. In the Figure 4 it is possible to see the growth of *L. paracasei* cells after electrospinning. Thereby, immediately after electrospinning of electrospun fibers still contained a high number of active *L. paracasei* as the survival rate of the viable cells, which is a remarkable result compared to the adverse effects of the electrospinning process and also of the polymeric solution used and tested for the first time for encapsulation of viable cells.

The initial amount of *L. paracasei* added was 8.705 (log (CFU/mL)) and after electrospinning was 5.837 (log (CFU/mL)). The remaining viable cells confirm that technique and polymer template can be explored to encapsulate *Lactobacillus* species. Heunis et al.[26] assert that encapsulation in electrospun fibers has a high viability rate.

Thus, electrospinning has been demonstrating a very useful and advantageous technique for the encapsulation of viable cells[22,27,34]. One of the factors may also have compensated the loss in viability that is the low diameter value of the fibers that favors the high surface to volume ratio of resulting material[26]. Besides that, the process of electrospinning may exclude environmental oxygen,

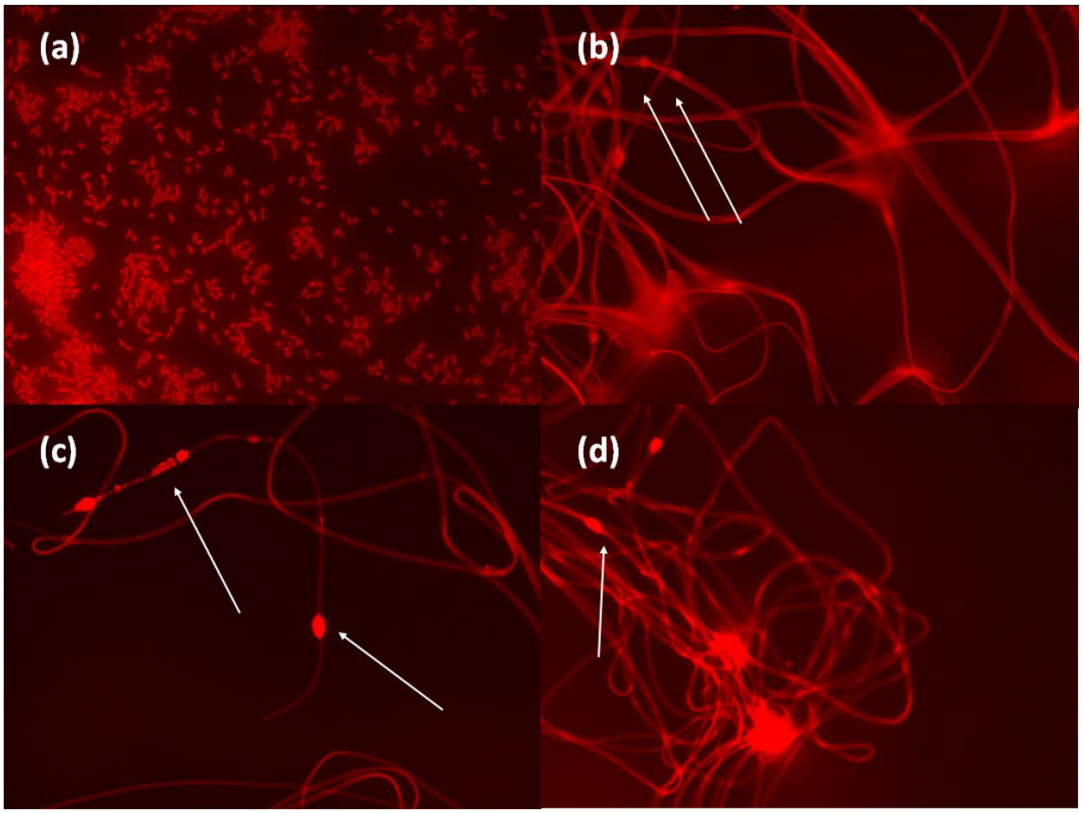

Figure 3. Fluorescence images for samples: (a) *L. paracasei* treated with AO, (b, c and d) EDGT-*L.paracasei* treated with AO.

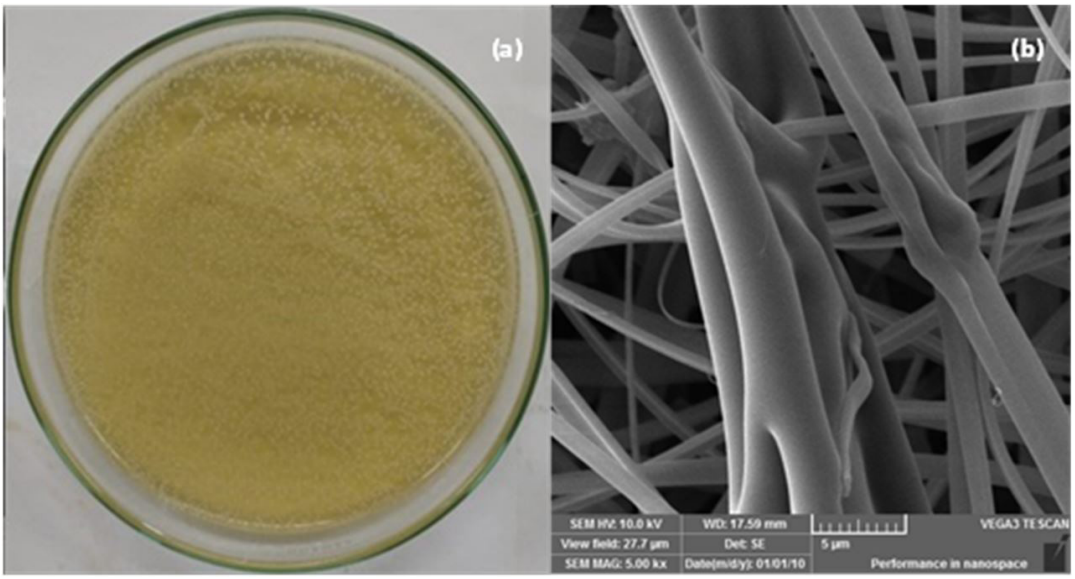

Figure 4. Growth of *L. paracasei* cells (a) after the release of encapsulated species loaded on electrospun fibers (b).

contributing to the stability of *Lactobacillus* species[23]. Thus, although the decrease in water activity decreases during the electrospinning, a reduced oxygen level can improve storage stability[51].

In general, the effects observed in the literature for encapsulation of probiotics refer to the increase in the number of viable cells at prolonged contact with the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)[21]. Mojaveri et al.[19] reported that nanoencapsulation by electrospinning affects the number of viable cells (in 1-log reduction) as a result of extreme conditions (high electric field for synthesis). Despite this effect, the strong protection

provided by the fibers improves the viability rate of cells under SIF and SGF conditions. While a 3-log reduction is observed for free cells in an extreme environment, the production of the electrospun fibers reduces the number of viable cells from 8.37-8.44 log CFU/mL to 7.25-7.31 CFU/mL, revealing the potential of electrospun fibers.

As shown from comparison with data reported in the literature, superior performance in terms of survivability degree of cells during the electrospun procedure and the lower reduction in the viable cells at SGF/ SIF conditions is reached for binary systems, such as introduced as Yilmaz et al.[17] that associated conventional polymer matrix and sodium alginate.

The improvement in the survivability for experimental systems based on EDGT-based electrospun mats depends on adequate interaction of probiotics and polymer support with a third component that can be an alginate polymer, that acts as an extra layer to protect cells against high electric field and an barrier for controlled release under specific pH. The incorporation of additives for electrospun mats represents an important trend for this work, in a posterior step that tends to improve not only the retention of viable cells under electrospinning but also at adverse conditions (low pH) for long time assays.

4. Conclusions

The electrospinning technique demonstrated to be a successful strategy applied in the encapsulation of *L. paracasei* by Eudragit® L100. Although some factors interfere in cell viability, the results revealed that Eudragit® L100 electrospun fibers offer a hydrophobic environment that provides adequate protection of *L. paracasei* cells against oxygen – preserving its viability. Viable cells were identified by fluorescence microscopy before and after release from controlled conditions, confirming that strategy of encapsulation of probiotics in enteric polymer-based electrospun fibers has been successfully established under acidic pH for following the release of viable cells – that remain protected against adverse conditions – and optimizing the characteristics of probiotics for prolonged action. In summary, these findings open new possibilities for use of a simple experimental system (alcoholic solution of EDGT) for encapsulation of probiotics in a promising template that can be enriched by the incorporation of additives such as sodium alginate in binary electrospun mats.

5. Acknowledgements

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES, Financiadora de Estudos e Projetos – FINEP, Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq and Fundação de Amparo a Ciência e Tecnologia do Estado de Pernambuco – FACEPE.

6. References

1. Akman, P. K., Uysal, E., Ozkaya, G. U., Tornuk, F., & Durak, M. Z. (2019). Development of probiotic carrier dried apples for consumption as snack food with the impregnation of *Lactobacillus paracasei*. *Lebensmittel-Wissenschaft + Technologie*, *103*, 60-68. http://dx.doi.org/10.1016/j.lwt.2018.12.070.
2. Wu, Z., Wu, J., Cao, P., Jin, Y., Pan, D., Zeng, X., & Guo, Y. (2017). Characterization of probiotic bacteria involved in fermented milk processing enriched with folic acid. *Journal of Dairy Science*, *100*(6), 4223-4229. http://dx.doi.org/10.3168/jds.2017-12640. PMid:28434721.
3. Le, B., & Yang, S. H. (2018). Efficacy of *Lactobacillus plantarum* in prevention of inflammatory bowel disease. *Toxicology Reports*, *5*, 314-317. http://dx.doi.org/10.1016/j.toxrep.2018.02.007. PMid:29854599.
4. He, D., Wang, Y., Lin, J., Xing, Y.-F., Zeng, W., Zhu, W.-M., Su, N., Zhang, C., Lu, Y., & Xing, X.-H. (2020). Identification and characterization of alcohol-soluble components from wheat germ-apple fermented by Lactobacillus sp. capable of preventing ulcerative colitis of dextran sodium sulfate-induced mice. *Journal of Functional Foods*, *64*, 103642. http://dx.doi.org/10.1016/j.jff.2019.103642.
5. Santos, S. C., Konstantyner, T., & Cocco, R. R. (2020). Effects of probiotics in the treatment of food hypersensitivity in children: a systematic review. *Allergologia et Immunopathologia*, *48*(1), 95-104. http://dx.doi.org/10.1016/j.aller.2019.04.009. PMid:31477401.
6. Cavalcante, R. G. S., Albuquerque, T. M. R., Luna Freire, M. O., Ferreira, G. A. H., Carneiro dos Santos, L. A., Magnani, M., Cruz, J. C., Braga, V. A., Souza, E. L., & Brito Alves, J. L. (2019). The probiotic *Lactobacillus fermentum* 296 attenuates cardiometabolic disorders in high fat diet-treated rats. *Nutrition, Metabolism, and Cardiovascular Diseases*, *29*(12), 1408-1417. http://dx.doi.org/10.1016/j.numecd.2019.08.003. PMid:31640890.
7. Riaz Rajoka, M. S., Mehwish, H. M., Fang, H., Padhiar, A. A., Zeng, X., Khurshid, M., He, Z., & Zhao, L. (2019). Characterization and anti-tumor activity of exopolysaccharide produced by *Lactobacillus kefiri* isolated from Chinese kefir grains. *Journal of Functional Foods*, *63*, 103588. http://dx.doi.org/10.1016/j.jff.2019.103588.
8. Vos, P., Faas, M. M., Spasojevic, M., & Sikkema, J. (2010). Encapsulation for preservation of functionality and targeted delivery of bioactive food components. *International Dairy Journal*, *20*(4), 292-302. http://dx.doi.org/10.1016/j.idairyj.2009.11.008.
9. Kailasapathy, K. (2009). Encapsulation technologies for functional foods and nutraceutical product development. *Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, *4*(33), 1-19. http://dx.doi.org/10.1079/PAVSNNR20094033.
10. Sanhueza, E., Paredes-Osses, E., González, C. L., & García, A. (2015). Effect of pH in the survival of *Lactobacillus salivarius* strain UCO_979C wild type and the pH acid acclimated variant. *Electronic Journal of Biotechnology*, *18*(5), 343-346. http://dx.doi.org/10.1016/j.ejbt.2015.06.005.
11. Kaushik, J. K., Kumar, A., Duary, R. K., Mohanty, A. K., Grover, S., & Batish, V. K. (2009). Functional and probiotic attributes of an indigenous isolate of *Lactobacillus plantarum*. *PLoS One*, *4*(12), e8099. http://dx.doi.org/10.1371/journal.pone.0008099. PMid:19956615.
12. Xu, M., Gagné-Bourque, F., Dumont, M.-J., & Jabaji, S. (2016). Encapsulation of Lactobacillus casei ATCC 393 cells and evaluation of their survival after freeze-drying, storage and under gastrointestinal conditions. *Journal of Food Engineering*, *168*, 52-59. http://dx.doi.org/10.1016/j.jfoodeng.2015.07.021.
13. Silva, T. M., Deus, C., Souza Fonseca, B., Lopes, E. J., Cichoski, A. J., Esmerino, E. A., Bona da Silva, C., Muller, E. I., Moraes Flores, E. M., & Menezes, C. R. (2019). The effect of enzymatic crosslinking on the viability of probiotic

bacteria (*Lactobacillus acidophilus*) encapsulated by complex coacervation. *Food Research International*, *125*, 108577. http://dx.doi.org/10.1016/j.foodres.2019.108577. PMid:31554127.

14. Dimitrellou, D., Kandylis, P., Lević, S., Petrović, T., Ivanović, S., Nedović, V., & Kourkoutas, Y. (2019). Encapsulation of *Lactobacillus casei* ATCC 393 in alginate capsules for probiotic fermented milk production. *Lebensmittel-Wissenschaft + Technologie*, *116*, 108501. http://dx.doi.org/10.1016/j.lwt.2019.108501.
15. Yucel Falco, C., Amadei, F., Dhayal, S. K., Cardenas, M., Tanaka, M., & Risbo, J. (2019). Hybrid coating of alginate microbeads based on protein-biopolymer multilayers for encapsulation of probiotics. *Biotechnology Progress*, *35*(3), e2806. http://dx.doi.org/10.1002/btpr.2806. PMid:30884190.
16. Librán, C. M., Castro, S., & Lagaron, J. M. (2017). Encapsulation by electrospray coating atomization of probiotic strains. *Innovative Food Science & Emerging Technologies*, *39*, 216-222. http://dx.doi.org/10.1016/j.ifset.2016.12.013.
17. Yilmaz, M. T., Taylan, O., Karakas, C. Y., & Dertli, E. (2020). An alternative way to encapsulate probiotics within electrospun alginate nanofibers as monitored under simulated gastrointestinal conditions and in kefir. *Carbohydrate Polymers*, *244*, 116447. http://dx.doi.org/10.1016/j.carbpol.2020.116447. PMid:32536387.
18. Tanganurat, P. (2020). Probiotics encapsulated fruit juice bubbles as functional food product. *International Journal of GEOMATE*, *19*(72), 145-150. http://dx.doi.org/10.21660/2020.72.5640.
19. Mojaveri, S. J., Hosseini, S. F., & Gharsallaoui, A. (2020). Viability improvement of Bifidobacterium animalis Bb12 by encapsulation in chitosan/poly(vinyl alcohol) hybrid electrospun fiber mats. *Carbohydrate Polymers*, *241*, 116278. http://dx.doi.org/10.1016/j.carbpol.2020.116278. PMid:32507203.
20. Alfaro-Galarza, O., López-Villegas, E. O., Rivero-Perez, N., Tapia- Maruri, D., Jiménez-Aparicio, A. R., Palma-Rodríguez, H. M., & Vargas-Torres, A. (2020). Protective effects of the use of taro and rice starch as wall material on the viability of encapsulated *Lactobacillus paracasei subsp. Paracasei. Lebensmittel-Wissenschaft + Technologie*, *117*, 108686. http://dx.doi.org/10.1016/j.lwt.2019.108686.
21. Akanny, E., Bourgeois, S., Bonhommé, A., Commun, C., Doleans-Jordheim, A., Bessueille, F., & Bordes, C. (2020). Development of enteric polymer-based microspheres by spray-drying for colonic delivery of *Lactobacillus rhamnosus GG. International Journal of Pharmaceutics*, *584*, 119414. http://dx.doi.org/10.1016/j.ijpharm.2020.119414. PMid:32438040.
22. Nagy, Z. K., Wagner, I., Suhajda, Á., Tobak, T., Harasztos, A. H., Vigh, T., Sóti, P. L., Pataki, H., Molnár, K., & Marosi, G. (2014). Nanofibrous solid dosage form of living bacteria prepared by electrospinning. *Express Polymer Letters*, *8*(5), 352-361. http://dx.doi.org/10.3144/expresspolymlett.2014.39.
23. Fung, W. Y., Yuen, K. H., & Liong, M. T. (2011). Agrowaste-based nanofibers as a probiotic encapsulant: fabrication and characterization. *Journal of Agricultural and Food Chemistry*, *59*(15), 8140-8147. http://dx.doi.org/10.1021/jf2009342. PMid:21711050.
24. Ceylan, Z., Meral, R., Karakaş, C. Y., Dertli, E., & Yilmaz, M. T. (2018). A novel strategy for probiotic bacteria: ensuring microbial stability of fish fillets using characterized probiotic bacteria-loaded nanofibers. *Innovative Food Science & Emerging Technologies*, *48*, 212-218. http://dx.doi.org/10.1016/j.ifset.2018.07.002.
25. Liu, Y., Rafailovich, M. H., Malal, R., Cohn, D., & Chidambaram, D. (2009). Engineering of bio-hybrid materials by electrospinning polymer-microbe fibers. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(34), 14201-14206. http://dx.doi.org/10.1073/pnas.0903238106. PMid:19667172.
26. Heunis, T. D., Botes, M., & Dicks, L. M. (2010). Encapsulation of *Lactobacillus plantarum* 423 and its bacteriocin in nanofibers. *Probiotics and Antimicrobial Proteins*, *2*(1), 46-51. http://dx.doi.org/10.1007/s12602-009-9024-9. PMid:26780900.
27. Lancuški, A., Abu Ammar, A., Avrahami, R., Vilensky, R., Vasilyev, G., & Zussman, E. (2017). Design of starch-formate compound fibers as encapsulation platform for biotherapeutics. *Carbohydrate Polymers*, *158*, 68-76. http://dx.doi.org/10.1016/j.carbpol.2016.12.003. PMid:28024544.
28. Škrlec, K., Zupančič, Š., Prpar Mihevc, S., Kocbek, P., Kristl, J., & Berlec, A. (2019). Development of electrospun nanofibers that enable high loading and long-term viability of probiotics. *European Journal of Pharmaceutics and Biopharmaceutics*, *136*, 108-119. http://dx.doi.org/10.1016/j.ejpb.2019.01.013. PMid:30660693.
29. Hu, X., Liu, S., Zhou, G., Huang, Y., Xie, Z., & Jing, X. (2014). Electrospinning of polymeric nanofibers for drug delivery applications. *Journal of Controlled Release*, *185*, 12-21. http://dx.doi.org/10.1016/j.jconrel.2014.04.018. PMid:24768792.
30. Correia, D. M., Ribeiro, C., Botelho, G., Borges, J., Lopes, C., Vaz, F., Carabineiro, S. A. C., Machado, A. V., & Lanceros-Méndez, S. (2016). Superhydrophilic poly(l-lactic acid) electrospun membranes for biomedical applications obtained by argon and oxygen plasma treatment. *Applied Surface Science*, *371*, 74-82. http://dx.doi.org/10.1016/j.apsusc.2016.02.121.
31. Wu, J., & Hong, Y. (2016). Enhancing cell infiltration of electrospun fibrous scaffolds in tissue regeneration. *Bioactive Materials*, *1*(1), 56-64. http://dx.doi.org/10.1016/j.bioactmat.2016.07.001. PMid:29744395.
32. Saallah, S., Naim, M. N., Lenggoro, I. W., Mokhtar, M. N., Abu Bakar, N. F., & Gen, M. (2016). Immobilisation of cyclodextrin glucanotransferase into polyvinyl alcohol (PVA) nanofibres via electrospinning. *Biotechnology Reports*, *10*, 44-48. http://dx.doi.org/10.1016/j.btre.2016.03.003. PMid:28352523.
33. Moustafine, R. I. (2014). Role of macromolecular interactions of pharmaceutically acceptable polymers in functioning oral drug delivery systems. *Russian Journal of General Chemistry*, *84*(2), 364-367. http://dx.doi.org/10.1134/S1070363214020388.
34. Ceylan, Z., Uslu, E., İspirli, H., Meral, R., Gavgalı, M., Yilmaz, M. T., & Dertli, E. (2019). A novel perspective for *Lactobacillus reuteri*: nanoencapsulation to obtain functional fish fillets. *Lebensmittel-Wissenschaft + Technologie*, *115*, 108427. http://dx.doi.org/10.1016/j.lwt.2019.108427.
35. Hu, M. X., Li, J. N., Guo, Q., Zhu, Y. Q., & Niu, H. M. (2019). Probiotics biofilm-integrated electrospun nanofiber membranes: a new starter culture for fermented milk production. *Journal of Agricultural and Food Chemistry*, *67*(11), 3198-3208. http://dx.doi.org/10.1021/acs.jafc.8b05024. PMid:30838858.
36. Cai, Y. (1999). Identification and characterization of *Enterococcus* species isolated from forage crops and their influence on silage fermentation. *Journal of Dairy Science*, *82*(11), 2466-2471. http://dx.doi.org/10.3168/jds.S0022-0302(99)75498-6. PMid:10575614.
37. Almeida, W. L. G., Jr., Ferrari, Í. S., Souza, J. V., Silva, C. D. A., Costa, M. M., & Dias, F. S. (2015). Characterization and evaluation of lactic acid bacteria isolated from goat milk. *Food Control*, *53*, 96-103. http://dx.doi.org/10.1016/j.foodcont.2015.01.013.
38. Fredricks, D. N., & Relman, D. A. (1998). Improved amplification of microbial DNA from blood cultures by removal of the PCR inhibitor sodium polyanetholesulfonate. *Journal of Clinical Microbiology*, *36*(10), 2810-2816. http://dx.doi.org/10.1128/JCM.36.10.2810-2816.1998. PMid:9738025.
39. Gudiña, E. J., Rocha, V., Teixeira, J. A., & Rodrigues, L. R. (2010). Antimicrobial and antiadhesive properties of a biosurfactant isolated from *Lactobacillus paracasei* ssp.

paracasei A20. *Letters in Applied Microbiology*, *50*(4), 419-424. http://dx.doi.org/10.1111/j.1472-765X.2010.02818.x. PMid:20184670.

40. Feng, K., Zhai, M. Y., Zhang, Y., Linhardt, R. J., Zong, M. H., Li, L., & Wu, H. (2018). Improved viability and thermal stability of the probiotics encapsulated in a novel electrospun fiber. *Journal of Agricultural and Food Chemistry*, *66*(41), 10890-10897. http://dx.doi.org/10.1021/acs.jafc.8b02644. PMid:30260640.
41. Araújo, E. S., Costa, B. P., Oliveira, R. A. P., Libardi, J., Faia, P. M., & Oliveira, H. P. (2016). TiO2/ZnO hierarchical heteronanostructures: synthesis, characterization and application as photocatalysts. *Journal of Environmental Chemical Engineering*, *4*(3), 2820-2829. http://dx.doi.org/10.1016/j.jece.2016.05.021.
42. Santos, T. M. M., Oliveira, P. H., Jr., Ribeiro, L. A. A., & Oliveira, H. P. (2014). Drug/magnetite - loaded enteric particles: the influence of localized magnetic field on controlled release of nifedipine. *Asian Journal of Biochemical and Pharmaceutical Research*, *4*(1), 63-71. Retrieved in 2020, March 13, from https://saepub.com/acc.php?journal_name=AJBPR&volume=4&issue=1
43. Illangakoon, U. E., Yu, D. G., Ahmad, B. S., Chatterton, N. P., & Williams, G. R. (2015). 5-Fluorouracil loaded Eudragit fibers prepared by electrospinning. *International Journal of Pharmaceutics*, *495*(2), 895-902. http://dx.doi.org/10.1016/j.ijpharm.2015.09.044. PMid:26410755.
44. Shah, A., Gani, A., Ahmad, M., Ashwar, B. A., & Masoodi, F. A. (2016). β-Glucan as an encapsulating agent: effect on probiotic survival in simulated gastrointestinal tract. *International Journal of Biological Macromolecules*, *82*, 217-222. http://dx.doi.org/10.1016/j.ijbiomac.2015.11.017. PMid:26562556.
45. Vodnar, D. C., Socaciu, C., Rotar, A. M., & Stãnilã, A. (2010). Morphology, FTIR fingerprint and survivability of encapsulated lactic bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) in simulated gastric juice and intestinal juice. *International Journal of Food Science & Technology*, *45*(11), 2345-2351. http://dx.doi.org/10.1111/j.1365-2621.2010.02406.x.
46. Karwoski, M., Venelampi, O., Linko, P., & Mattila-Sandholm, T. (1995). A staining procedure for viability assessment of starter culture cells. *Food Microbiology*, *12*, 21-29. http://dx.doi.org/10.1016/S0740-0020(95)80075-1.
47. Seo, E.-Y., Ahn, T.-S., & Zo, Y.-G. (2010). Agreement, precision, and accuracy of epifluorescence microscopy methods for enumeration of total bacterial numbers. *Applied and Environmental Microbiology*, *76*(6), 1981-1991. http://dx.doi.org/10.1128/AEM.01724-09. PMid:20097826.
48. Yu, W., Dodds, W. K., Banks, M. K., Skalsky, J., & Strauss, E. A. (1995). Optimal staining and sample storage time for direct microscopic enumeration of total and active bacteria in soil with two fluorescent dyes. *Applied and Environmental Microbiology*, *61*(9), 3367-3372. http://dx.doi.org/10.1128/AEM.61.9.3367-3372.1995. PMid:16535124.
49. Cao-Hoang, L., Marechal, P. A., Le-Thanh, M., Gervais, P., & Wache, Y. (2008). Fluorescent probes to evaluate the physiological state and activity of microbial biocatalysts: a guide for prokaryotic and eukaryotic investigation. *Biotechnology Journal*, *3*(7), 890-903. http://dx.doi.org/10.1002/biot.200700206. PMid:18481263.
50. Powless, A. J., Prieto, S. P., Gramling, M. R., Conley, R. J., Holley, G. G., & Muldoon, T. J. (2019). Evaluation of acridine orange staining for a semi-automated urinalysis microscopic examination at the point-of-care. *Diagnostics*, *9*(3), 122. http://dx.doi.org/10.3390/diagnostics9030122. PMid:31540364.
51. Kurtmann, L., Carlsen, C. U., Risbo, J., & Skibsted, L. H. (2009). Storage stability of freeze-dried Lactobacillus acidophilus (La-5) in relation to water activity and presence of oxygen and ascorbate. *Cryobiology*, *58*(2), 175-180. http://dx.doi.org/10.1016/j.cryobiol.2008.12.001. PMid:19111715.

Received: Mar. 13, 2020
Revised: June 30, 2020
Accepted: Aug. 11, 2020