Use of red onion (*Allium cepa* L.) residue extract in the co-microencapsulation of probiotics added to a vegan product

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Abstract

This study aimed to develop a functional strawberry pulp containing the combination of *Lactobacillus casei* and bioactive compounds from red onion peel extract into the microparticles formulations to improve bacteria survival during storage and product

consumption. To achieve this goal, the addition of different concentrations of red onion peel extract added to the microparticles was evaluated: 5, 20 and 40%. Microparticles were morphologically characterized and the encapsulation efficiency of the bioactive compounds were evaluated. The physicochemical and microbiological characteristics of the fruit pulp were within the required standards, regardless of the formulation evaluated. As for the pulp added from the microparticles, their physicochemical and microbiological features and probiotic survival under simulated gastrointestinal conditions and storage were analyzed; the size of the microparticles ranged from 136.00 to 305.00 µm. The encapsulation efficiency of both, probiotics and compounds was satisfactory over the different treatments. Indeed, the results pointed out values in the range from 77.77 to 92.11% for probiotic bacteria; from28.88 to 50.18% for reducing compounds; 35.72 to 69.01% for flavonoids; and 25.39 to 60.00% for total monomeric anthocyanins. The formulations of alginate microparticles and alginate + 5% extract had the best results of *L. casei* probiotic viability in strawberry pulp under simulated gastrointestinal conditions and during storage at -18 °C for 60 days. In conclusion, red onion peel extract at low concentrations can help the survival of the probiotic *L. casei* under different conditions.

Keywords: microparticles, antioxidant**,** co-encapsulation, external gelation, probiotic.

1. Introduction

The search for a diet that transcends the need for nutrients and aims at awareness regarding environmental causes and balance with nature is pivotal, as veganism is no longer a trend, given it is growing exponentially and becoming a relevant market. Moreover, to

achieve a better quality of life, the search for healthy and bioactive foods has also become part of the dietary pattern of the vegan and general population.

According to Kleerebezem et al. (2019), probiotic foods are widespread and consumed by millions of people every day. Nonetheless, most of the probiotic products available today are of dairy origin (Reque & Brandelli, 2021); therefore, there is an undeniable need to expand the development of probiotic products and multiply the number of alternatives to meet the demands of consumer groups.

As part of a healthy diet, it is recommended to consume 400 g or five servings of fruits and vegetables every day (WHO, 2003; Mondini, Moraes, Freitas, & Gimeno, 2010). Fruits have numerous vitamins, fiber, minerals, and polyphenols (Harborne & Williams, 2000; Jaime, Figueiredo, Moura, & Malta, 2009), which decrease the incidence of diseases such as cancer and cardiovascular diseases (Williamson, 1996). Thus, developing new fruit-based probiotic products is a healthy alternative with high acceptability for all audiences, including vegans, vegetarians, and people who are intolerant or allergic to milk.

Strawberries are capable of exerting antioxidant, diuretic, and anti-inflammatory effects; these fruits improve muscle development and regeneration and exert positive effects on the skin, teeth, and bones, and act in collagen formation in the body (Rocha, Abreu, Corrêa, Santos, & Fonseca, 2008; Andrade, Diniz, Neves, & Nóbrega, 2002; Nunes & Novello, 2020). However, strawberries have low perishability due to their high post-harvest metabolic rates and must be consumed within a week (Alves, Alencar, Ferreira, Silva, & Ribeiro, 2019). With this in mind, the use of fruit pulps reduces losses and enables all harvest fruits to be fully taken advantage of (Dantas et al., 2012). The fruit pulp is defined as the unfermented product, not concentrated, not diluted, and obtained from pulpy fruit through the appropriate technological process, with a minimum content of total solids from the edible part

of the fruit (Brazil, 2000); the most common way to preserve fruit pulp nowadays is by freezing (Carvalho, Mattietto, & Beckman, 2017).

Given that it presents low pH, high acidity, and antimicrobial compounds, inserting probiotic bacteria into strawberry pulp is challenging because these microorganisms are highly sensitive. In order to form a protective barrier that prevents probiotics from interacting with food, microencapsulation presents itself as a highly effective alternative. Associated with this, the use of bioactive extracts in the process of forming microparticles may prove effective in increasing probiotic survival.

Numerous studies have already reported quercetin and its ability to modulate intestinal microbiota (Etxeberria et al., 2015; Porras et al., 2017). Onions stand out as one of the vegetables with the highest levels of flavonoids, especially quercetin (Santiago et al., 2020; Campone et al., 2018), and higher quantities of this compound are found in the peel than in any other part of the vegetable (Shim, Yi, & Kim, 2011). In the red onion peel, reducing compounds and anthocyanins are also found, which, like quercetin, have high antioxidant properties. The use of onion peel would generate added value and create a useful destination for this material, which is normally treated as waste (Mekar Saptarini & Herawati, 2018).

Given this scenario, this study aimed to evaluate the influence of a red onion (*Allium cepa* L.) peel extract at different concentrations (5, 20, and 40%) on probiotic microparticle viability added to frozen strawberry pulp.

2. Materials and Methods

The red onions were purchased locally in Santa Maria (Rio Grande do Sul State, southern Brazil). The onion peels were manually removed and sanitized with hypochlorite (200 mg L^{-1} / 20 min); the peels were then dried in an oven at 50 °C (± 1) for 24 h, ground in

a knife mill (Mill MA 630/1-Marconi; 5 rpm for 10 s), and stored in a freezer at -18 °C until analyses. Organic strawberries were purchased in Agudo (Brazil).

The probiotics of the species *Lactobacillus casei* LC03 were kindly provided by the company Coana (Florianópolis, Brazil). For culture activation, 1 g was incubated in 100 mL of MRS broth (Himedia) for 15 to 18 h at 37 ± 1 °C. It was then centrifuged at 4670 × g for 15 min at 4 ± 1 °C and washed with saline (0.85%).

2.1 Obtaining the red onion peel extract

Hydroalcoholic extracts were prepared from 3 g of ground red onion peel and then mixed with 60 mL of 80% ethanol at 25 °C under stirring; the extraction time was 120 min according to Viera et al. (2017). Afterward, ethanol was evaporated from the extract using a rotary evaporator, and its volume was corrected with distilled water.

2.2 Red onion peel extract reducing capacity

The reducing capacity was evaluated using the Folin-Ciocalteu method according to Roesler et al. (2007). The absorbances obtained in the reaction were read in a spectrophotometer at a wavelength of 765 nm, and the content of reducing compounds was expressed as milligrams of gallic acid/g of dry sample (mg GAE g^{-1})

2.3 Red onion peel extract total flavonoid content

The total flavonoid content was evaluated according to Zhishen, Mengcheng, & Jianming (1999). The absorbances obtained in the reaction were read in a spectrophotometer at a wavelength of 510 nm, and the flavonoid content was expressed as mg quercetin (QE) equivalent per g of dry sample (mg QE/g).

2.4 Red onion peel extract total monomeric anthocyanin content

The total monomeric anthocyanin content was performed according to Giusti & Wrolstad (2001) using the differential pH method. The absorbances obtained in the reaction were read at the wavelengths of 520 and 700 nm, and the total monomeric anthocyanin content was calculated using the following equation:

Anthocyanin content (mg/100 g dry matter) = A x MW x DF⁄ (ε x W)

Where A is the absorbance (520 – 700 nm) pH 1.0 - (520 – 700 nm) pH 4.5, MW is the molecular weight of cyanidin-3glucoside ($C_{15}H_{11}O_6$, 449.2), DF is the dilution factor, ε is the molar absorptivity (26900), and W is the sample weight (g).

2.5 Red onion peel extract antioxidant capacity

The oxygen radical absorbance capacity (ORAC) was analyzed as proposed by Ou et al. (2001), and the results were expressed as μmol equivalents of Trolox per gram of red onion peel (μmol of Trolox/g).

2.6 Microparticle production by external ionic gelation

The microparticles were produced using the method described by Etchepare et al. (2016), with adaptations. For this, 100 mL of a solution containing 2% alginate was prepared along with the activated probiotic *L. casei* LC03 and red onion peel extract. A dual fluid atomizer nozzle (0.3 mm) at 0.125 kgf/cm was used, and this mixture was sprayed in 0.1 M $CaCl_2$, where it remained under stirring for 30 min. From this method, four microparticle formulations were obtained: 2% alginate microparticles (AM), 2% alginate microparticles + 5% extract (AME5), 2% alginate microparticles + 20% extract (AME20), and 2% alginate microparticles + 40% extract (AME40).

2.7 Microparticle characterization

2.7.1 Optical microscopy and particle size

Optical microscopy was performed using an optical microscope (Carl Zeiss Axio Scope A1, Oberkochen, Germany) equipped with an Axio Cam MRc digital camera (Carl Zeiss). The size of the microparticles was measured by laser diffraction using the Mastersizer 2000 particle size analyzer (Malvern, UK).

2.7.2 Encapsulation efficiency of reducing compounds, flavonoids, and total monomeric anthocyanin content

The extraction of reducing compounds, flavonoids, and total monomeric anthocyanin content from inside the microparticles was performed according to Robert et al. (2010), with adaptations. Briefly, 0.5 mL of acetonitrile and 0.5 mL of methanol:acetic acid:water (50:8:42 mL/mL/mL) and 1 g of microparticles were vortexed for 4 min and then sonicated for 60 min. Afterward, the supernatant was collected and centrifuged at 5000 rpm for 15 min. From this liquid, the encapsulation efficiency of reducing compounds, flavonoids, and total monomeric anthocyanin content were determined as described in sections 2.2, 2.3, and 2.4, respectively, by calculating the percentage of reducing compounds, flavonoids, and total monomeric anthocyanin content encapsulated over the total of these compounds in the initial alginate solution.

2.7.3 Probiotic encapsulation efficiency

The probiotic encapsulation efficiency (EE%) was calculated as proposed by Martin, Lara-Villoslada, Ruiz, & Morales (2013) in Eq. 1.

173

174 $$Eq.\ 1.\ EE = (N/N0) \times 100$$

175

176 Where N is the number of viable cells (log CFU g^{-1}) released by breaking down the
177 microparticles using a sterile phosphate buffer solution (pH 7.5; 25 g/225 mL) according to
178 item 2.9, and N0 is the number of viable cells (log CFU g^{-1}) in the cell concentrate before
179 microencapsulation.

180

181 **2.8 Preparation of the frozen strawberry pulp with added microparticles containing *L.***
182 ***casei* and red onion (*Allium cepa* L.) peel extract**

183 The strawberries were washed with running water, processed in a blender, and sieved
184 to prepare the pulp. Then, 5% microparticles (5 g/100 g) were added to the pulp and
185 homogenized. The pulp was then packed in polyethylene bags and stored at -18 °C for further
186 analysis.

187

188 **2.9 Microbiological analysis in the frozen strawberry pulp**

189 Microbiological analyses to detect *Salmonella* sp., total coliforms, thermotolerant
190 coliforms, and molds and yeasts were performed according to Brazil (2003). Probiotic
191 quantification was performed according to Sheu, Marshall, & Heymann (1993), with
192 modifications. The probiotics were released from the microparticles using a sterile phosphate
193 buffer solution (pH 7.5; 25 g/225 mL) with stirring for 10 minutes. Serial decimal dilutions
194 were then performed in 0.1% peptone water with plating of 1.0 mL of the dilutions in
195 triplicate onto sterile Petri dishes, followed by adding MRS Agar. The plates were then
196 incubated at 37 ± 1 °C for 72 h under anaerobic conditions.

197

198 **2.10 Physicochemical analysis in the frozen strawberry pulp**

199 For the physicochemical characterization of the pulp, the pH, soluble solids, moisture,
200 and titratable acidity were evaluated according to the method of the Adolfo Lutz Institute
201 (IAL, 2009).

202

203 **2.11 Resistance of free and microencapsulated probiotics in strawberry pulp under**
204 **simulated gastrointestinal conditions**

205 Simulation of esophagus/stomach (pH 2.0/90 min in the presence of pepsin),
206 duodenum (pH 5.0/20 min in the presence of pancreatin and bile salts), and ileum (pH 7.5/90
207 min) conditions were performed according to the method described by Madureira, Amorim,
208 Gomes, Pintado, & Malcata (2011) with adaptations. The analysis was performed in a TE 421
209 shaker (Tecnal, Piracicaba, São Paulo, Brazil) at 37 ± 2 °C to simulate body temperature. At
210 the end of each simulation stage, aliquots were collected and plated according to item 2.9.

211

212 **2.12 Evaluation of the shelf life of free and microencapsulated probiotics in the**
213 **strawberry pulp during storage and under freezing conditions**

214 The probiotic viability of the strawberry pulp was evaluated for 60 days at -18 ± 1 °C
215 (freezing conditions). Counts were performed every 15 days according to item 2.9.

216

217 **2.13 Statistical analyses**

218 Data were submitted to analysis of variance (ANOVA) using the Statistica 7.0
219 software (Statsoft Inc., Tulsa, OK, USA), followed by Tukey's test for comparison of means,
220 considering a significance level of 5% ($p<0.05$). Tests were performed in triplicate, and data
221 were expressed as mean ± standard deviation.

222

3. Results and discussion

3.1 Optical microscopy and microparticle size

Based on the morphological analyses of the microparticles (Figure 1), all treatments showed spherical or elliptical shapes and an intact structure without visible cracks or fissures. The advantages of obtaining spherical-shaped microparticles include easier consumption, manufacturing, and packaging (Nami, Lornezhad, Kiani, Abdullah, & Haghshenas, 2020).

Furthermore, we observed that adding the red onion peel extract altered the color of the microparticles, as shown in Figures A, B, C, and D, which correspond to treatments AM, AME5, AME20, and AME40, respectively. The higher the extract concentration added to the microparticles, the darker the coloration. This coloration, which can vary from red to purple, is due to anthocyanins in the red onion peel extract; anthocyanins are natural pigments characterized as phenolic compounds of the flavonoid family and confer coloration to a plethora of flowers, fruits, and plants (Chopra & Panesar, 2010; Nascimento et al., 2008). Moreover, incorporating anthocyanins in foods grants them bioactive and medicinal properties given their high antioxidant activity (Maran, Priya, & Manikandan, 2014).

As for the size of the microparticles, there was a variation from 136.00 to 305.00 µm, with the smallest size being obtained in the AM treatment and the largest size in the AME20 treatment (Table 1). Treatments AME5 and AME40 showed 214.00 and 205.00 µm sizes, respectively. The sizes obtained in this study are advantageous because, in the micrometer range, the microparticles allow the food to remain smooth, while in the millimeter range, the particles confer a sandy texture to the product (Mohammad Ali Khosravi Zanjani, 2012)

Nami, Lornezhad, Kiani, Abdullah, & Haghshenas (2020) employed an extrusion technique and reported even larger particles (860-1130 µm) using 2% alginate. Nevertheless, the authors did not observe any adverse effect on the texture and structure of orange juice

mixed with these particles. Similarly, Batista de Oliveira et al. (2021) maintained the average acceptance for the consistency of chocolate milk with microencapsulated *Spirulina sp*. LEB 18 and reported microparticles that varied from 616.60 to 680.75 µm.

It was also possible to conclude that the composition of the wall material interferes with the size of the microparticles, as shown in Table 1. The addition of the red onion peel extract at different concentrations (5, 20, and 40%) significantly increased the size of the microparticles compared to the treatment that only contained alginate (AM). It is known that changes in the viscosity of the encapsulating solution can have an effect on the size of the microparticles (Haghshenas et al, 2015).

3.2 Probiotic encapsulation efficiency, reducing compounds, flavonoids, and total monomeric anthocyanin content

Encapsulation efficiency (EE%) above 77 % indicates the successful entrapment of the probiotic *L. casei* inside the microparticles prepared in the different formulations (AM, AME5, AME20, and AME40; Table 1). These high EE% values are pivotal so that when they are inserted into food, the microparticles contain a sufficiently high number of probiotics that survive the processing and storage conditions of the product and reach their site of action in adequate amounts. Microencapsulation, in general, is one of the best ways to protect sensitive bioactive compounds; in external ionic gelation, other authors have reported EE% >90% (Angélica Andrade Lopes et al., 2020), as well as in other microencapsulation techniques, including complex coacervation with EE% >90.9% (da Silva et al., 2019), internal emulsification/ionic gelation with EE% >66.2% (Raddatz et al., 2020), and spray drying with EE% = 94.61% (Rosolen et al., 2019).

Among the different microparticle formulations, AME40 showed the highest EE% compared to the others, reaching a value above 92%. As for the compounds present in the

extract, before microencapsulation, the content of reducing compounds was 653.75 ± 32.78 mg/g, the flavonoid content was 78.49 ± 0.34 mg/g, the total monomeric anthocyanin content was 47.09 ± 7.65 mg/g, and the antioxidant capacity was 1096.45 μmol Trolox/g (data not tabulated). Onions contain the highest amount of flavonoids and organosulfur compounds (Pareek, Sagar, Sharma, & Kumar, 2017). Viera et al. (2017) obtained values of 640.8, 35.2, and 30.9 mg/g of total reducing compounds, flavonoids, and monomeric anthocyanin content, respectively, when producing red onion peel extracts using this extraction method.

As for the EE% of the reducing compounds, the AME20 treatment stood out, with 50.18 ± 0.01%. Arriola et al.(2018) obtained EE% of total phenolic compounds above 60% when using the extrusion/external ionic gelation technique to encapsulate stevia extract. The use of alginate has also already demonstrated excellent EE% results when encapsulating anthocyanins (~89%) and phenolic compounds (~98%) extracted from jabuticaba fruit (*Plinia cauliflora* (Mart.) Kausel) and Tubuna (*Scaptotrigona bipunctata*) stingless bee propolis peels by ionotropic gelation (Dallabona et al., 2020).

As for the EE% of flavonoids, AME5 obtained the highest values (69.01 ± 0.01%); this result is similar to the data reported by Milea et al. (2019), who achieved 66.46 ± 0.18% as the best result of EE% of flavonoids from yellow onion peel, although the authors employed freeze-drying. Lastly, the EE% of total monomeric anthocyanin content was higher in the AME20 and AME40 treatments with values of 60.00 ± 0.08 and 59.95 ± 0.05, respectively. Enache et al. (2020) obtained EE% of anthocyanins from blackcurrant fruits of 95.46 ± 1.30% and 87.38 ± 0.48% from the probiotic *Lactobacillus casei* when encapsulating them into a single matrix, albeit the authors used microencapsulation via freeze-drying.

According to Belščak- Cvitanović et al, (2015), lower concentrations of active compound tend to result in less entrapment of some compounds.

3.3 Physicochemical and microbiological characteristics of fruit pulp containing microencapsulated *L. casei* in the different treatments

The physicochemical evaluations in the strawberry pulps containing the free or microencapsulated *L. casei* probiotic are listed in Table 2. According to the general technical regulation to set the identity and quality standards for fruit pulp set by legislation (Brazil, 2001), the strawberry pulp is the product obtained from the edible part of the strawberry (*Fragaria x. ananassa* Duchesne, *Fragaria chiloensis Duchesne* x *Fragaria virginiana* Duchesne) through an appropriate technological process.

The regulation establishes the minimum values of 3.3 for pH, 6.5 for soluble solids in °Brix at 20 °C, and 0.8 for titratable acidity (g/100 g). All the samples evaluated were above these minimum values required. In addition, the moisture content of the different samples was evaluated and ranged from 90.69 to 91.12%, with no statistical differences among the five treatments. Fruits have high moisture content with large variations among them. In addition, the water supply to the plant and growing conditions interfere with the moisture content of fruit (Chitarra & Chitarra, 2005).

The results of the microbiological evaluations of pathogenic or spoilage bacteria in strawberry pulp containing free or microencapsulated *L. casei* probiotic are described in Table 3. The analyses were based on the standards required by RDC No. 12 (Brazil, 2001), which establishes the microbiological standards for foods in Brazil.

According to the results presented herein, the strawberry pulps analyzed are suitable for consumption, since no salmonella, fecal coliforms, and total coliforms were observed in all samples evaluated (Table 3), with these results being in agreement with the legislation, which has a maximum tolerable limit of 10^2 NMP/g of fecal coliforms and absence of *Salmonella* spp. in 25 g of sample. The thermotolerant coliforms may signal contamination of fecal origin or by other enteric microorganisms, which are associated with risks to human

health (Verma, Saharan, Nimesh, & Singh, 2018). *Salmonella sp.* is one of the main microorganisms that cause food outbreaks worldwide, causing economic losses due to its high morbidity (Sharma & Carlson, 2000).

The National Sanitary Surveillance Agency (ANVISA) does not determine limits for molds and yeasts in fruit pulps; nonetheless, these microorganisms were evaluated based on Normative Instruction No. 4, which establishes maximum counts of $5x10^3$/g for *in natura* pulp, frozen or not (Brazil, 2018). All samples evaluated were in compliance with current legislation, with counts of $1.06 \times 10^2 \pm 5.77$, $1.56 \times 10^2 \pm 23.09$, $4.50 \times 10^2 \pm 36.30$, $8.40 \times 10^2 \pm 22.81$, $8.93 \times 10^2 \pm 13.85$ for free culture, AM, AME5, AME20, and AME40, respectively. High mold and yeast counts represent a potential spoilage capacity as well as a public health risk, considering that some filamentous fungi are toxin-producing organisms (Moraes & Machado, 2021).

Therefore, the data obtained in the microbiological evaluations indicate that the strawberry pulps were prepared under adequate hygienic and sanitary conditions and did not represent a threat to the consumers.

3.4 Survival of free and microencapsulated *L. casei* in fruit pulp after simulating gastrointestinal conditions.

The survival of the free or microencapsulated *L. casei* probiotic in strawberry pulp under simulated gastrointestinal conditions is listed in Table 4. Stress conditions such as digestive enzymes and low pH have negative and inhibitory effects on probiotic performance and viability. In the duodenum, the gallbladder and pancreas release bile and pancreatic juices, respectively. These, responsible for the digestion of lipids, proteins and sugars, end up also affecting the proteins, lipids and sugars present in the structure of microorganisms, thus, a second barrier against bacteria, after the acidic environment of the stomach and causing few

348 species of *Lactobacillus* survive (Guyton & Hall, 2002; Nutrition and Health Collection, 1998
349 and 1999). Overcoming these challenges is paramount for probiotics to reach the ileum (their
350 site of action) and colonize it to promote beneficial effects to the host organism (Zeashan et
351 al., 2020). As shown in Table 4, the strawberry pulp containing the free culture lost its
352 viability in the first stage of the simulated gastrointestinal conditions, reaching the
353 esophagus/stomach with only 3.66 ± 0.03 log CFU/g^{-1}. This result demonstrates the need for a
354 system that ensures that the probiotic *L. casei* reaches the ileum without remaining in direct
355 contact with gastric fluids or the product in which it is inserted.

356 The microparticles are designed to remain intact as they pass through the
357 stomach/esophagus and duodenum. This is possible because the alginate that makes up the
358 wall of the microparticles only dissolves at a pH close to 7.5, thus, the dissolution of the
359 microparticles with consequent release of the probiotics only occurs in the ileum (pH 7.5),
360 where it is the site of action of probiotics and, in the other stages (esophagus/stomach and
361 duodenum) the probiotics must remain intact inside the microparticles.

362 Dimitrellou et al., (2016), Dimitrellou et al., (2019) and Li, (2011) showed that
363 microencapsulation favors the survival of the probiotic L. casei in food matrices, when
364 subjected to simulated gastric juice and salt solutions bile.

365 We observed that the microencapsulation system used in this study met such a need,
366 with the strawberry pulps containing the microparticles from treatments AM and AME5
367 releasing the probiotic cells in sufficient quantities and at the appropriate time (6.51 ± 0.48
368 and 6.73 ± 0.23 log CFU/g^{-1}, respectively). Alginate is extremely efficient in releasing trapped
369 compounds at neutral pH, such as intestinal pH (Annan, Borza, & Hansen, 2008; Gul &
370 Dervisoglu, 2017). Higher probiotic bacteria viability was also reported by Chandramouli,
371 Kailasapathy, Peiris, & Jones (2004) at pH 2.0 when alginate was used for encapsulation.

Other microencapsulation methods, such as freeze-drying, are also efficient in protecting *L. casei* probiotics from simulated gastrointestinal conditions, with increased survival of the encapsulated probiotic, while there was a loss of viability of the free probiotic (Li et al., 2019). The strawberry pulps that contained the probiotic microparticles together with the highest concentrations of red onion skin extract (AME20 and AME40) did not reach the ileum in the necessary amounts (6 log CFU/g).

Reza Gheisari, Davar, & Shahram Shekarforoush (2018) also evaluated microencapsulation process efficiency on the probiotic *L. casei* under simulated gastrointestinal conditions when added to mango juice. The microencapsulation was performed by extrusion with calcium alginate and chitosan, and similarly to our study, the authors observed higher probiotic survival than the free microorganism. The same occurred in the study of Marcial-Coba, Saaby, Knøchel, & Nielsen (2019) for *Akkermansia muciniphila* and *L. casei* added in chocolate in free form or microencapsulated by extrusion followed by lyophilization after gastric passage *in vitro*, with higher survival of microencapsulated strains.

3.5 Viability of free and microencapsulated *L. casei* added to strawberry pulp stored at freezing temperatures

The survival of the free and microencapsulated *L. casei* LC03 probiotic in the strawberry pulp during storage at -18°C was evaluated, and the results are presented in Figure 2. To develop new probiotic foods, an important factor is to verify how they adapt to the food matrix as probiotics must be consumed regularly, and in order to obtain their health benefits, their presence in foods must be in sufficient quantities (at least 10^6 CFU/g or mL^{-1}) during the shelf life (Abadía-García et al., 2013).

Probiotic bacteria are recommended to be inserted into food formulations with relatively high pH, around 5.5 to 6.5 (Cruz, Antunes, Sousa, Faria, & Saad, 2009). In

addition, freezing conditions may cause cellular damage to these microorganisms, leading to a reduction of up to 1 log CFU/g (Cruz et al., 2011). It was possible to observe that the free culture was not resistant to the acidity, low pH, and freezing conditions of the strawberry pulp since, in less than 15 days, its counts were already below the required standard (6 log CFU/g^{-1}) for the product to be considered a probiotic (Figure 2). Despite the low pH of the strawberry pulp developed in this study (3.48-3.85), we noted that the microencapsulation allowed the survival of the microorganism *L. casei* LC03, highlighting the AM treatment, which kept the probiotics viable until the end of 60 days (6.92 log CFU/g), followed by the AME5 treatment, with viability between 45 and 60 days. The AME20 treatment lost viability after 30 days of storage, while AME40 remained viable for less than 15 days.

In addition to this study, Afzaal et al. (2020) also reported significant improvements in the viability of microencapsulated *Lactobacillus casei* added to ice cream for 80 days at -20 °C, in addition to higher resistance under simulated gastrointestinal tract conditions compared to the free microorganism in the same product. In another study, microencapsulation also proved effective in protecting the probiotic *Lactobacillus casei* ATCC 334, since, when added to cashew ice cream at -18 °C for 150 days, the probiotic microencapsulated by extrusion with sodium alginate and chitosan showed higher survival than the free microorganism (Farias, 2017).

As for the concentration of red onion (*Allium cepa* L.) peel extract in the microparticles, we observed that the higher the extract concentration, the greater the loss of probiotic viability over the 60 days. Thus, the lowest concentration of extract (5%) proved to be the most effective in maintaining the shelf life of the probiotic *L. casei* LC03. The bioactive compounds present in onions have, among their various properties, antibacterial action (Griffiths, Trueman, Crowther, Thomas, & Smith, 2002), which may explain the fact that the highest concentrations of extract used in this study (20 and 40%) resulted in

unfavorable effects on the viability of the probiotic *L. casei* LC03. The way plant extracts act is not yet completely defined; according to several studies, the compounds in the extracts may lead to the permeabilization or rupture of the cytoplasmic membrane of microorganisms, exposing the contents of the cytoplasm, besides being able to produce inhibitory effects on the ATPase enzyme and, consequently, causing cell death (Gill & Holley, 2006; Zhang et al., 2016).

When associating the results of probiotic viability at -18ºC (Figure 2), with the survival of the probiotic in simulated gastrointestinal conditions (Table 4), it is possible to verify that the sum of the entire period of storage under freezing with the contact with the different sections of the gastrointestinal tract, induces to severe drops in the viability of the probiotic *L.casei*, causing it to reach the duodenum in adequate amounts (above 6 log CFU) only in strawberry pulps containing AM and AME5 microparticles formulations. The other treatments showed insufficient cell count for this.

Sagdic, Ozturk, Cankurt, & Tornuk (2011) supplemented ice cream containing *L. casei* Shirota with ellagic acid, gallic acid, grape seed extract, pomegranate peel extract, and peppermint essential oil and investigated the interaction of the probiotics with these compounds after 60 days at -18 °C. The phenolic compounds were found to benefit the survival of the probiotics.

Lastly, as described herein, it was possible to obtain a polyfunctional product by successfully applying the probiotic *L. casei* with a red onion peel extract to a highly nutritious food matrix that numerous vitamins, fibers, minerals, and polyphenols.

4. Conclusions

The red onion peel extract can be efficiently used to microencapsulate the probiotic *L. casei* LC03 to ensure viability and stability in new functional foods, such as the strawberry

pulp. It was possible to observe that low extract concentrations are enough to achieve a protective and functional effect in the microcapsule, with probiotic viability under simulated gastrointestinal conditions and storage at -18 °C with only 5% extract. Therefore, this study allowed the development of a new probiotic product capable of satisfying the most diverse publics, including vegetarians, vegans, and those intolerant to gluten and lactose.

5. Referências

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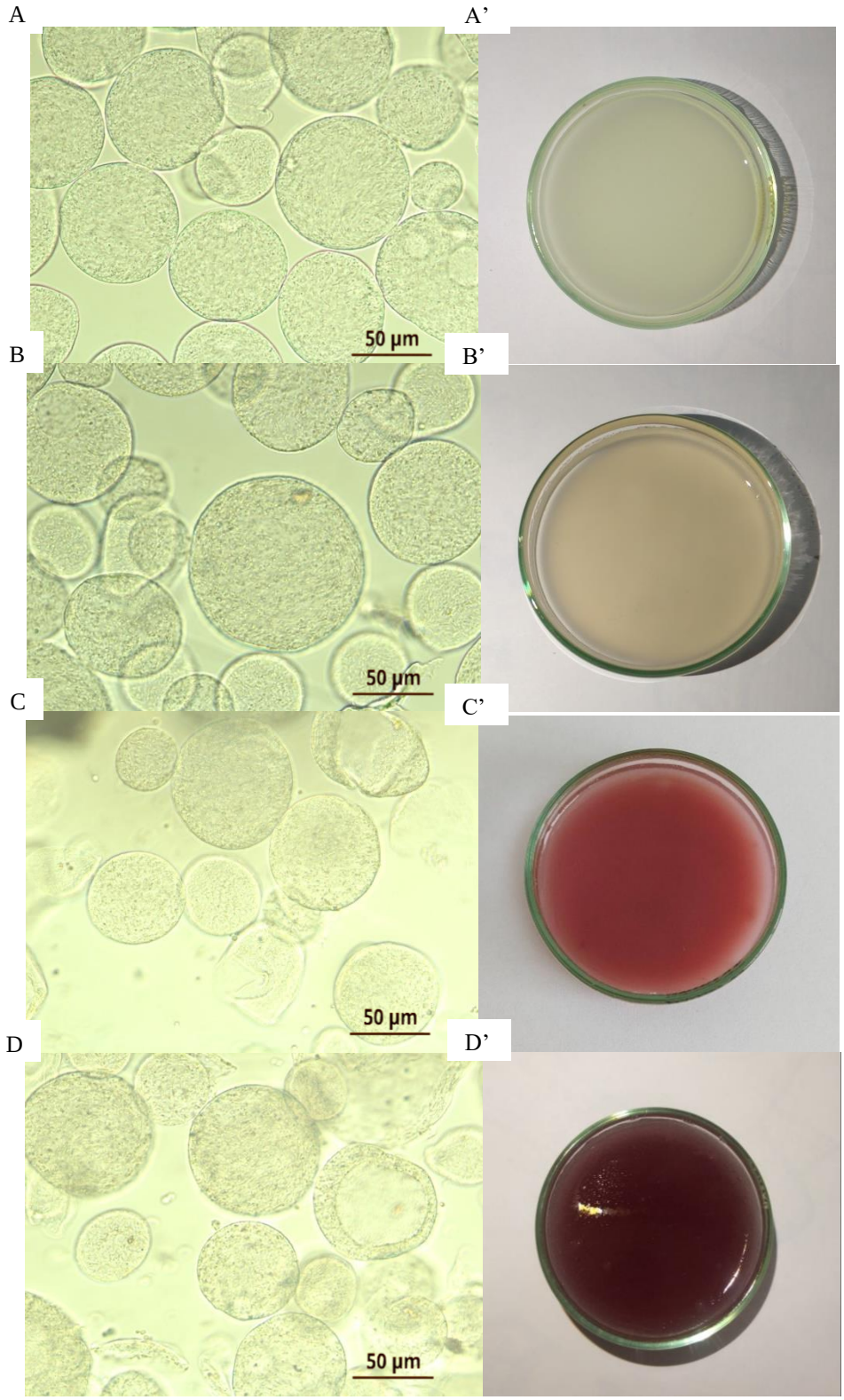

Fig 1. Optical microscopy of alginate microparticles with different concentrations of red onion peel extract containing *L. casei* obtained by external ionic gelation. A = 2% alginate microparticles (AM) (40x); B = 2% alginate microparticles + 5% extract (AME5) (40x); C = 2% alginate microparticles + 20% extract (AME20) (40x); D = 2% alginate microparticles + 40% extract (AME40) (40x).
A', B', C' and D' = Treatments AM, AME5, AME20 and AME40, respectively, observed without microscope.

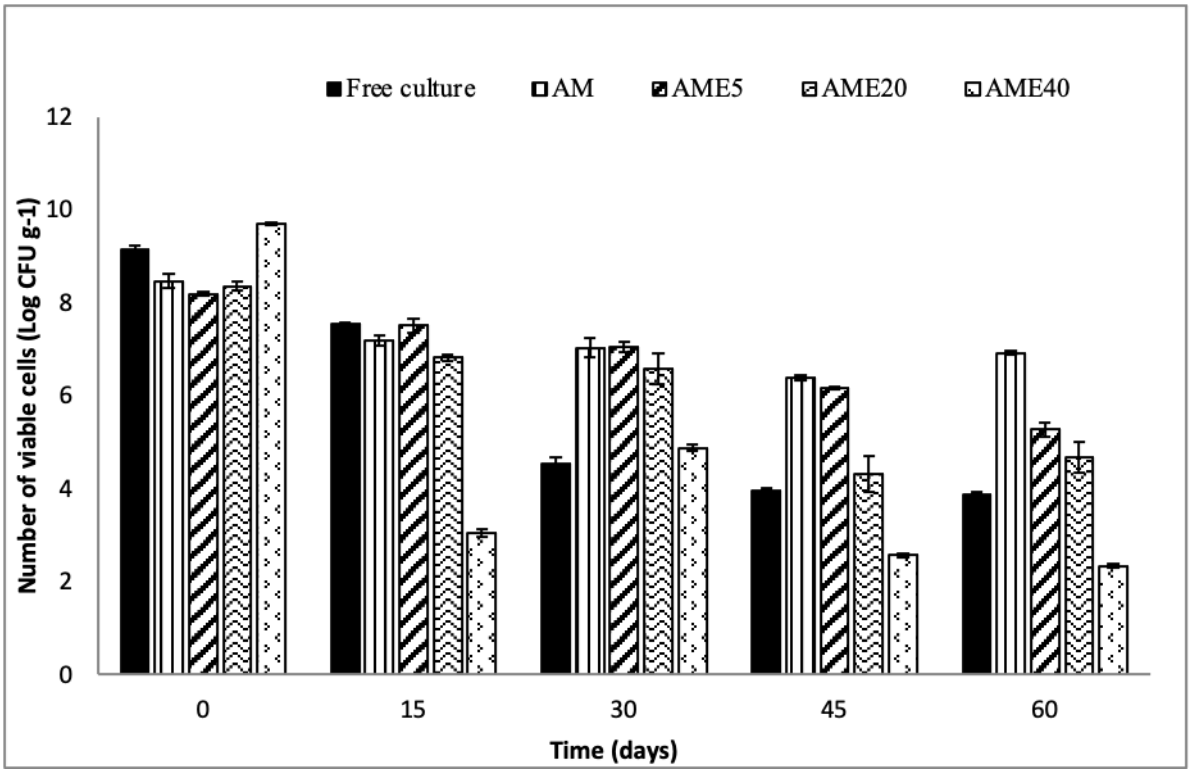

Figure 2 – Effect of temperature (-18 °C) on the viability of *L. casei* (free and immobilized) in strawberry pulp stored for 60 days..

Free culture = Strawberry pulp containing free probiotics.

AM = 2% alginate particles; AME5 = particles of 2% alginate + 5% extract; AME20 = particles of 2% alginate + 20% extract; AME40 = particles of 2% alginate + 40% extract.

Table 1 – Characteristics of particles containing *L. casei* obtained by external ionic gelation with alginate and red onion peel extract at different concentrations.

Treatment	Particle size (µm)	EE% probiotics	EE% reducing compounds	EE% flavonoids	EE% total monomeric anthocyanins
AM	136.00	80.30 ± 1.42^{b}	-----	-----	------
AME5	214.00	77.77 ± 0.43^{c}	30.10 ± 0.06^{b}	69.01 ± 0.01^{a}	25.39 ± 0.05^{b}
AME20	305.00	79.35 ± 0.92^{bc}	50.18 ± 0.01^{a}	35.72 ± 0.00^{c}	60.00 ± 0.08^{a}
AME40	205.00	92.11 ± 0.23^{a}	28.88 ± 0.01^{b}	41.28 ± 0.00^{b}	59.95 ± 0.05^{a}

AM = 2% alginate particles; AME5 = particles of 2% alginate + 5% extract; AME20 = particles of 2% alginate + 20% extract; AME40 = particles of 2% alginate + 40% extract. EE%= percentage of encapsulation efficiency. The means followed by the same lowercase letter in the column do not differ statistically from each other by Tukey's test, with a significance of 5%. Means found in triplicate.

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Table 2 - Physicochemical characterization of whole strawberry pulp with or without alginate microparticles with different concentrations of red onion peel extract containing *L. casei* obtained by external ionic gelation.

Treatment	pH	Soluble Solids / Brix	Moisture	Titratable Acidity
Free culture	3.61 ± 0.01^{bc}	7.80 ± 0.17^{a}	91.12 ± 0.13^{a}	1.03 ± 0.05^{a}
AM	3.62 ± 0.01^{bc}	7.40 ± 0.17^{ab}	90.69 ± 0.68^{a}	0.86 ± 0.05^{a}
AME5	3.85 ± 0.12^{a}	7.50 ± 0.20^{ab}	91.09 ± 0.09^{a}	1.0 ± 0.00^{a}
AME20	3.48 ± 0.00^{c}	7.33 ± 0.10^{b}	90.75 ± 0.51^{a}	0.86 ± 0.05^{a}
AME40	3.70 ± 0.02^{ab}	7.43 ± 0.15^{ab}	90.73 ± 0.28^{a}	1.0 ± 0.1^{a}

AM = 2% alginate particles; AME5 = particles of 2% alginate + 5% extract; AME20 = particles of 2% alginate + 20% extract; AME40 = particles of 2% alginate + 40% extract. The means followed by the same lowercase letter in the column do not differ statistically from each other by the Tukey test, with a significance of 5%. Means found in triplicate.

Free culture = Strawberry pulp containing free probiotics.

Table 3 – Microbiological characteristics of strawberry pulp added with microparticles containing *L. casei* obtained by external ionic gelation with alginate and red onion peel extract at different concentrations.

Treatment	Molds and Yeasts	Total Coliforms	Thermotolerant Coliforms	Salmonella
Free culture	$1.06 \times 10^2 \pm 5.77$	Absent	Absent	Absent
AM	$1.56 \times 10^2 \pm 23.09$	Absent	Absent	Absent
AME5	$4.50 \times 10^2 \pm 36.30$	Absent	Absent	Absent
AME20	$8.40 \times 10^2 \pm 22.81$	Absent	Absent	Absent
AME40	$8.93 \times 10^2 \pm 13.85$	Absent	Absent	Absent

AM = 2% alginate particles; AME5 = particles of 2% alginate + 5% extract; AME20 = particles of 2% alginate + 20% extract; AME40 = particles of 2% alginate + 40% extract. The means followed by the same lowercase letter in the column do not differ statistically from each other by the Tukey test, with a significance of 5%. Means found in triplicate.

Free culture = Strawberry pulp containing free probiotics.

	Free culture	AM	AME5	AME20	AME40
Initial count	9.14±0.06^{aB}	8.45±0.15^{aC}	8.19±0.04^{aD}	8.35±0.09^{aCD}	9.70±0.02^{aA}
Esophagus / stomach 90 min / pH 2.0	3,66±0.03^{bB}	4.66±0.07^{cA}	4.28±0.07^{cA}	4.72±0.04^{cA}	4.31±0.36^{cA}
Duodenum 20 min / pH 5.0	3,58±0.05^{bD}	4.51±0.03^{cB}	4.17±0.09^{cC}	4.43±0.12^{dB}	5.31±0.00^{bA}
Ileum 90 min / pH 6.5	2,91±0.31^{cD}	6.51±0.48^{bA}	6.73±0.23^{bA}	5.22±0.10^{bB}	3.71±0.02^{dC}

Table 4 – Survival of free and encapsulated *L. casei* in different treatments after each stage of the simulation of gastrointestinal conditions.

AM = 2% alginate particles; AME5 = particles of 2% alginate + 5% extract; AME20 = particles of 2% alginate + 20% extract; AME40 = particles of 2% alginate + 40% extract.

Free culture = strawberry pulp containing free *L. casei*.

The means followed by the same lowercase letter in the column and uppercase in the row do not differ statistically from each other by the Tukey test, with significance set at 5%. Means found in triplicate. Values in log CFU / g.