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Co-encapsulation of guaraná extracts and probiotics increases probiotic survivability and simultaneously delivers bioactive compounds in simulated gastrointestinal fluids

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ABSTRACT

Co-encapsulation addresses the protection of multiple compounds from harmful conditions. However, the release of entrapped bioactive compounds and probiotics in the gut is required to achieve their health benefits. This study focused on the co-encapsulation of probiotics and guaraná extracts by complex coacervation using gelatin and gum Arabic, evaluating the release of encapsulated probiotics and bioactive compounds during *in vitro* digestion. The antioxidant activity of guaraná extracts and their stimulatory effect on probiotic populations were also investigated. Guaraná seed extract (GSE) showed more significant antioxidant activity. Concerning the influence of guaraná extracts on the growth of probiotics, guaraná peel extract (GPE) prolonged their growth. The release of encapsulated phenolic compounds from GSE was higher in simulated gastric fluid (SGF), reaching at least 80% of accumulative release after 2 h. In contrast, the maximum release of encapsulated carotenoids from GPE was around 90% in simulated intestinal fluid (SIF). Furthermore, the co-encapsulation of probiotics and guaraná extracts improved the final number of cells found in SIF, which was around 7 log CFU/mL. Therefore, co-encapsulation by complex coacervation is efficient for simultaneous delivery of bioactive compounds and probiotics to the gut, extending their benefits by this combination.

1. Introduction

The current concern about health has increased consumers' interest in the supplementation of bioactive compounds and probiotics due to their beneficial effects. For instance, the consumption of phenolic compounds and carotenoids may reduce degenerative and cardiovascular diseases, respectively (Majhenic, Skerget, & Knez, 2007; Rodriguez-Amaya, 2019), while probiotic supplementation aids gastrointestinal and immune health (Hill et al., 2014). In this way, the combination of bioactive compounds and probiotics, whether through food or supplements, could be a valuable strategy to extend their health benefits. In addition, there is some evidence that probiotics and polyphenols may interact positively in gut health (Valdés et al., 2015). For instance, polyphenols present antimicrobial activity, reducing the proliferation of pathogenic microorganisms in the gut (Zhao & Shah, 2015). Also, plant polyphenols are emerging prebiotics due to their stimulatory effect on the growth of beneficial gut microbiota, which consequently enhances the bioavailability of polyphenols (Gibson et al., 2017; Kawabata et al., 2019). Thus, plant phenolic compounds stand out from carbohydrate-based prebiotics for their antioxidant activity and synergistic effect with probiotics.

The incorporation of probiotics in plant food matrices, such as passion fruit juice (Dias et al., 2018) and jussara sorbet (Marinho, Silva, Mazzocato, Tulini, & Favaro-Trindade, 2019), has been explored to develop healthier products. However, it can be a challenge to maintain the viability of some probiotic strains when they are applied in food matrices, considering the physical-chemical characteristics of the food matrix, such as low pH, the presence of oxygen, and storage temperature. Thus, a potential technology for combining these bioactive compounds and probiotics is co-encapsulation, which is based on packaging the materials of interest to protect them from environmental conditions (Comunian & Favaro-Trindade, 2016). Furthermore, the microcapsules

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can be used as a supplement or added to the food matrix. However, the choice of microencapsulation technique and polymers for packaging the bioactive compounds and probiotics drives their release during digestion.

Among the encapsulation techniques that have been used to investigate the simultaneous protection of probiotics and bioactive compounds are emulsification/internal gelation (Gaudreau, Champagne, Remondetto, Gomaa, & Subirade, 2016), spray drying (Vásquez-Maldonado et al., 2020), and complex coacervation (Eratte et al., 2015). Coacervates are produced after the phase separation of charged polymers, mainly with the adjustment of pH, providing electrostatic interaction between the opposing charges of polymers surrounding the core material (Gouin, 2004; Timilsena, Akanbi, Khalid, Adhikari, & Barrow, 2019). Several proteins and polysaccharides are explored for encapsulation by complex coacervation, but the pair of polymers gelatin/gum Arabic is often used. Furthermore, comprehension of properties associated with biopolymers is relevant for encapsulation, such as molecular weights, concentrations, and ionic charges, which significantly affect coacervates production (Eghbal & Choudhary, 2018; Timilsena et al., 2019). Although complex coacervation has been widely explored for the protection of hydrophobic materials, some recent studies have demonstrated its potential to preserve phenolic compounds and probiotics (Holkem & Favaro-Trindade, 2020; Silva, Mesquita, Rubio, Thomazini, & Favaro-Trindade, 2022). This method can protect food and cosmetic ingredients, natural repellents, and others (Timilsena et al., 2019). Release studies of coacervates loaded simultaneously with bioactive compounds and probiotics in simulated gastrointestinal fluids are relevant for understanding the influence of antioxidant compounds in increasing the number of viable cells that reaches the gut.

Guaraná is an Amazonian fruit which has been investigated due to the caffeine and phenolic compounds in its seeds (Santana & Macedo, 2018), which are used as a supplement and to produce energy beverages. Guaraná peel is discarded during processing and used as a fertilizer with no aggregate value. However, guaraná peel is a source of bioactive compounds, such as β-carotene and lutein (Pinho et al., 2021). The full use of guaraná would add value to the fruit since carotenoids and phenolic compounds have antioxidant properties. For this reason, the combination of guaraná extracts with probiotics is promising for reducing the oxidative stress of cells and extending their viability during stress conditions, such as the digestion process.

Therefore, the overall aims of this study were (i) to evaluate the antioxidant activity of guaraná seed extract (GSE) and guaraná peel extract (GPE); (ii) to verify the prebiotic potential of GSE and GPE during *in vitro* incubation with probiotics, and the hydrophobicity of probiotic cells; (iii) to co-encapsulate guaraná extracts and probiotics by complex coacervation; (iv) to assess the release of phenolic compounds, carotenoids, and probiotics during an *in vitro* digestion assay.

2. Materials and methodology

2.1. Materials

Lacticaseibacillus paracasei BGP-1 and *Bifidobacterium animalis* subsp. *lactis* BLC-1 were donated by Sacco (Campinas, Brazil). Guaraná fruits were provided by the Executive Commission of the Rural Economic Recuperation Plan in Cacao (Taperoá, Brazil). Type A swine gelatin (Gelnex, Itá, Brazil) and gum Arabic (Nexira, São Paulo, Brazil) were used as encapsulating materials. Concerning the *in vitro* digestion test, a pepsin from porcine gastric mucosa (≥250 units/mg, EC 3.4.23.1), pancreatin from porcine pancreas (8 x USP specifications, EC 232-468-9) and bile salts were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Methodology

2.2.1. Production of guaraná extracts

First, guaraná fruit was washed, separating the seeds and peel, followed by drying of these parts at 40 °C for 24 h to avoid contamination during storage and to facilitate the recovery of bioactive compounds. GSE was produced as proposed by Silva et al. (2022) using 30% (v/v) ethanol at 60 °C and mechanical agitation for recovery of phenolic compounds. GSE was atomized using an MSD 1 spray-dryer (LabMaq, Brazil) containing a 1.2 mm diameter atomizer nozzle, inlet air temperature at 150 °C, and 10 mL/min feed flow rate.

Concerning the preparation of carotenoid-rich extract from guaraná, peel was crushed using a blender. Crushed peel was put in contact with absolute ethanol in the proportion of 1–10 to extract carotenoids. The GPE was produced using a water bath at 50 °C for 2 h and mechanical stirring. Then, GPE was concentrated using a rotary evaporator (TE-211, Tecnal, Piracicaba, Brazil) at 40 °C until the ethanol was removed. For this, 3% (w/w) of sunflower oil was added into the GPE to preserve the carotenoids during evaporation, obtaining an oil with carotenoids.

2.2.2. Antioxidant activity (ABTS, DPPH, and ORAC) of guaraná extracts

The antioxidant activity of spray-dried GSE (without carrier) and GPE was evaluated by DPPH and ABTS free radical scavenging activity and peroxyl radical scavenging capacity (ORAC).

DPPH (2,2-diphenyl-1-picrylhydrazyl radical; Sigma-Aldrich) free radical scavenging activity was evaluated according to Brand-Williams, Cuvelier, and Berset (1995) and Melo et al. (2015). For this, 66 μL of sample and 134 μL of DPPH solution (150 μM) were added to a 96-well microplate, followed by mixing. Then, the microplate was protected from light to allow the reaction to proceed for 45 min, then the absorbance was read at 517 nm at room temperature.

ABTS free radical scavenging activity was performed as described by Re et al. (1999) and Al-Duais, Müller, Böhm, and Jetschke (2009). First, the ABTS radical was diluted with potassium phosphate buffer to obtain an approximate absorbance of 0.7 at a wavelength of 734 nm. About 20 μL of extract and 220 μL of the ABTS radical solution were added to a 96-well microplate and maintained protected from the light. After the addition of ABTS radical solution, samples were kept for 6 min to finalize the reaction before the absorbance was read at 734 nm at room temperature.

ORAC was conducted using the methodology suggested by Melo et al. (2015) and Prior, Wu, and Schaich (2005). A microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) was set up for kinetic reading each minute for 2 h ($\lambda_{emission} = 582$ nm and $\lambda_{excitation} = 485$ nm) and controlled temperature at 37 °C. Approximately 30 μL of the sample, 60 μL of fluorescein solution (508.25 nM), and 110 μL of dihydrochloride (AAPH) were added to a 96-well plate.

All antioxidant activities of GSE and GPE were expressed as μmol Trolox equivalents/g sample since Trolox was used as a standard for these methods.

2.2.3. Probiotic inoculum

Preparation of probiotic suspension was performed by adding 50 mg of freeze-dried probiotic cells to 10 mL of MRS broth for incubation at 37 °C for 18 h. Then, the probiotic suspension was added to 100 mL of MRS broth, followed by incubation. Cells were collected by centrifugation and resuspended in 2% sodium citrate for further analysis.

2.2.4. Enumeration of probiotic

Aliquots of 100 μL were withdrawn and serially diluted, followed by the inoculation of bacterial suspension into De Man, Rogosa, and Sharpe (MRS) agar. Enumeration of viable probiotics was performed after the incubation of plates at 37 °C in an anaerobic jar for 48 h. Results were expressed as the number of colony-forming units (CFU) per mL or gram.

2.2.5. In vitro antimicrobial activity of guaraná extracts

The *in vitro* antimicrobial activity of guaraná extracts against probiotic cells was determined by inoculation of guaraná extracts onto MRS agar according to Martin et al. (2012) with modification. The probiotic inoculum was prepared as described in section 2.3.3, diluting the bacterial pellet in 2% sodium citrate for a final population of 8 log CFU/mL. Approximately 1 mL of probiotic inoculum was added to 100 mL of the previously cooled semi-solid MRS agar composed of 3.7% (w/v) MRS agar and 1.8% (w/v) MRS broth. The semi-solid MRS agar was placed in Petri dishes and 5 mm wells were made after 60 min. Then, 40 μL of pure guaraná extracts was pipetted onto MRS agar and incubated at 37 °C for 48 h. Also, 40 μL of diluted guaraná extracts (1:1, 1:2, 1:3) was assessed. In addition, distilled water and tetracycline solution (50 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) were used as negative and positive controls, respectively.

2.2.6. Influence of guaraná extracts on the growth of probiotics

Interaction between probiotics and guaraná extracts was evaluated following the methodology proposed by China et al. (2012) with modification. Approximately 0.5 g of spray-dried GSE was rehydrated in 20 mL of distilled water and filter-sterilized (0.22 μm), whereas concentrated GPE, previously filter-sterilized (0.22 μm), was evaluated. Then, supplemented MRS broth was prepared using 4 mL of MRS broth and 1 mL of GSE or GPE, referred to as MRS broth supplemented with 20% (v/v) of guaraná extracts. In addition, 10% (v/v) and 5% (v/v) guaraná extract supplementation in MRS broth was evaluated using 0.5 or 0.25 mL of guaraná extract, respectively. The final volume was maintained at 5 mL using sterilized water. Finally, 10 μL of probiotic inoculum (8 log CFU/mL), previously prepared as described in section 2.3.3, was inoculated in MRS broth. As a control, probiotic was added to MRS broth without guaraná extracts. The probiotic suspension was incubated at 37 °C for 96 h. Then, an aliquot of 100 μL of each sample was withdrawn at the initial time and after 24, 48, 72, and 96 h of incubation, diluted in 2% sodium citrate solution, and enumerated onto MRS agar, as described in section 2.3.4.

2.2.7. Hydrophobicity of probiotics

Cell surface hydrophobicity was determined following the methodology proposed by Vinderola, Medici, and Perdigón (2004). Probiotic inoculum was produced as described previously in section 2.3.3. In addition, the supplementation of MRS broth with 10% guaraná extracts was evaluated to check whether the cell hydrophobicity changed during incubation. After incubation, probiotic cells were washed three times with phosphate-buffered saline (PBS) solution. Initially, the optical density (OD) of probiotic cells was adjusted to 0.8 at 560 nm, maintaining the cells suspended in PBS. After that, 0.6 mL of n-hexadecane was added to 3 mL of cell suspension. Tubes were vortexed for 2 min; after 1 h with complete phase separation, the aqueous phase was removed for reading at 560 nm in a spectrophotometer. The percentage hydrophobicity was calculated using Equation (1).

$$H(\%) = \left[(A_0 - A)/_{A_0} \right] \times 100 \quad (1)$$

where A_0 and A are the OD of the aqueous phase before and after contact with n-hexadecane, respectively.

2.2.8. Simultaneous encapsulation of probiotics and guaraná extracts

Control coacervates were produced without the addition of guaraná extracts according to Silva et al. (2018) with some modifications. Approximately 1.5 g of probiotic pellet was dispersed in 150 mL of gelatin solution (2.5% w/w) at 6000 rpm for 1 min using an Ultra Turrax (IKA, Staufen, Germany), followed by the addition of 150 mL of gum Arabic solution (2.5% w/w) and adjustment of pH to 4 using citric acid (5 M). Finally, 600 mL of distilled water was added to the mixture and maintained under magnetic stirring until the temperature reached 10 °C.

To produce the coacervates containing probiotics and GPE, approximately 1.5 g of probiotic pellet was dispersed in 1.5 g of GPE at 3600 rpm for 1 min. Then, 150 mL of gelatin solution (2.5% w/w) was added to the mixture at 6000 rpm for 60 s. Approximately 150 mL of gum Arabic solution (2.5% w/w) was added to the mixture and kept under magnetic stirring, then the pH was adjusted to 4.2 using citric acid (5 M). Approximately 600 mL of distilled water was added to the mixture and magnetic stirring was stopped only after reduction of the temperature to 10 °C.

Probiotics and GSE were encapsulated as described by Souza et al. (2018) with modification and Silva et al. (2022). First, about 3.75 g of spray-dried GSE was rehydrated in 600 mL of deionized water at 6000 rpm for 2 min. Next, aqueous GSE was added to 150 mL of gelatin solution (5% w/w) and homogenized at 12,000 rpm for 2 min using an Ultra Turrax. In the last minute of homogenization, approximately 1.5 g of probiotic pellet was put into the mixture. Then, 150 mL of gum Arabic solution (5% w/w) was added to the mixture and kept under magnetic stirring, then the pH was adjusted to 3.8 using citric acid (5 M). The mixture was maintained under magnetic stirring until the temperature reached 10 °C.

All coacervates were kept in the refrigerator at 7 °C overnight for decantation. Finally, coacervates were freeze-dried in a lyophilizer (LC 1500, Terroni, Brazil) for 48 h.

2.2.9. Characterization of coacervates

2.2.9.1. Particle size and morphology. The particle size of wet coacervates was evaluated by laser diffraction (Shimadzu Sald-201V particle size analyzer, Kyoto, Japan). Thus, coacervates were dispersed in distilled water.

The morphology of wet coacervates was qualitatively analyzed using a confocal laser scanning microscope (Leica Microsystems GmbH, SP5, Germany) with an objective of 63 × (1.4 aperture and oil immersion). The coacervates were dyed with a live/dead kit (Abcam, Waltham, MA, USA) to evaluate the resistance of probiotic cells after encapsulation. Live probiotics were excited with an argon laser at 488 nm, and the emitted light was recorded between 500 and 550 nm. Dead probiotics were excited with a HeNe laser at 543 nm, and the emitted light was recorded between 588 and 682 nm. Besides that, Nile red dye (excitation: 488 nm, emission: 515–645 nm) was used to verify the encapsulation of GPE. These analyses were done at the Multi-User Laboratory for Confocal Microscopy (LMMC) at the Faculdade de Medicina de Ribeirao Preto (FMRP-USP).

After freeze-drying of coacervates, the morphology of formulations was evaluated using a scanning electron microscope (TM3000 Tabletop Microscope, Japan). First, freeze-dried powders were accommodated on double-faced carbon tapes (Ted Pella, Inc., Redding, CA, USA) attached to aluminum stubs. Images were captured at a voltage of 5 kV and magnification of 500 × .

2.2.9.2. Enumeration of probiotics loaded in coacervates. Encapsulated probiotics were released by adding 2% sodium citrate solution and agitating in a vortex for 10 min. Then, enumeration of viable probiotics was performed as described in section 2.3.4.

2.2.9.3. Evaluation of total phenolic content (TPC) loaded in coacervates. TPC in coacervates was evaluated as described by Souza, Thomazini, Chaves, Ferro-Furtado, and Favaro-Trindade (2020). First, 0.1 g of freeze-dried coacervate was added to 2.5 mL of NaOH solution (0.1 M) and 5 mL of 0.5% (v/v) acetic acid in acetone solution. The release was performed in a Multi Reax Vortex (Heidolph, Schwabach, Germany) for 30 min, followed by centrifugation at 6603*g* for 5 min. The aqueous phase was collected to quantify the TPC as described in section 2.3.9.4.

2.2.9.4. Quantification of TPC. TPC was evaluated according to

Singleton, Orthofer, and Lamuela-Raventos (1999) with modification. Thus, 0.25 mL of sample was added to 2 mL of distilled water and 0.25 mL of the Folin–Ciocalteu reagent. After that, 0.25 mL of saturated sodium carbonate was added to the mixture which was vortexed and incubated in a water bath at 37 °C for 30 min to complete the reaction. Measurement of the absorbance at 750 nm was carried out using a UV–Vis Genesys 10s spectrophotometer (Thermo Scientific, Waltham, MA, USA) and gallic acid was applied as a reference for the calculation of TPC.

2.2.9.5. Evaluation of total carotenoids (TC) loaded in coacervates. TC in coacervates were determined by adding 0.1 g of freeze-dried coacervate to 2.5 mL of 2% sodium citrate solution and incubating at 40 °C for 5 min. Then, 5 mL of hexane was added to this mixture, and samples were sonicated for 5 min. The organic phase was separated by centrifugation at 2935*g* for 5 min. An exhaustion extraction procedure was performed three times to effect complete removal of the yellow color of coacervates, and the organic phase was collected to quantify the TC as described in section 2.3.9.6.

2.2.9.6. Quantification of TC. The absorbance of the organic phase was measured in a spectrophotometer, at 450 nm. TC was calculated using Equation (2) (Rodriguez-Amaya & Kimura, 2004, p. 58).

$$TCC\ (\mu g\ /\ ml) = \frac{Absorbance \times Volume(ml) \times 10^4}{Absorption\ coef \times Sample\ weight(g)} \quad (2)$$

where the absorption coefficient is 2500, which is recommended for mixtures (Rodriguez-Amaya & Kimura, 2004, p. 58).

2.2.9.7. Encapsulation efficiency (EE). Concerning EE, the content of phenolics on the surface of coacervates was evaluated as suggested by Souza et al. (2018). Approximately 0.1 g of freeze-dried coacervate was added to 5 mL of distilled water and mixed in a vortex for 1 min. Samples were centrifuged at 6603 *g* for 5 min. The quantification of TPC was performed in the supernatant as described in section 2.3.9.4.

The content of carotenoids on the surface of freeze-dried coacervates was evaluated for EE. Then, 0.1 g of freeze-dried coacervates was added to 3 mL of hexane and mixed for 1 min, followed by centrifugation at 2935*g* for 5 min. Quantification of TC was carried out in the supernatant as described in section 2.3.9.6.

The EE for phenolics from GSE and carotenoids from GPE was calculated using Equation (3).

$$EE(\%) = \left(\frac{Bioactive_{Coacervates} - Bioactive_{Surface}}{Bioactive_{Initial}}\right) \times 100 \quad \text{(Eq. 3)}$$

where: $Bioactive_{Coacervates}$ – total content of phenolics or carotenoids in coacervates; $Bioactive_{Surface}$ – total content of phenolics or carotenoids on the surface of coacervates; $Bioactive_{Initial}$ – total content of phenolics or carotenoids added to the polymers for encapsulation.

2.2.10. Release of probiotics and bioactive compounds in simulated gastrointestinal conditions

The composition of simulated gastrointestinal fluids followed the recommended electrolytic concentration described by Minekus et al. (2014). Approximately 0.5 g of freeze-dried coacervate was added to 1.5 mL of distilled water and 2 mL of simulated salivary fluid for 5 min. Sequentially, about 4 mL of simulated gastric fluid (SGF) was added to the tubes, followed by adjustment of pH to 3 using HCl (5 M) and the addition of 0.2 mL of pepsin solution (2000 U/mL). Tubes were incubated at 37 °C under agitation at 200 rpm for 120 min. After that, simulated intestinal fluid (SIF) was added to tubes, and the pH was changed to 7 using NaOH (1 M). Approximately 2 mL of pancreatin solution (100 U/mL) and 1 mL of bile salts (10 mM) were added to the mixture; thus, tubes were incubated at the same conditions described. Samples were evaluated after 30, 60, and 120 min, referring to the SGF, and 130, 180, and 240 min, referring to the SIF.

2.2.10.1. Evaluation of probiotic release in simulated gastrointestinal fluids. To evaluate the release of probiotics in SGF and SIF, an aliquot of sample was centrifuged (6603 g, 5 min) for sedimentation of remained coacervates. Then, supernatant was collected to perform the serial dilution in 2% sodium citrate solution and the sequential inoculation onto MRS agar. The incubation conditions were described in section 2.3.4. The percentage of probiotics released in SGF and SIF was calculated considering the initial count of probiotics entrapped in coacervates.

2.2.10.2. Evaluation of TPC and TC release in simulated gastrointestinal fluids. To quantify the TPC in SGF and SIF, approximately 0.5 mL of supernatant was previously centrifuged (6603 g, 5 min). The quantification of TPC released was performed as described in section 2.3.9.4. In addition, the initial TPC in coacervates, before their addition in the experiment, was considered for calculation of the percentage of release (Eq. (4) below).

Concerning the TC released in simulated gastrointestinal fluids, the supernatant containing carotenoids was collected after centrifugation (2935 g, 5 min). Subsequently, TC was quantified as described in section 2.3.9.6. Also, the initial TC in coacervates was analyzed. The percentage of bioactive compound released was calculated using Equation (4).

$$Release\,(\%) = \left(\frac{Amount\ of\ bioactive\ released\ in\ simulated\ gastrointestinal\ fluid}{Amount\ of\ bioactive\ in\ coacervates}\right) \times 100 \quad \text{(Eq. 4)}$$

In addition, the morphology of coacervates were also observed using an optical microscope (DM500, Leica Microsystems GmbH, Germany) with an objective of 10 × .

2.3. Statistical analysis

All experiments were performed as independent triplicates, and the results were presented in terms of mean and standard deviation. Data were evaluated by analysis of variance (ANOVA) followed by Tukey's post-test (95% confidence interval), using the SAS v9.1.3 program (Statistic Analysis Software, SAS Institute Inc., USA).

3. Results and discussion

3.1. Antioxidant activity of guaraná extracts

The antioxidant activity of the guaraná extracts can be seen in Table 1. GSE presented the highest antioxidant activity for all tests performed when compared to GPE values. This result can be correlated with the higher content of phenolic compounds such as catechins found in guaraná seeds (Silva et al., 2019). Despite the fact that GPE showed less antioxidant potential, its application in food products is an interesting approach not only as a color additive due to the presence of carotenoids but also for the sustainability of using this food by-product.

Table 1
Antioxidant activity of guaraná seeds extract (GSE) and guaraná peels extract (GPE).

Extract	DPPH (μmol eq. de Trolox/g)	ABTS (μmol eq. de Trolox/g)	ORAC (μmol eq. de Trolox/g)
GSE	3218 ± 50^{A}	9534 ± 70^{A}	$11{,}293 \pm 14^{A}$
GPE	22 ± 2^{B}	68 ± 2^{B}	127 ± 6^{B}

Values are mean ± standard error (SE) (n = 4 analytical replicates). Values with the same upper case letter in a column are not statistically different ($p > 0.05$).

Santana and Macedo (2019) reported higher values of DPPH antioxidant activity for GSE, approximately 25,000 μmol eq. Trolox/g. However, this difference probably occurred due to the origin of the guaraná used in the experiments (Maués, Amazônia) and the different extraction conditions.

Kang et al. (2012) verified that the antioxidant activity of açaí pulp, which is another superfruit from the Amazon region, by the ORAC and DPPH methods was approximately 7700 and 320 μmol eq. Trolox/g, respectively. Another study evaluated the antioxidant activity of 30 plant water extracts, showing that the antioxidant activity of cinnamon extract (*Cinnamomum zeylanicum*) by ORAC was around 8500 μmol eq. Trolox/g (Dudonné, Vitrac, Coutière, Woillez, & Mérillon, 2009). In this way, GSE showed greater antioxidant potential than açaí pulp and water extract of cinnamon.

Hence, the GSE has high antioxidant potential to be used in food systems. This result was already expected, considering the content of catechins present in the extract. In addition, Grzesik, Naparło, Bartosz, and Sadowska-Bartosz (2018) demonstrated that catechins have a high capacity for sequestering the ABTS radical compared to other flavonoids. However, they indicated that these compounds display poor bioavailability. Microencapsulation could be an alternative to solve this problem since the application of some polymers may improve the solubility of the bioactive compounds and prevent their degradation in stress conditions, consequently improving their bioavailability.

3.2. Antimicrobial activity and effect of guaraná extracts on the growth of probiotics

Some phenolic-rich plant extract can display antimicrobial potential, which could cause probiotic death. For this reason, the antimicrobial activity of guaraná extracts against the studied probiotics was determined using the agar diffusion technique. In this test, TPC of GSE ranged from 130 to 520 mg Gallic acid equivalent - GAE/mL and TC of GPE varied from 55 to 222 μg carotenoids/mL. No inhibition of either probiotic was observed using pure guaraná extracts and diluted fractions. Based on these results, the simultaneous loading of pure guaraná extracts and probiotics for microencapsulation is feasible to maintain viable probiotic cells.

Another study reported that guaraná extract exhibited inhibitory activity against *Pseudomonas aeruginosa* and *Escherichia coli*, which are two pathogenic and undesirable bacteria in food production (Basile et al., 2005). However, no other study has evaluated the antimicrobial activity of guaraná extracts against probiotics. Similarly, Marinho et al. (2019) reported that jussara pulp did not show inhibitory activity against *Lactobacillus acidophilus* LA3 and *L. paracasei* BGP-1, despite its high content of phenolics and anthocyanins.

Table 2 displays the enumeration of probiotics in MRS broth (control) and MRS broth supplemented with different concentrations of guaraná extracts, evaluating their effect on the growth of probiotics under conditions of prolonged incubation. Regarding L. *paracasei* in the presence of MRS broth without extract, its populations were reduced by approximately 1.5 log CFU/mL after 96 h of incubation at 37 °C. However, the population of *L. paracasei* grown in MRS broth containing 10% GPE increased significantly when incubated for 96 h. On the other hand, the counts of *L. paracasei* in MRS broth containing GSE were similar to those found for *L. paracasei* in MRS broth without extract.

The growth of *B. animalis* subsp. *lactis* BLC-1 in MRS broth without extract was not efficient for maintaining its population during prolonged incubation. However, supplementation of 10% and 20% GPE stimulated the probiotic population, the highest count being obtained after 96 h of incubation. In contrast, the probiotic population in the presence of GSE was maintained until 72 h of incubation.

The different chemical compositions of GSE and GPE are correlated with these findings. GSE is a source of catechins and procyanidins (Silva et al., 2019). In contrast, GPE contains carotenoids, such as lutein and beta-carotene (Pinho et al., 2021), and the addition of sunflower oil to GPE provides tocopherols, albeit in a small quantity. The antioxidant action of phenolics and carotenoids differs, which could explain the differences in the growth of probiotics. For instance, phenolics can act as antioxidants in several ways, mainly as good hydrogen donors (Pereira et al., 2009). Carotenoids can quench oxygen radicals and the sensitized triplet state and avoid lipid peroxidation (Hudson, 1990). In addition, it is essential to highlight the presence of sunflower oil in GPE, albeit in a small quantity, which could also contribute to probiotic survivability due to the presence of tocopherols.

Some studies have asserted that the effect of phenolic compounds on the growth of probiotics depends on the probiotic strains, the chemical structure of polyphenols, and concentration (Campos, Couto, & Hogg, 2003), which supports these variable results found during the growth of probiotics. Similar results were noticed by Hervert-Hernández, Pintado, Rotger, and Goñi (2009) who evaluated the effect of grape pomace extract on the growth of *Lactobacillus acidophilus* CECT 903, reporting a significant increment of probiotic population in the presence of extract. The authors also investigated the effect of pure catechin (standard), verifying that a concentration greater than 96 mg/mL must cause the stimulatory effect. Spray-dried GSE contained around 130 mg of catechins/g of powder (Silva et al., 2019), which probably positively influenced the growth of probiotics. However, another study reported a negative effect of flavonoid aglycones, inhibiting intestinal bacteria (Duda-Chodak, 2012). Thus, not all phenolic compounds and derivatives stimulate the growth of probiotics.

There is a lack of studies evaluating the influence of carotenoids on probiotic growth. GPE had a positive effect on the growth of the probiotic population during incubation, but other compounds extracted,

Table 2
Enumeration of L. *paracasei* BGP-1 (LP) and *B. animalis* subsp. *lactis* BLC-1 (B) populations cultured in MRS broth (control) and MRS broth supplemented by guaraná seed extract (GSE) and guaraná peel extract (GPE).

Time	Control	5% GSE	10% GSE	20% GSE	5% GPE	10% GPE	20% GPE
L. paracasei BGP-1 (LP)							
0 h	5.65 ± 0.07^{Ad}	5.34 ± 0.12^{Ac}	5.54 ± 0.09^{Ac}	5.89 ± 0.27^{Ac}	6.22 ± 0.37^{Ab}	5.95 ± 0.07^{Ab}	6.07 ± 0.32^{Ac}
24 h	8.82 ± 0.31^{Aa}	9.07 ± 0.16^{Aa}	9.00 ± 0.06^{Aa}	8.74 ± 0.06^{Aa}	8.83 ± 0.02^{Aa}	8.59 ± 0.27^{Aa}	8.61 ± 0.52^{Aab}
48 h	8.54 ± 0.09^{Aab}	8.85 ± 0.21^{Aa}	8.72 ± 0.34^{Aa}	9.05 ± 0.14^{Aa}	7.91 ± 1.17^{Aab}	8.85 ± 0.21^{Aa}	9.06 ± 0.08^{Aa}
72 h	8.06 ± 0.03^{Ab}	7.84 ± 0.09^{ABb}	7.66 ± 0.11^{ABb}	7.92 ± 0.11^{ABb}	7.92 ± 0.02^{ABab}	7.93 ± 0.12^{ABab}	8.06 ± 0.05^{Aab}
96 h	7.41 ± 0.02^{Ac}	7.54 ± 0.09^{Ab}	7.63 ± 0.21^{Ab}	7.77 ± 0.10^{Ab}	7.50 ± 0.28^{Aab}	8.44 ± 1.04^{Aa}	7.63 ± 0.22^{Ab}
B. animalis subsp. *lactis* BLC-1 (B)							
0 h	6.11 ± 0.10^{Ac}	6.18 ± 0.26^{Ac}	6.14 ± 0.13^{Ac}	6.02 ± 0.25^{Ac}	6.22 ± 0.09^{Abc}	6.09 ± 0.06^{Ab}	6.16 ± 0.06^{Aa}
24 h	8.16 ± 0.02^{Aa}	8.42 ± 0.03^{Aa}	8.36 ± 0.05^{Aa}	8.19 ± 0.02^{Aab}	7.51 ± 0.05^{Aab}	6.04 ± 0.62^{Bb}	6.35 ± 0.18^{Ba}
48 h	8.31 ± 0.11^{Aa}	8.19 ± 0.02^{Aa}	8.21 ± 0.24^{Aa}	8.59 ± 0.16^{Aa}	8.86 ± 0.68^{Aa}	5.81 ± 0.05^{Bb}	6.22 ± 0.54^{Ba}
72 h	8.36 ± 0.15^{ABa}	8.22 ± 0.06^{ABa}	8.77 ± 0.10^{Aa}	8.36 ± 0.08^{ABa}	7.80 ± 0.02^{ABa}	6.35 ± 0.06^{Bb}	7.22 ± 1.48^{ABa}
96 h	7.27 ± 0.18^{BCb}	7.57 ± 0.10^{ABb}	7.58 ± 0.03^{ABb}	7.77 ± 0.08^{ABb}	6.00 ± 0.31^{Cc}	8.98 ± 0.28^{Aa}	8.16 ± 0.87^{ABa}

In this table: 5% GSE or GPE is the 5% (v/v) GSE or GPE supplementation in MRS broth; 10% GSE or GPE is the 10% (v/v) GSE or GPE supplementation in MRS broth; 20% GSE or GPE is the 20% (v/v) GSE or GPE supplementation in MRS broth. Values are expressed as log CFU/mL. Values are mean ± standard error (SE) (n = 3 analytical replicates). Values with the same upper case letter in a row and values with the same lower case letter in a column are not statistically different ($p > 0.05$).

such as phenolics or even the small quantity of tocopherols due to the presence of sunflower oil, could influence the growth. On the other hand, GSE was pertinent to the increment of the probiotic population in the first 24 h of incubation, after which it was maintained until 72 h of incubation. Also, different concentrations of GSE could be beneficial for increasing probiotic viability. Based on these promising results, further studies could elucidate the stimulatory mechanisms of guaraná extracts, considering differences in the chemical composition. Also, the addition of other species in the media to mimic the human gut microbiota could verify the prebiotic potential of guaraná extracts since it should allow the proliferation only of beneficial species, avoiding the growth of pathogenic ones.

3.3. Hydrophobicity of probiotics

Comprehension of the physical-chemical properties of the probiotic cell wall is relevant because hydrophobic cells present a high capacity to adhere to the intestine (Rijnaarts, Norde, Bouwer, Lyklema, & Zehnder, 1993), as well as affecting the attachment of probiotics in coacervates depending on the encapsulating materials applied.

Results related to the hydrophobicity of the studied probiotics are shown in Fig. 1. Of the probiotics activated in control MRS broth, *L. paracasei* BGP-1 was more hydrophobic (48%) than *B. animalis* subsp. *lactis* BLC-1 (25%). In this context, correlating the characteristics of encapsulant materials, *L. paracasei* would probably be better entrapped or immobilized in a lipid matrix, while *B. animalis* subsp. *lactis* may be protected in a hydrophilic polymer matrix. However, both could be encapsulated in amphiphilic substances, such as proteins, gum Arabic, some modified starches, and others.

Holkem and Favaro-Trindade (2020) investigated the hydrophobicity of the same probiotic strains, reporting values of around 29% and 37% for *L. paracasei* and *B. animalis* subsp. *lactis*, respectively. The differences in hydrophobicity found in this study may be correlated with the duration of incubation of probiotic strains in MRS broth, control of the temperature, and the pH of media.

The same microorganisms grown in the presence of the GSE showed less affinity for n-hexadecane, considering the low hydrophobicity values. On the other hand, incubation in broth supplemented with GPE increased the hydrophobicity of *B. animalis* subsp. *lactis* BLC-1, probably due to the presence of carotenoids, known as hydrophobic antioxidants, in this extract. In addition, some phenolic molecules may affect the cell surface hydrophobicity of probiotics (Santos et al., 2019). Therefore, the supplementation of guaraná extracts in MRS broth during the growth of probiotics affected the hydrophobicity of cells, which could interfere

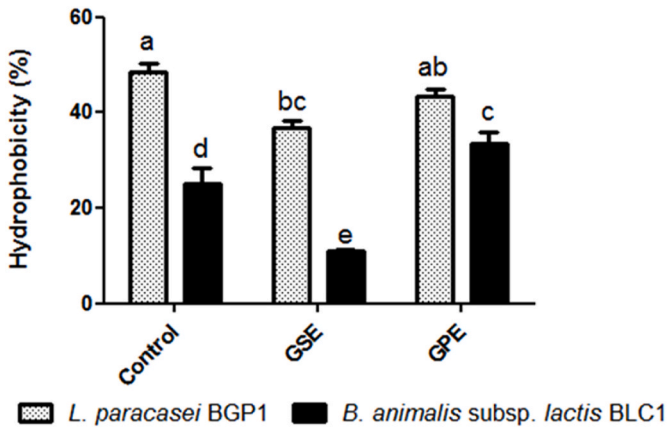

Fig. 1. Hydrophobicity of probiotics cultured in MRS broth or supplemented MRS broth with 10% (v/v) of guaraná seeds extract (GSE), or guaraná peels extract (GPE).
Values are mean ± standard error (SE) (n = 3 analytical replicates). Bars with the same lower case letter in a column are not statistically different ($p > 0.05$).

with the EE and attachment of probiotics in the gut for temporary colonization.

3.4. Characterization of coacervates: morphology and particle size

Fig. 2 displays the morphology of the coacervates, as well as the aspect of powders obtained after freeze-drying. All formulations studied resulted in coacervates with a spherical shape and defined walls, due to the electrostatic interaction between gelatin and gum Arabic.

Besides that, coacervates containing probiotics (Fig. 2 A) and coacervates containing probiotics and GSE (Fig. 2 C) were treated with a live/dead cell viability kit, distinguishing live, compromised membrane and dead cells. Changes of probiotic viability can be related to experimental parameters used in microencapsulation, such as mechanical stress, harmful temperature, pH, etc. In this context, live and dead cells fluoresce as blue and red, respectively. Probiotics entrapped in control coacervates exhibited few red cells and mainly blue cells, indicating the presence of mostly viable probiotics. This finding was expected since probiotic cells are very sensitive. For example, Silva et al. (2018) reported a reduction of probiotic viability, around 2 log CFU/g, after microencapsulation by complex coacervation. Concerning the co-encapsulation of probiotic and GSE, it was noticed mainly blue cells entrapped in this coacervate and outside. It is worth emphasizing that probiotics were also observed outside the coacervates for all samples, even if they were stood out only in the coacervate containing probiotics and GSE.

In contrast, GPE was previously stained with Nile Red instead of live/dead cell viability kit to verify the microencapsulation of lipid extract. In this way, the red droplets inside the coacervates in Fig. 2 (B) confirms the entrapment of GPE. Also, probiotics were not evidenced in this image since this coacervate was not treated with live/dead viability kit.

These results are supported by other studies that encapsulated probiotics by complex coacervation and used a high melting point vegetable fat as a core (Holkem & Favaro-Trindade, 2020; Silva et al., 2018). Lipids enhanced the attachment of probiotics in the core of coacervates, which could increase the protection of probiotic cells by the double layer of polymers. However, the application of lipids in coacervates is limited since they can alter the sensory attributes of food matrices.

Wet coacervates loaded with *L. paracasei* BGP-1 or *B. animalis* subsp. *lactis* BLC-1 had particle sizes of 108.33 ± 0.10 and 131.03 ± 0.15 μm, respectively. On simultaneous loading of the same probiotics and GSE, coacervates maintained their average particle size (112.46 ± 0.31 and 129.46 ± 0.26 μm, respectively). However, the particle sizes of coacervates containing GPE and *L. paracasei* BGP-1 or *B. animalis* subsp. *lactis* BLC-1 were increased significantly: 141.42 ± 0.13 and 139.18 ± 0.12 μm, respectively. Thus, the particle size of wet coacervates ranged from 108 to 141 μm, showing potential for application as supplements in food. Although the desirable size for microcapsules for application in food matrices is above 100 μm, to preserve their textural and sensory properties (Cruxen et al., 2017icrocapsules with an average size of 140 μm in yogurt did not affect the consumers' sensory perception (Silva et al., 2022). Other possibilities for the incorporation of larger particles are solid food matrices, such as cereal bars, peanut butter, and chocolate, since their composition and texture could be favorable to mask them.

In order to improve the viability of probiotics during storage, coacervates were freeze-dried. Fig. 2 shows the aspect of powders, presenting aggregation, which is typical of freeze-dried materials. Furthermore, GPE and GSE provided the powders with yellowish and brownish colors, due to the presence of carotenoids and phenolic compounds, respectively.

Concerning the morphology of freeze-dried coacervates (Fig. 2), similar micrographs were observed in other studies using gelatin and gum Arabic to encapsulate bioactive compounds by complex coacervation. Freeze-dried coacervates formed a net, which may have been caused by encapsulating materials remaining in the middle of the

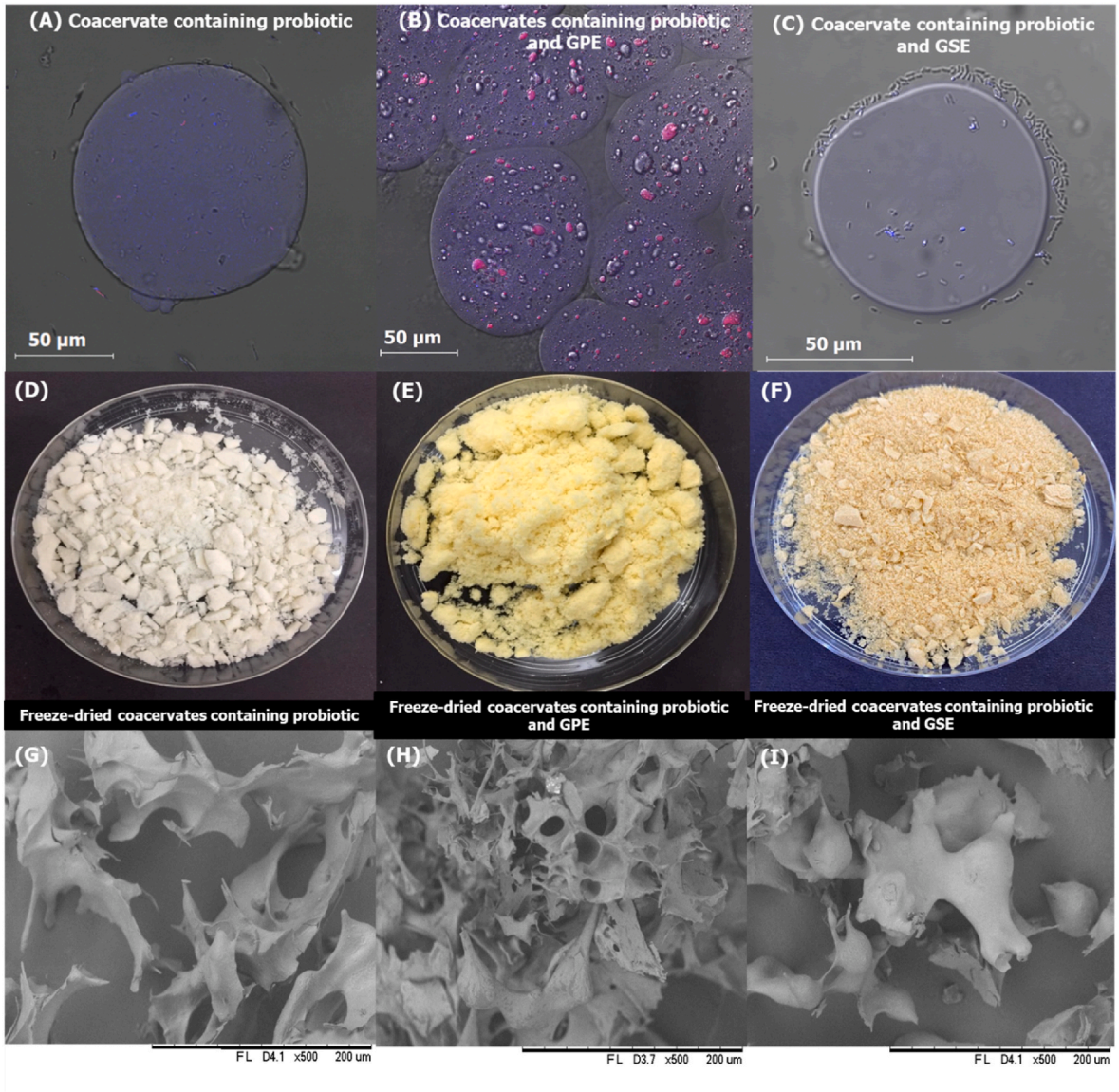

Fig. 2. Confocal microscopy of coacervates (A, B, C), followed by the aspect of powders after freeze-drying (D, E, F) and their micrographs by scanning electron microscopy (G, H, I). In this figure: A, D, G are coacervates containing probiotic (control), B, E, H are coacervates containing probiotic and guaraná peel extract (GPE), C, F, I are coacervates containing probiotic and guaraná seed extract (GSE). Scale bar for confocal microscopy is shown in the lower left of images, corresponding to 50 μm. Scale bar for scanning electron microscopy is shown in the lower right of images, corresponding to 200 μm.

Table 3
Characterization of freeze-dried coacervates containing probiotics *L. paracasei* BGP-1 (LP) or *B. animalis* subsp. *lactis* BLC-1 (B), or freeze-dried coacervates loaded simultaneously with probiotic and guaraná seed extract (GSE) or guaraná peel extract (GPE).

Parameters	Coacervate loaded with LP	Coacervate loaded with LP and GSE	Coacervate loaded with LP and GPE	Coacervate loaded with B	Coacervate loaded with B and GSE	Coacervate loaded with B and GPE
Enumeration of probiotics (log CFU/g of coacervate)	8.33 ± 0.13^{bc}	8.54 ± 0.08^{bc}	8.71 ± 0.05^{ab}	8.13 ± 0.07^{cd}	7.73 ± 0.18^{d}	9.12 ± 0.16^{a}
Total phenolic content (mg GAE/g of coacervate)	–	54.73 ± 3.21^{a}	1.42 ± 0.84^{b}	–	52.94 ± 4.26^{a}	1.69 ± 0.70^{b}
Encapsulation efficiency of phenolics (%)	–	78.12 ± 4.15^{a}	–	–	76.78 ± 3.58^{a}	–
Total carotenoids (μg carotenoids/g of coacervate)	–	–	37.67 ± 1.27^{b}	–	–	43.03 ± 2.40^{a}
Encapsulation efficiency of carotenoids (%)	–	–	82.35 ± 2.88^{a}	–	–	85.72 ± 3.19^{a}

Values are mean ± standard error (SE) (n = 4 analytical replicates). Values with the same lower case letter in a row are not statistically different ($p > 0.05$).

particles, as also observed by Silva et al. (2018).

3.5. Enumeration of probiotics, EE, and quantification of TPC and TC in freeze-dried coacervates

Initial enumerations of *L. paracasei* and *B. animalis* subsp. *lactis* were 10.0 ± 0.2 and 9.9 ± 0.4 log CFU/g of inoculum (data not shown). Depending on the formulation of freeze-dried coacervates, the final counts ranged from 7.7 to 9.1 log CFU/g (Table 3), demonstrating that complex coacervation and freeze-drying were mild processes since the reduction of probiotic populations varied from 2 to 1 log CFU/g. The simultaneous loading of GPE and probiotics in coacervates provided greater viability of probiotics after encapsulation and freeze-drying processes, which could be correlated with their prebiotic potential as mentioned before. On the other hand, the concentration of GSE applied in coacervates loaded with probiotics did not enhance the resistance of probiotics to the process of encapsulation and dehydration since the counts were similar to the control formulations (without guaraná extracts).

Regarding the TPC of freeze-dried coacervates loaded with GSE and the EE for phenolics, values were around 54 mg GAE/g and 78%, respectively. Thus, coacervates may entrap hydrophilic materials, such as phenolic compounds, without the necessity of producing a previous emulsion with oil. It is worth mentioning that guaraná seeds are not a source of carotenoids, only caffeine and phenolic compounds, as reported by other studies (Silva et al., 2019, 2022).

Souza et al. (2018), who encapsulated cinnamon extract by complex coacervation using different polymer pairs, reported similar EE for phenolic compounds. Another study used vegetable fat to immobilize probiotics and cinnamon extract followed by complex coacervation, reporting a TPC of 33–83 mg GAE/g of microcapsules, depending on the proportion of cinnamon extract added to the formulation (Holkem & Favaro-Trindade, 2020). In contrast, these authors reported higher EE of phenolics, varying from 99.9% to 120%, indicating a great advantage of using vegetable fat in complex coacervation for entrapment of hydrophilic materials such as phenolic compounds.

TC and TPC in freeze-dried coacervates loaded with GPE varied from 38 to 43 μg of carotenoids/g and below 2 mg GAE/g, respectively, confirming that extracting solution was efficient to recover mainly carotenoids from guaraná peels. The difference of TC between the coacervates formulations can be related to some experimental drawbacks since lipids and carotenoids could adhere in tubes during quantification. The EE of carotenoids in coacervates was around 84%. These results were expected since complex coacervation shows high EE for hydrophobic materials such as carotenoids. For instance, shrimp lipid extract encapsulated by complex coacervation using gelatin and gum Arabic displayed a high EE of around 93.5% (Gomez-Estaca, Comunian, Monteiro, & Favaro-Trindade, 2018). Another study recovered the carotenoids from tomato peel followed by complex coacervation, showing 83.6% EE for lycopene, and TC of around 44 mg/g in coacervates (Gheonea et al., 2021). Thus, complex coacervation successfully entrapped the bioactive compounds from guaraná extracts and probiotics.

3.6. Release of probiotics in simulated gastrointestinal fluids

Microencapsulation is a potential technology to improve probiotic survivability during harsh conditions. For instance, other studies reported a significant reduction of final counts, around 4–5 log CFU/g, for unencapsulated *B. animalis* subsp. *lactis* BLC-1 and *Lacticaseibacillus paracasei* BGP-1 in simulated gastrointestinal fluids (Holkem, Favaro-Trindade, & Lacroix, 2020; Matos-Jr et al., 2019; Silva et al., 2017). In this sense, microencapsulation is required to increase the preservation of probiotic viability during *in vitro* digestion tests. Furthermore, efficient microcapsules protect probiotics in harsh conditions but release them into the intestine for colonization. Most studies about the encapsulation of probiotics investigate the release or survival of probiotics in simulated gastrointestinal fluids. A release study evaluates the viable probiotics released into simulated gastrointestinal fluids, while a survival study also verifies the viable probiotics that remain in microcapsules. Fig. 3 displays the release of probiotics in simulated gastrointestinal fluids, expressed as a percentage. Initially, the counts for freeze-dried coacervates loaded with probiotics were around 8.5 log CFU/g.

All coacervates released around 45% of probiotic cells at the beginning of the *in vitro* digestion test, which can be correlated with the probiotics attached to the surface of coacervates or the early release of probiotics due to their hydrophilicity. In addition, according to the composition of SGF, low pH and pepsin may facilitate the release of probiotics, as demonstrated by the increment of viable probiotics in SGF after 120 min of the *in vitro* test. For instance, control coacervates loaded with *B. animalis* subsp. *lactis* showed the maximum percentage of release, around 75%, at 120 min. However, coacervates loaded with GPE showed a lower release of probiotics in SGF, demonstrating that GPE probably enhanced the entrapment of probiotic cells in coacervates. Thus, producing a previous emulsion containing probiotics may reduce this premature release of probiotics in SGF.

There was a similar probiotic release for coacervates loaded with only *L. paracasei* BGP-1 (LP – Control) and coacervates loaded simultaneously with *L. paracasei* BGP-1 and GSE at the end of SGF. In this context, a fast release of probiotics in SGF is not desirable since it could cause probiotic death by the harsh conditions. However, the presence of phenolic compounds from GSE could be positive about maintaining the viability of prematurely released probiotics in the middle, indicating a potential advantage of co-encapsulating probiotics with phenolic compounds from plant extracts.

Among the formulations, coacervates loaded with GPE showed a gradual release in simulated gastrointestinal fluids, increasing after applying SIF. The pH adjustment to 7 in SIF, pancreatin, and bile salts may have contributed to the complete dissociation of polymers, delivering the probiotics with more affinity with GPE. In this way, the association of carotenoid-rich extract with probiotics in coacervates was adequate to increase the number of probiotic cells reaching the intestinal phase.

The final release of probiotics was at least 60% in SIF, as can be seen in Fig. 3. Among the coacervates studied, the final counts of probiotics simultaneously encapsulated with guaraná extracts were higher, about

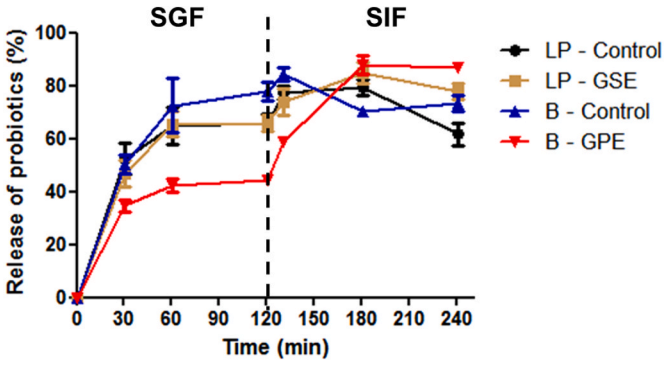

Fig. 3. Release of probiotics in simulated gastric fluid (SGF) for 120 min, sequentially added to the simulated intestinal fluid (SIF) until complete 240 min of the assay, expressed as percentages (%), considering the initial number of viable cells in coacervates. In this Figure: the black line is the control coacervate loaded with *L. paracasei* BGP-1 (LP); the brown line is the coacervate loaded with LP and guaraná seed extract (GSE); the blue line is the control coacervate loaded with *B. animalis* subsp. *lactis* BLC-1 (B); the red line is the coacervate loaded with B and guaraná peel extract (GPE).
Values are mean ± standard error (SE) (n = 3 analytical replicates). . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

6.5–7.2 log CFU/mL. In contrast, the final counts of probiotics released by control coacervates were 5–6 log CFU/mL. For this reason, the co-encapsulation of probiotic and guaraná extracts clearly preserved the viability of probiotics in simulated gastrointestinal fluid. One possible explanation is that the physical-chemical characteristics of guaraná extracts could delay the release of probiotics, probably because bioactive compounds (e.g., phenolics) interacted with the polymers that constitute the coacervates, fortifying their structure. Another study demonstrated that phenolic compounds from GSE may inhibit enzymes, which may favor the probiotic survivability in this middle (Silva et al., 2019). However, further studies evaluating the antioxidant capacity of encapsulated bioactive compounds in simulated gastrointestinal fluids could elucidate the role of phenolics and carotenoids in decreasing the oxidative stress of probiotics.

3.7. Release of phenolic compounds from GSE and carotenoids from GPE in simulated gastrointestinal fluids

3.7.1. Gastric phase

Fig. 4 illustrates the release of phenolic compounds (A) and carotenoids (B) in simulated gastrointestinal fluids, as well as the micrographs obtained during the *in vitro* assay. The cumulative release of phenolic compounds in SGF was around 60%, proving their fast release at low pH and susceptibility of hydrophilic materials encapsulated by complex coacervation. Also, micrographs exhibited the aggregation of coacervates containing GSE in SGF, but the integrity of coacervates was maintained. In this way, the premature release of phenolic compounds from GSE can be correlated with their attachment on the surface of coacervates, facilitating the quick release. Although micrographs could reveal interesting aspects of microcapsules during *in vitro* digestion tests,

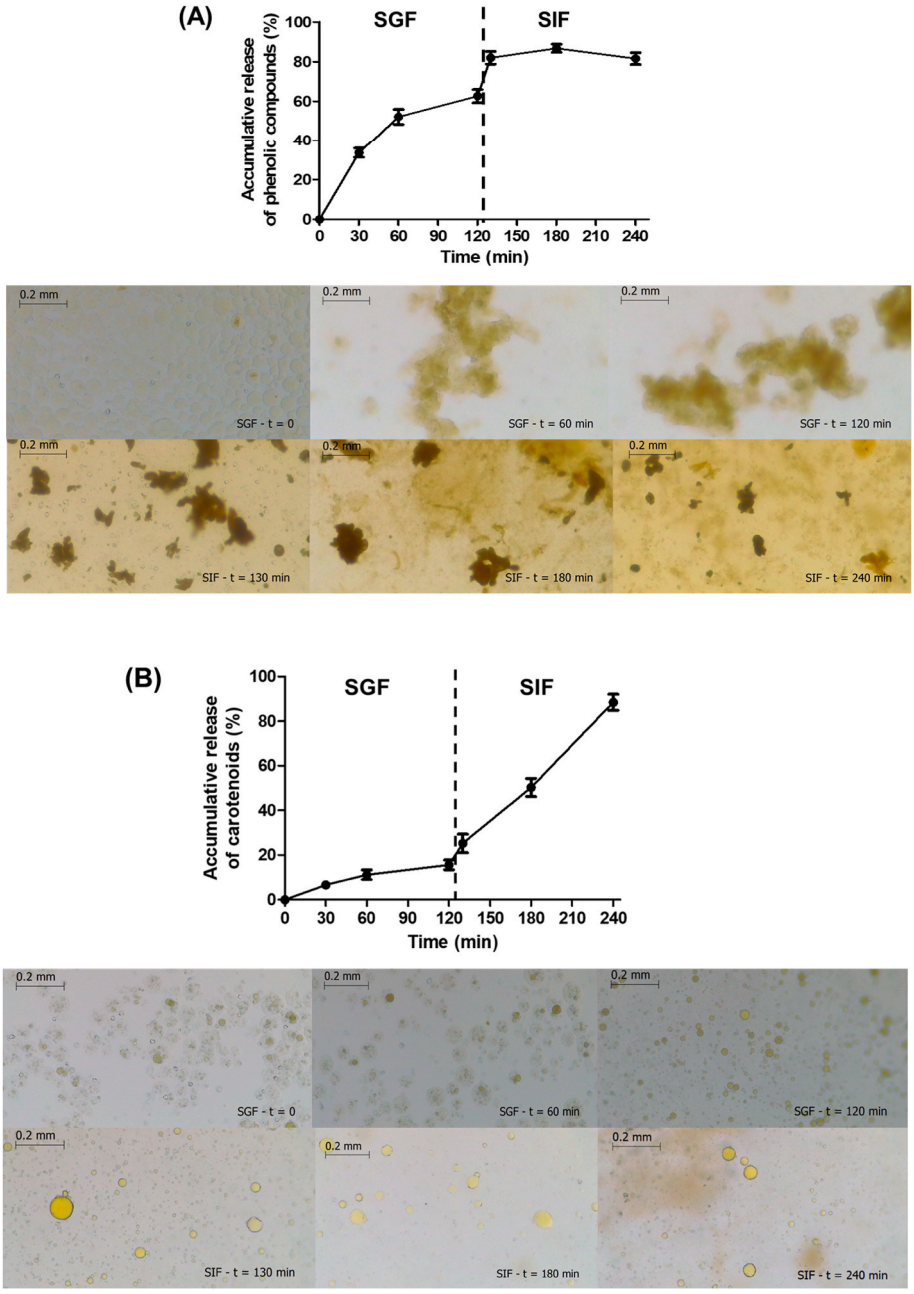

Fig. 4. Micrographs and accumulated release of phenolic compounds from guaraná seed extract (GSE) (A) and carotenoids from guaraná peel extract (GPE) (B) in simulated gastric fluid (SGF) for 120 min, sequentially adding the simulated intestinal fluid (SIF), completing 240 min of the assay. Values are mean ± standard error (SE) (n = 3 analytical replicates).

few studies evaluated the modifications of the morphology of coacervates by optical microscopy (Souza, Comunian, Kasemodel, & Favaro-Trindade, 2019; Holkem et al., 2020).

The microencapsulation of GSE is relevant since another study reported that phenolic compounds from free GSE were sensitive in SGF during *in vitro* digestion (Silva et al., 2019). Furthermore, after the microencapsulation of GSE by spray chilling using vegetable fat, the resulting lipid particles were highly hydrophobic and reduced the quick release of phenolic compounds in SGF (Silva et al., 2019). Similarly, phenolics from cinnamon extract co-encapsulated with probiotics by complex coacervation using vegetable fat, whey protein concentrate, and gum Arabic were mainly released in SIF (Holkem et al., 2020), indicating the beneficial role of lipids for controlled release of phenolics. Thus, the production of a previous emulsion of GSE or its immobilization in vegetable fat could protect phenolics in SGF and control their release in SIF.

Differently, coacervates loaded with carotenoids from GPE showed a lower release of carotenoids, about 13% in SGF. The composition of SGF with low pH and pepsin solution did not trigger the release of carotenoids, maintaining GPE entrapped in coacervates. This finding was confirmed by optical microscopy since most of the coacervates were intact, as seen by the well-delimited edges around the yellow multinucleate. In this sense, complex coacervation was efficient to protect GPE in SGF, proving to be an excellent encapsulation technique for protecting lipophilic compounds.

Similar results were reported by Neagu et al. (2020), who encapsulated oleoresin extract from sea buckthorn by complex coacervation, showing around 9% release of carotenoids in SGF. Thus, complex coacervation was efficient for preventing the premature release of carotenoids in SGF.

3.7.2. *Intestinal phase*

The application of SIF did not significantly affect the release of phenolic compounds from GSE since most phenolics had been released previously, which could make these compounds more susceptible to degradation. However, after the addition of SIF, micrographs displayed an increase in the brownish coloration of the middle, which was composed of electrolytic, pancreatin, and bile salts solutions at pH 7. This modification of color in the middle, which mimics SIF, may partly occur due to the addition of pancreatin solution, which has this characteristic color. Additionally, phenolic compounds could interact with the enzymes and bile salts, changing the color of the remaining coacervates to dark brown. Likewise, Souza et al. (2019) evaluated the combination of double emulsion and complex coacervation using gelatin and gum Arabic for encapsulation of lactase, indicating that aggregates at the end of SIF could be an association of digested polymers.

Another study evaluated the kinetics of release for coacervates loaded with a double emulsion containing anthocyanin extract in simulated gastrointestinal fluids (Kanha, Regenstein, Surawang, Pitchakarn, & Laokuldilok, 2021). Although freeze-dried coacervates showed a cumulative release of around 40% of anthocyanins in SGF at 120 min, the application of SIF released about 80% of anthocyanins (Kanha et al., 2021). These authors indicated an erosion mechanism of release for freeze-dried coacervates.

Concerning the coacervates loaded with GPE, the change of pH by adding SIF and the bile salts and pancreatin solution facilitated the release of carotenoids. In addition, the choice of gum Arabic as encapsulating material can improve micellization due to its emulsifying capacity, consequently facilitating the carotenoid absorption (Montero, Calvo, Gómez-Guillén, & Gómez-Estaca, 2016).

These results can be correlated with the micrographs shown in Fig. 4 (B) since the coacervates loaded with GPE displayed their rupture after the addition of SIF by the increase of oil droplets in the middle containing electrolytic, pancreatin, bile salts solutions with pH adjusted to 7. Furthermore, the presence of varied oil droplet size at the end of SIF is associated with the action of bile salts and pancreatin, which are responsible for lipid digestion. Similarly, another study reported that SIF was decisive to release bioactive compounds and probiotics from coacervates, confirmed by optical microscopy (Holkem, Favaro-Trindade, & Lacroix, 2020).

Based on these results, the chemical characteristics of bioactive compounds and the adjustment of pH by adding simulated gastrointestinal fluids affected the electrostatic interaction of the polymers, releasing guaraná extracts. Furthermore, gelatin and gum Arabic have a negative charge at pH 7, and repulsive force may dissociate the coacervates. These results are in accordance with Zhou et al. (2018) who reported the dissociation of microcapsules loaded with astaxanthin oleoresin obtained by complex coacervation using whey protein and gum Arabic after a change of pH and application of digestive enzymes.

At the end of the *in vitro* digestion test, the total release of carotenoids and phenolic compounds was about 90% and 80%, respectively, demonstrating that microencapsulation successfully released bioactive compounds from guaraná extract and probiotics in simulated gastrointestinal fluids. In this context, the simultaneous release in the gut could provide synergistic interactions between them, enhancing their health benefits.

4. Conclusions

The use of guaraná seeds and peel to extract bioactive compounds originated two extracts with different chemical profiles and with antioxidant potential. In addition, the supplementation of MRS broth with GPE positively affected the growth of probiotics, demonstrating a potential prebiotic activity of this guaraná by-product. Although coacervates displayed fast release of probiotics and phenolic compounds from GSE in gastric phase, a considerable quantity of phenolics and probiotics reached the intestinal phase. However, complex coacervation protected carotenoids from GPE and probiotics, delaying their release in simulated gastrointestinal fluids. Therefore, co-encapsulation improved the protection of probiotics in simulated gastrointestinal fluids since probiotic counts were higher than for coacervates loaded only with probiotics. Also, these freeze-dried coacervates could be applied in food for supplementation of bioactive compounds and probiotics, providing simultaneous release in simulated gastrointestinal fluids.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Marluci Palazzolli Silva: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft. **Milena Martelli-Tosi:** Methodology, Investigation, Data curation. **Adna Prado Massarioli:** Methodology, Investigation, Data curation. **Priscilla Siqueira Melo:** Methodology, Investigation, Data curation. **Severino Matias Alencar:** Methodology, Investigation, Data curation. **Carmen S. Fávaro-Trindade:** Conceptualization, Supervision, Writing – review & editing.

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