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MICROENCAPSULATION OF *LACTOBACILLUS PLANTARUM* IN W/O EMULSIONS OF OKARA OIL AND BLOCK-COPOLYMERS OF POLY(ACRYLIC ACID) AND PLURONIC USING MICROFLUIDIC DEVICES

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ABSTRACT

Okara oil is a by-product remaining from defatting okara, the solid residue generated after extracting the aqueous fraction of grounded soybeans in the elaboration of soy beverages. The goal of this work was to encapsulate the probiotic *Lactobacillus plantarum* CIDCA 83114 into W/O emulsions composed of a block-copolymer constituted of pluronic® and acrylic acid (PPP12) and okara oil, prepared in microfluidic devices. For comparative purposes, alginate was also included as a second dispersed phase. *Lactobacillus plantarum* CIDCA 83114 was suspended in PPP12 or alginate giving rise to dispersed phases with different compositions, named I, II, III and IV. Controls were prepared by suspending microorganisms in water as dispersed phase. 6-carboxyfluorescein was added as bacterial marker in all the emulsions. The presence of green dyed bacteria in the dispersed phases, inside the droplets of the emulsions and the absence of fluorescence outside them, confirmed the complete encapsulation of bacteria in the dispersed phases. After being prepared, emulsions were freeze-dried. The exposure to gastric conditions did not lead to significant differences among the emulsions containing polymers. However, in all cases bacterial counts were significantly lower than those of the control. After exposing emulsions to the simulated intestinal environment, bacterial counts in assays I, II and III (emulsions composed of only one dispersed phase or of two dispersed phases with bacteria resuspended in the PPP12 one) were significantly greater than those of the control (p<0.05) and no detectable microorganisms were observed for assay IV (emulsions composed of two dispersed phases with bacteria resuspended in the alginate one). In particular, bacterial cultivability in emulsions corresponding to assay I (only PPP12 as dispersed phase) exposed to the intestinal environment was 8.22±0.02 log CFU/mL (2 log CFU higher than the values obtained after gastric digestion). These results support the role of PPP12 as an adequate co-polymer to protect probiotics from the gastric environment, enabling their release in the gut, with great potential for food or nutraceutical applications.

Keywords: microfluidics; pluronic-poly(acrylic) acid co-polymer; okara oil; gastro-intestinal digestion; lactobacilli release.

Abbreviations

PAA: poly(acrylic acid)

PLU: pluronic®

PPP12: PAA-PLU block-copolymer

^{1}H NMR: hydrogen nuclear magnetic resonance

PDMS: poly(dimethylsiloxane)

PBS: phosphate saline buffer

MRS: de Man, Rogosa, Sharpe culture medium

CFU: colony forming units

CF: carboxyfluorescein

W/O: water/oil

1. INTRODUCTION

Okara oil is a by-product remaining from defatting okara, the solid residue generated after extracting the aqueous fraction of grounded soybeans in the elaboration of soy beverages (Stanojevic, Bara, Pesic et al., 2013; Scialabba, 2014). It is mainly composed of C18:2 and C18:1 fatty acids, representing altogether *ca.* 75% of the total fatty acids, with lower amounts of C18:3 fatty acid (Quintana, Gerbino, & Gomez-Zavaglia, 2018), phytosterols and phenolic compounds (*e.g.,* isoflavones, genistein, daidzein). Because of their nutritional relevance, okara oil components can be recovered and applied in the cosmetic, pharmaceutical and food industries (Quitain, Oro, Kato et al., 2006; Borhan, Gani, & Shamsuddin, 2014; Quintana, Gerbino & Gomez-Zavaglia, 2018) for drug delivery, butter production, salad dressing or in the formulation of pharmaceutical or food emulsions (Quintana et al., 2018; Latheef, 2012).

Lactic acid bacteria are extensively used as starters in the development of food and probiotic products. The increasing demand of the food industry for functional foods containing probiotic bacteria underlines the need of implementing appropriate processes ensuring bacterial counts fitting the requirements of the international regulation organisms [*e.g.*, EFSA, FDA >6-7 log colony forming units (CFU)/g] (Aquilina, Bach, Bampidis et al., 2013). Encapsulation of probiotics is an extended strategy to maintain their mechanical integrity during production (*e.g.,* oxidation, shear, exposure to high/low temperatures) and dehydration processes (packaging and environmental conditions, including moisture, oxygen, temperature, etc), as well as when exposed to gastro-intestinal conditions (Heidebach, Först, & Kulozik, 2010; Sandoval-Castilla, Lobato-Calleros, Garcia-Galindo et al., 2010). The encapsulation of probiotic bacteria into emulsion droplets of different compositions (Pimentel-Gonzalez, Campos-Montiel, Lobato-Calleros et al., 2009) followed by dehydration (*e.g.* freeze-drying, spray-drying) has been successfully used to stabilize different bacterial strains (Rokka & Rantamäki, 2010; Zhang, Lin, & Zhong, 2015; Rodriguez-Huezo, Estrada-Fernandez, Garcia-Almendarez et al., 2014) into microcapsules of different types and sizes (Champagne & Fustier, 2007).

Among the methods employed to prepare emulsions, microfluidic technology allows the control of small amounts of fluids through micrometer-scale channels, enabling the production of droplet-based biomaterials (Francesko, Cardoso, & Lanceros-Mendez, 2019). It offers an alternative and versatile way to obtain emulsions with an exquisite control over the size, number and properties of each droplet, which are formed one-by-one (Utada, Lorenceau, Link et al., 2005; Chu, Utada, Shah, et al., 2007). Microfluidics has been used for the preparation of monodispersed vesicles constituted of particles with different internal structures or for precisely incorporating specific materials inside emulsions (Vinner, Vladisavljević, Clokie, et al., 2017; Bazban-Shotorbani, Dashtimoghadam, Karkhaneh et al., 2016; Håti, Basssett, Ribe et al., 2016; Marquis, Davy, Cathala, et al., 2015). As microfluidic devices have micrometrical dimensions comparable to the size of cells, this approach represents an invaluable tool to investigate complex cellular systems (Niu & de Mello, 2012). In spite of that, no attempts have been made to use it for the encapsulation of lactic acid bacteria.

Taking advantage of microfluidic devices, using them for encapsulating probiotic bacteria would not only allow their incorporation into the droplets with greater accuracy, but it would also offer the possibility of incorporating additional materials to better protect microorganisms during the processing and exposure to gastro-intestinal conditions. In this respect, the incorporation of probiotics into polymers that are not

hydrolyzed along the gastro-intestinal tract (*e.g.*, pectins, inulin, synthetically engineered polymers) represents an important advantage to enable their safe arrival to their target, that is, the gut (Ghibaudo, Gerbino, Copello et al., 2019; Ghibaudo, Gerbino, Hugo et al., 2018; Ghibaudo, Gerbino, Campo Dall´Orto et al., 2017; Cassani, Gomez-Zavaglia & Simal-Gandara, 2020). Poly(acrylic acid) (PAA) is a biocompatible and biodegradable anionic polyelectrolyte (pKa: 4.95), and pluronic® (PLU) is composed of triblock copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) (PEO–PPO–PEO) (Figure 1). The pluronic PEO block is hydrophilic and water soluble while the PPO block is hydrophobic and water insoluble, thus giving Pluronics an amphiphilic attribute (Quintana, Simões, Hugo et al., 2017). PAA-PLU-based co-polymers (PPP) have demonstrated to overcome gastro-intestinal environment without decomposing because of the amphiphilic properties of PLU and the resistance of PAA to the acid gastric environment. For this reason, they have been used for the controlled release of drugs whose target is the gut (Barreiro-Iglesias, Bromberg, Temchenko et al., 2005; Kabanov, Batrakova, & Alakhov, 2002). Besides such structural and physiological advantages, PPP is less viscous than other biocompatible polymers, which can facilitate the preparation of emulsions in microfluidic devices. In spite of these advantages, PPP has been scarcely used to encapsulate probiotics, whose target is also the gut (Quintana et al., 2017; Feng, Wei, Hu et al., 2020; Seifert, Kashi & Livney, 2019).

Taking into account the importance of adding value to okara oil, the precision of microfluidics for the formulation of emulsions, and the structural and physiological advantages of PPP, the goal of this work was to encapsulate the probiotic strain *Lactobacillus plantarum* CIDCA 83114 into O/W emulsions composed of okara oil and PPP. To achieve this goal, four different compositions of emulsions have been prepared using microfluidic devices, and then, freeze-dried. As alginate is generally used as dispersed phase in microfluidic devices, it was also employed as a second dispersed phase to compare the encapsulating capabilities of PPP (Campaña, Sotelo, Oliva et al., 2020; Håti, Basssett, Ribe et al., 2016; Marquis, Davy, Cathala, et al., 2015; Chen, Kim, Zhang et al., 2013; Ma, Pacan, Wang et al., 2012; Kalvanaraman, Retterer, McKnight et al., 2009). The efficiency of encapsulation and the droplets' size were determined by optical and fluorescence microscopy. The protective effect of PPP on microorganisms was assessed after exposing the emulsions to simulated gastro-intestinal conditions.

2. MATERIALS AND METHODS

2.1. Materials

Okara was obtained from Soyana S.H. (San Martín, Argentina); low fat milk (Difco, NJ, USA); MRS broth (Biokar Diagnostics, Paris, France); agar (Parafarm, Buenos Aires, Argentina); potassium chloride (Anedra, Buenos Aires, Argentina); sodium bicarbonate (Biopack, Buenos Aires, Argentina); calcium chloride (Panreac, Barcelona, Spain); sodium biphosphate (Anedra, Buenos Aires, Argentina); potassium biphosphate (J.T. Baker, NJ, USA); magnesium chloride (Riedel-de Haen, Heidelberg, Germany); hydrochloric acid (Fisher Scientific, Waltham, MA, USA); petroleum ether (Biopack, Buenos Aires, Argentina); ethyl ether (Biopack, Buenos Aires, Argentina); hexane (VWR Chemicals, Radnor, PA, USA), elastomer (SYLGARD™ 184 Silicone Elastomer Base, Midland, MI, USA); curing agent (SYLGARD™ 184 Silicone Elastomer Curing Agent, Midland, MI, USA); lecithin (Scharlab, Barcelona, Spain); sodium alginate (Alfa Aesar, Tewksbury,

MA, USA), 6-carboxyfluorescein (Fluka, BioChemika, Barcelona, Spain), fine-caliber polyethylene tubing (Portex, Smiths medical, Keene, NH, USA). All the following reagents were from Sigma-Aldrich (St. Louis, MO, USA): sodium chloride, ammonium chloride, mucin (Type II), pepsin (P7125), pancreatin (P1625), lipase (Type II), bile salts (P48305), Pluronic acid F68, acrylic acid, Tween 80, lauroyl peroxide, 4,4'-azobis (4-cyanovaleric acid), nonane and trichloro (1H,1H,2H,2H-perfluorooctyl)silane.

2.2. Methods

2.2.1. Extraction of oil from okara

Once received, okara was centrifuged 5 times to remove the excess of water. The solid sediment was frozen at -80°C for 48 hours and freeze-dried in a Heto FD4 (Heto Lab Equipment, Denmark) equipment for 48 hours (temperature of the condenser: -50°C; chamber pressure: 0.04 mbar). Okara oil was extracted with diethyl ether solvent (boiling point 34.6 °C) for 2 hours in a Soxhlet system (AOAC, 1995).

2.2.2. Synthesis of PPP copolymer

PPP was synthesized through polymerization by emulsion, according to Choo & Xue (2011) and Quintana et al. (2017). The reaction scheme is presented in Figure 1. Briefly, PLU F68 was dissolved in acrylic acid by addition of 0.5 mL of 5 M NaOH under constant shaking in a nitrogen stream. Acrylic acid was proportionally added taking into account the number of –OH terminal groups in PLU to obtain a copolymer with equal number of acrylic acid repeating units. Hundred mg of lauryl peroxide and 100 mg of 4-azobis-(4-cyanovaleric acid) were dissolved in 5 mL of acrylic acid, and then incorporated into the PLU solution. This final solution was introduced into a 500 mL round bottom 3-necked flask with 250 mL of Tween 80 solution in nonane (1% v/v) previously deoxygenated, for 1 hour under a constant nitrogen flow. The reaction was carried out at 70 °C for 10 hours. The obtained polymer, named PPP12, was washed in hexane and water to remove impurities, and dried in a vacuum oven at 40 °C for 48 hours. It was characterized by ^{1}H NMR using a Bruker Avance III 400 MHz spectrometer (Quintana et al., 2017), with theoretical molecular weight of 12 kDa.

2.2.3. Bacterial strain and culture conditions

Lactobacillus plantarum CIDCA 83114 was isolated from kefir grains (Garrote, Abraham, & De Antoni, 2001) and preserved at -80°C in 120 g/L of non-fat milk solids. Microorganisms were cultured twice in MRS broth (de Man, Rogosa, & Sharpe, 1960) at 37 ºC in aerobic conditions because of its microaerophilic character (Quintana et al., 2018). Cultures in the stationary phase (~1×10^{11} CFU/mL) were harvested by centrifugation at 8000 rpm (Hermle Centrifuge Z 326 K, Gosheim, Germany) for 10 minutes, washed twice with phosphate saline buffer (PBS, KH_2PO_4 0.144 g/L, NaCl 9 g/L, Na_2HPO_4 0.795 g/L, pH 7.2) and suspended in the corresponding dispersed phase (PPP12 or alginate as explained in ***section 2.2.5.2*** and Table 1) or in PBS (non-emulsified microorganisms), in the same volume as the original bacterial culture.

2.2.4. Building of microfluidic device (chips)

Microfluidic chips are micro-channels etched or molded into a material (elastomer), connected together to achieve the desired features of the fluids. For the fabrication of the poly(dimethylsiloxane) (PDMS) devices used in this work, a mixture of PDMS (PDMS, Sylgard 184) and cross-linker (ratio 10:1, w/w) were thoroughly mixed and further placed in a desiccator connected to a vacuum pump. Once completely degassed, the material was poured over a Petri dish where a master with the appropriated channel structure (obtained by soft lithographic techniques and kindly donated by Dr H. van Vliet from Delft University of Technology, Netherlands) was sealed. After a second degassing, the mold was covered and placed overnight in a stove at 65 °C for the curing process. After cooling down the molds to 25 °C, the area surrounding the quadrant, where the chips were located, was carefully peeled off with a scalpel. One mm diameter holes were drilled with a retractable hole puncher (50 or 200 μm) to define the inlet and outlet points. Finally, the chips were joined to a microscope glass slide and sealed with oxygen plasma in a clean room at the Autonomous University of Madrid (Spain) and flowed with trichloro (1H,1H,2H,2H-perfluorooctyl)silane in fluorinated oil HFE-7500 (1%, w/w; 3M) to provide a channel with hydrophobic surfaces (Zinchenko, Devenish, Kintses et al., 2014). After that, the chips were ready-to-use (Figure S1).

2.2.5. Formulation of emulsions

2.2.5.1. Assembling of equipment. Chips with 50 or 200 μm internal diameter channels and two inlet and one outlet (Figure S1A), and three inlet and one outlet points (Figure S1B) were used for generating the water-in-oil emulsions (W/O). The composition of the continuous and dispersed phases used for the different assays is shown in Table 1. Both the continuous and dispersed phases were injected with glass syringes (1 mL and 100 μL, respectively) using Nemesys Apparatus pumps controlled through a Nemesys software (Nemesys Interface, Madrid, Spain). Capillary polyethylene tubes (internal diameter: 0.38 mm) were used to connect the syringes to the chips' inlet and outlet points. W/O emulsions were collected off-chip. The formation of the emulsions in the chips was monitored in real time in an optical microscope (Optika, Ponteranica, Italy) connected to a "pike" camera using Vimba software (Vimba v3.1 ARM64, Germany).

2.2.5.2. Preparation of water/oil (W/O) emulsions. Okara oil containing 4% (w/v) of lecithin (incorporated as emulsifier) was used as continuous phase in all the assays. PPP12 prepared at 2% (w/v) in 0.5 M NaCl (pH 6) and sodium alginate prepared at 1.5% (w/v) in distilled water were used as dispersed phases. As mentioned in ***section 2.2.3***, *L. plantarum* CIDCA 83114 was suspended in the dispersed phases. 6-carboxyfluorescein (CF, 0.5 μM) was used as marker of the emulsions (Table 1).

2.2.6. Freeze-drying process and plate counting

The freshly obtained emulsions were frozen at -80 °C for 48 hours, freeze-dried in a Lyoquest -85 equipment (Telstar Technologies, S.L., Spain) at a continuous temperature of -85 °C for 48 hours, and stored at 4 °C in silica gel containing recipients. Non-emulsified microorganisms (section 2.2.3) were freeze-dried in PBS in the same conditions.

Microorganisms contained in the emulsions were plate counted before and after freeze-drying. To this aim, samples (powders) were rehydrated in PBS and then, serially diluted in PBS, plated on MRS agar and

incubated at 37 °C in aerobic conditions for 48 hours. Non-dehydrated bacteria (fresh cultures harvested and not freeze-dried) were directly diluted in PBS and plate counted as for freeze-dried ones. Results were expressed as log CFU/mL.

2.2.7. Fluorescence microscopy and droplets' size

Before and after freeze-drying, the emulsions stained with 6-carboxyfluorescein were observed in a fluorescence microscope (Nikon Model Eclipse TS100LED-F MV, Germany) using Vimba software (Vimba v3.1 ARM64, Germany). Fast Read 102^{R} plates composed of 10 counting chambers were used to observe the emulsions' droplets and determine their size (Fiji software, Madison, Java8, Germany) (Figure S2). An average of not less than 50 determinations was reported.

2.2.8. Simulated gastrointestinal digestion

After freeze-drying, the emulsions were digested in simulated gastrointestinal solutions (emulsion:fluid ratio = 1), according to Quintana, Spinola, Martins, et al. (2020). The detailed composition of digestive juices (gastric, intestinal and bile) is provided in Table 2. The process was divided into two stages, gastric and intestinal digestion. For experimental convenience, samples were divided into two batches, one of them only exposed to gastric digestion and the other one, exposed to the whole digestive process, and then used to perform determinations. The digestion process was executed as follows: both experimental batches of samples (assays I, II, III, IV and control; Table 1) were suspended in 1 mL of gastric solution and incubated for 2 hours at 37 °C under continuous shaking (190 rpm, MaxQ 4000, Thermo Scientific, USA), protected from light. Samples were then centrifuged at 4000 rpm for 10 minutes at 25 ºC to enable bacteria sedimentation. One of the sample batches was washed with PBS and kept for plate counting, and the second one was exposed to the simulated intestinal environment. To simulate the intestinal fluid, 0.5 mL of duodenal solution and 0.5 mL of biliary solution were added to the gastric digested emulsions (Table 2) and incubated for 2 hours at 37 °C under continuous shaking (190 rpm, MaxQ 4000, Thermo Scientific, USA). After that, samples were centrifuged again at 4000 rpm for 10 minutes at 25 ºC, and the bacterial pellets were washed once with PBS.

After each digestion stage, the samples were serially diluted in PBS and microorganisms were plate counted as described in ***section 2.2.6***. Results were expressed as log CFU/mL.

2.2.9. Statistical analysis

All experiments were conducted in triplicate and on independent bacterial cultures. Average values were used for data analysis. Analysis of variance (ANOVA) was carried out for all the assays, using the statistical program InfoStat 2008 (Infostat Group/FCA. National University of Cordoba. Ed. Brujas, Cordoba, Argentina). Comparison of means was tested using Tukey's method, and if $p<0.05$, the difference was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Formulation of W/O emulsions in microfluidic devices

The size of the inlet and outlet points of the chips was of great importance to formulate emulsions. Using 50 µm diameter chips led to major experimental limitations, mainly arising from obstruction problems due to the high viscosity of the continuous phase and dispersed phases (data not shown). For this reason, experiments were set-up using 200 µm diameter chips. This posed several advantages over the smaller ones, including the possibility of using greater flow rates and higher concentrations of PPP12, alginate and lecithin, the possibility of simultaneously using more than one polymer (*e.g.* PPP12 and alginate), and the avoidance of obstruction problems, thus enabling the re-utilization of chips. Table 1 depicts the flow rates and flow rate ratios of the dispersed and continuous phases employed in the different assays (I, II, III, IV and control). PPP12 and alginate were used in the highest concentrations enabling the work without experimental limitations arising from obstruction problems associated to the use of viscous solutions in microfluidic devices. Considering the emulsifying role of lecithin, the concentration used was that allowing a perfect dilution in the okara oil. In these experimental conditions, stable and uniformly size droplets were regularly generated, as determined by direct microscopic observation.

At the flow rates reported in Table 1, a steady flow of droplets in the T-junction (contact point of the continuous and dispersed phases) was achieved (Figure 2, images on the left -a-), and these droplets were transported through the channel of the chip to the outlet point (Figure 2, images on the right -b-).

The composition of the emulsions and the flow rates of the continuous and dispersed phases determined the cutting forces of the droplets at the moment of its formation (Vinner et al., 2017), which ultimately determined the characteristics of the emulsions formulated in the four assays. For alginate containing emulsions a longer dispersed phase was observed at cutting point (where the droplets are formed) (Figure S3). This behavior results from the high flow rates of the dispersed phase compared to those of the continuous phases, which prevents the continuous phase to break up the flow and form droplets (Figure S3). Besides the flow rates, the concentration of alginate can also cause the extension of the dispersed phases (Vinner et al., 2017; Mazutis, Vasiliauskas, & Weitz, 2015).

To avoid an extension of the disperse phase in alginate containing emulsions, different flow rate ratios had to be settled taking into account the composition of the disperse phases (Table 1). Three different flow rate ratios were settled for emulsions prepared in devices with two inlet points (assay I, III and control). The different viscosity of PPP12 and alginate strongly determined the set-up of a flow rate enabling a steady flow of droplets formation. In assay III (dispersed phase containing just alginate), a lower dispersed/continuous flow rates ratio (1:10) was required to enable droplets formation. In the control, the dispersed phase (water) did not have the same viscosity of alginate or PPP12 solutions, and the flow rate ratio was not a determinant factor to achieve a steady flow of droplets formation. Other authors also reported that changing the flow rates of dispersed and continuous phases leads to changes in the droplets' size (Eun, Utada, Copeland et al., 2011; Utech, Prodanovic, Mao et al., 2015).

For emulsions prepared in devices with three inlet points (assays II and IV) the same flow rate ratio was settled (1:6) regardless the composition of the dispersed phases (PPP12 or alginate), and regardless the polymer in the internal dispersed phase containing microorganisms. When increasing the flow rate of the dispersed

phase or decreasing that of the continuous phase, an extension of the dispersed phase was also observed (Figure S3).

Figure 3 depicts the images of fresh W/O emulsions obtained using microfluidic devices. The emulsions' droplets had spherical shapes surrounded by a ring in all the assays (I, II, III, IV). That ring was particularly evident in assays II and IV, probably due to the use of two different dispersed phases. The control showed steady and spherical droplets as well, but just surrounded by a thin crust (Figure 3C). *L. plantarum* CIDCA 83114 was observed in the dispersed phases of the emulsions (red arrows in Figure 3). The absence of bacteria outside the emulsions confirmed their correct encapsulation. The labelled emulsions looked like pretty similar and Figure 3F shows a representative image (corresponding to the control) without bacteria outside, which confirmed this observation. Other authors also reported similar rings surrounding the droplets of microfluidics-obtained emulsions and the absence of microorganisms outside them, although the composition of such emulsions was different (Barlow, Gozzi, Kelley et al., 2017; Eun et al., 2015).

Figure 4 shows the size of the droplets corresponding to assays I, II, III, IV before and after freeze-drying. The droplets corresponding to fresh emulsions were within 143 and 180 μm (gray bars, Figure 4) and no significant differences were observed among assays (I, II, III, IV) nor in the control. The droplets corresponding to the emulsions after freeze-drying were within 156 and 208 μm (dashed bars, Figure 4). No significant differences were observed for the droplets' size before and after freeze-drying, except for assay IV and for the control. Dehydration involved in the freeze-drying process could explain the increase in the control droplets' size because the removal of water in the absence of emulsions makes them prompter to aggregate. The increase in droplet size of assay IV follows the same principle. Samples corresponding that assay were the only ones composed of two dispersed phases, PPP12 being the last to be incorporated. The dehydration/hydration process changes the surrounding environment (salts concentration, pH) and therefore the polymer conformation, primarily of the PAA segment and concomitantly of the PPP as a whole, thus possibly contributing to change the droplet size.

3.2. Simulated gastro-intestinal digestion

In order to avoid thermal treatment with unknown effect on the polymers used, freeze-drying was selected as dehydrated method for the obtained emulsions, as it does not have negative effects on the polymers employed (Quintana et al., 2017). The protective effect of polymers on microorganisms was evaluated by determining the bacterial release after exposing the freeze-dried W/O emulsions (I, II, III, IV, control) to simulated gastro-intestinal conditions.

Figure 5 shows the log CFU/mL before and after each digestion stage. Before digestion, bacterial viability in polymers' containing emulsions was significantly lower than that corresponding to the control (9.28±0.06 log CFU/mL) (gray bars in Figure 5). After exposure to gastric conditions, bacterial counts for the control were significantly lower than those before digestion ($p<0.05$) but still greater than those corresponding to assays I, II, III and IV ($p<0.05$) (white bars in Figure 5). No significant differences in bacterial counts were observed among polymers' containing emulsions after exposure to gastric conditions ($p>0.05$).

After exposing the emulsions to the simulated intestinal environment, bacterial counts in assays I, II and III were significantly greater than those of the control (p<0.05) (dashed bars in Figure 5). No detectable microorganisms were observed for assay IV (containing two dispersed phases). The most remarkable result was that obtained for assay I (bacterial suspended in PPP12, Table 1). In assay I, bacterial counts were significantly higher than those observed for the other two assays (II and III). In fact, bacterial cultivability in assay I emulsions exposed to the intestinal environment was 8.22±0.02 log CFU/mL, that is, *ca.* 2 log CFU higher than the values obtained after gastric digestion. This indicates that PPP12 used as only dispersed phase resulted in the best formulation to protect microorganisms from gastric conditions and enable their release in the gut. The high concentration of released bacteria in assay I fits well within the requirements of the international organisms (EFSA, FDA) regarding probiotic containing products, stated as at least 6-7 log CFU of viable microorganisms per gram of product at the moment of being consumed (Aquilina et al., 2013).

In a previous work, it was pointed out that the bacterial surface has a negative charge (Quintana et al., 2017). It is therefore interesting that the first layer of polymer placed on these has a positive charge, to favor the stability. In gastric conditions, pH (*ca.* 3) was close to the pKa values of both polymers (PPP12, pKa= 4.95 and alginate pKa=3.5) (Cook, Tzortzis, Khutoryanskiy et al., 2013; Alves, Hugo, Tymczyszyn et al., 2013), but more so for alginate. This means that, at gastric pH, PPP12 has a higher percentage of positive charges than alginate. Therefore, the emulsions in which PPP12 was immediately deposited over bacteria should be more stable, as the repulsion should be lower. This explains the good results for the assay I, which allowed the retaining of more viable bacteria during the gastric phase than the other assays (Figure 5).

However, the result was not the same when alginate was incorporated as a second polymer, despite PPP12 was again immediately deposited over bacteria (assay II). Electrostatic repulsion between these two positively charged polymeric layers could generate a less stable emulsion reducing the number of viable bacteria reaching the gastric phase (Figure 5). This effect was more accentuated when alginate was used as the first layer and PPP12 as the second one (assay IV). In this case the repulsion between polymers is combined with a certain repulsion between the negative charges of the alginate (about 24% of the charges at pH 3) and those of the bacterial surface. Hence, the low counts observed for the assay IV in the intestinal phase (Figure 5). At intestinal pH (*ca.* 8.5), both polymers were negatively charged (>99%), and thus, the high electrostatic repulsion destabilized the emulsions, enabling the release of viable bacteria to this phase. Other authors also reported electrostatic repulsions in capsules composed of soy protein isolates and alginate exposed to different pH (Hadzieva, Mladenovska, Crcarevska et al., 2017), thus supporting the explanation provided above. In line with this explanation, Vinner et al., (2017) reported that by combining the pH responsive character of Eudragit1with alginate, phage CDKM9 was significantly protected from exposure to simulated gastric fluid at pH 2 and could thereafter be readily released upon exposure to pH 7. Duan, Lü, Gao, et al. (2016) found that the Eudragit1S100 coating enables resistance to the acidic environment of the stomach, whilst alginate provides muco-adhesive properties which prolong the residence time of microparticles in the body. Similar investigations carried out by Sookkasem, Chatpun, Yuenyongsawad et al. (2015) showed the release of curcumin in simulated intestinal fluid at pH 7.4.

4. CONCLUSIONS

Using microfluidic devices to formulate emulsions and encapsulate probiotic microorganisms brings several attractive advantages and a new perspective from economic, technological and environmental point of view in the food industry. In this context, this work proposed an innovative approach to add value to okara oil as continuous phase, enabling an appropriate control on the formation of emulsion droplets. In addition, using PPP12 as dispersed phase of emulsions provided an interesting system to control probiotic deliver to the gut, ensuring a bacterial release fitting the requirements of international organisms for probiotic containing products. The key role of this polymer also provided strong support for the application of PLU based co-polymers beyond intestinal drug delivery. The results obtained in this work constitute a promising contribution for the implementation of strategies originally developed for the pharmaceutical industry in the food/nutraceutical industries. Further investigations will be necessary to better characterize the particles and study their stability under different environmental conditions providing new strategies for their implementation at an industrial level.

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Authors contribution: G.Q. and E.G. did the experimental work (work with microfluidic devices, bacteria involving assays); P.A. and P.S., synthesized PPP12; M.L.R. and C.F. conceptualization, microfluidic equipment, discussion of results; A.G.-Z. conceptualization, analysis and discussion of results and written of the manuscript. All authors have approved the final version of the manuscript.

Captions to figures

Figure 1. Reaction scheme for the synthesis of PAA-PLU-PAA (PPP). x = number of ethylene oxide units; y = number of O-CH_3-CH_2 units; z = number of propylene oxide units, m, n = number of acrylic acid units.

Figure 2. Microscopic images from W/O emulsions obtained using microfluidic devices. White arrows represent the entrance of the dispersed phase; black arrows, the input of the continuous phase; red arrows indicate the input of dispersed 1 phase in emulsions II and IV (see Table 1 for definition). **a** indicates T-junctions of the two phases, and **b**: end point before the outlet. A scheme of the devices is shown on the right part of the figure.

A. Chips having two inlet (denoted as 1 and 2 in the scheme on the right) and one outlet (denoted as 3) points (used in assays I, III and control);

B. Chips having three inlet (1, 2, 3) and one outlet (4) points (used in assays II and IV).

Figure 3. Microscopic images of W/O emulsions containing *L. plantarum* CIDCA 83114. I, II, III, IV represent the different assays defined in Table 1. C: control, F: CF labeled emulsions. Red arrows denote encapsulated microorganisms. Images corresponding to samples I, II, II, IV and C were taken with a 40× magnification, and image corresponding to F, with a 20× magnification. Red circle in script C denotes the crust.

Figure 4. Droplet size of W/O emulsions obtained using microfluidic devices. Gray bars: fresh emulsions; dashed bars: emulsions after freeze-drying. An average of 50 determinations is plotted. Different letters indicate statistically significant differences ($p<0.05$).

Figure 5. Bacterial release after exposing freeze-dried W/O emulsions to simulated gastro-intestinal conditions. Gray bars: before digestion, white bars: after exposure to the gastric environment, dashed bars: after exposure to intestinal digestion. Different letters indicate statistically significant differences ($p<0.05$). *no growth. Bacterial viability before freeze-drying (average all assays): 11.56±0.34 log CFU/mL; viability for microorganisms freeze-dried in PBS (non-emulsified): 5.43±0.42 log CFU/mL.

Supplementary material

Figure S1. Scheme of the different chips used in this work:

A. Chip with two inlet points. I1: Inlet point of the continuous phase; I2: Inlet point of the dispersed phase; O: Outlet point;

B. Chip with three inlet points. I1: Inlet of the continuous phase; I2: Outlet of the first dispersed phase 2; I3: Inlet of the second dispersed phase 1; O: Outlet point.

In the middle, a picture of the chips.

Figure S2. **A.** Fast Read 102 plate consisting of 10 counting chambers to observe emulsions;

B. Fast Read plate gang allowing the measurement of emulsions inside the quadrant.

Figure S3. Extension of the dispersed phase in:

A. emulsions containing alginate;

B. emulsions not containing alginate.

REFERENCES

Alves, P., Hugo, A. A., Tymczyszyn, E. E., Ferreira, A. F., Fausto, R., Perez, P. F., Coelho, J.F.J., Simões P.N., & Gómez-Zavaglia, A. (2013). Effect of cholesterol-poly (N, N-dimethylaminoethyl methacrylate) on the properties of stimuli-responsive polymer liposome complexes. *Colloids and Surfaces B: Biointerfaces*, *104*, 254-261.

Aquilina, G., Bach, A., Bampidis, V., Bastos, M. L., Flachowsky, G., Gralak, M. A., Hogstrand, Ch., Leng, L., López-Puente, S., Martelli, G., Mayo, B., Renshaw, D., Rychen, G., Saarela, M., Sejrsen, K., Van Beelen, P., Wallace, R. J. & Westendorf, J. (2013). Scientific Opinion on the safety and efficacy of Probiotic LACTINA® (*Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Lactobacillus bulgaricus*, *Lactobacillus lactis*, *Streptococcus thermophilus* and *Enterococcus faecium*) for chickens for fattening and piglets. *EFSA Journal, 11*, 3170-3183.

Association of Official Analytical Chemists [AOAC] (1995). *Official Methods of Analysis Association of Official Analytical Chemists, 2 Vol. 16th Edn*. Washington, DC: Association of Official Analytical Chemists.

Barlow, J., Gozzi, K., Kelley, C. P., Geilich, B. M., Webster, T. J., Chai, Y., Sridhar, S., & van de Ven, A. L. (2017). High throughput microencapsulation of Bacillus subtilis in semi-permeable biodegradable polymersomes for selenium remediation. *Applied Microbiology and Biotechnology*, *101*(1), 455-464.

Barreiro-Iglesias, R., Bromberg, L., Temchenko, M., Hatton, T. A., Alvarez-Lorenzo, C., & Concheiro, A. (2005). Pluronic-g-poly (acrylic acid) copolymers as novel excipients for site specific, sustained release tablets. *European Journal of Pharmaceutical Sciences*, *26*(5), 374-385.

Bazban-Shotorbani, S., Dashtimoghadam, E., Karkhaneh, A., Hasani-Sadrabadi, M. M., & Jacob, K. I. (2016). Microfluidic directed synthesis of alginate nanogels with tunable pore size for efficient protein delivery. *Langmuir*, *32*(19), 4996-5003.

Borhan, F. P., Gani, S. S. A., & Shamsuddin, R. (2014). The use of D-optimal mixture design in optimising okara soap formulation for stratum corneum application. *The Scientific World Journal* 2014, Article ID 173979, 8 pages.

Campaña, A. L., Sotelo, D. C., Oliva, H. A., Aranguren, A., Ornelas-Soto, N., Cruz, J. C., & Osma, J. F. (2020). Fabrication and characterization of a low-cost microfluidic system for the manufacture of alginate–lacasse microcapsules. *Polymers*, *12*(5), 1158-1174.

Cassani, L., Gomez-Zavaglia, A., & Simal-Gandara, J. (2020) Technological strategies ensuring the safe arrival of beneficial microorganisms to the gut: From food processing and storage to their passage through the gastrointestinal tract. *Food Research International, 129,* art. no. 108852. doi: 10.1016/j.foodres.2019.108852.

Champagne, C. P., & Fustier, P. (2007). Microencapsulation for the improved delivery of bioactive compounds into foods. *Current Opinion in Biotechnology*, *18*(2), 184-190.

Chen, W., Kim, J. H., Zhang, D., Lee, K. H., Cangelosi, G. A., Soelberg, S. D., Furlong, C. E., Chung, J. H., & Shen, A. Q. (2013). Microfluidic one-step synthesis of alginate microspheres immobilized with antibodies. *Journal of The Royal Society Interface*, *10*(88), 20130566.

Choo, E. S. G., Yu, B., & Xue, J. (2011). Synthesis of poly (acrylic acid)(PAA) modified Pluronic P123 copolymers for pH-stimulated release of doxorubicin. *Journal of Colloid and Interface Science*, *358*(2), 462-470.

Chu, L. Y., Utada, A. S., Shah, R. K., Kim, J. W., & Weitz, D. A. (2007). Controllable monodisperse multiple emulsions. *Angewandte Chemie International Edition*, *46*(47), 8970-8974.

Cook, M. T., Tzortzis, G., Khutoryanskiy, V. V., & Charalampopoulos, D. (2013). Layer-by-layer coating of alginate matrices with chitosan–alginate for the improved survival and targeted delivery of probiotic bacteria after oral administration. *Journal of Materials Chemistry B*, *1*(1), 52-60.

de Man, J. O., Rogosa, M., & Sharpe, M. E. (1960) A medium for the cultivation of *lactobacilli*. *Journal of applied Bacteriology, 23*, 130-135.

Duan, H., Lü, S., Gao, C., Bai, X., Qin, H., Wei, Y., Wu, X., & Liu, M. (2016). Mucoadhesive microparticulates based on polysaccharide for target dual drug delivery of 5-aminosalicylic acid and curcumin to inflamed colon. *Colloids and Surfaces B: Biointerfaces*, *145*, 510-519.

Eun, Y. J., Utada, A. S., Copeland, M. F., Takeuchi, S., & Weibel, D. B. (2011). Encapsulating bacteria in agarose microparticles using microfluidics for high-throughput cell analysis and isolation. *ACS Chemical Biology*, *6*(3), 260-266.

Feng, K., Wei, Y.-S., Hu, T.-G., Linhardt, R.J., Zong, M.-H., & Wu, H. (2020). Colon-targeted delivery systems for nutraceuticals: A review of current vehicles, evaluation methods and future prospects. *Trends in Food Science and Technology*, *102*, 203-222.

Francesko, A., Cardoso, V. F., & Lanceros-Mendez, S. (2019). Lab-on-a-chip technology and microfluidics. In: Microfluidics for pharmaceutical applications from nano/micro systems fabrication to controlled drug delivery. pp 3-36. Santos, H. A., Liu, D., & Zhag, H. eds. Elsevier. The Netherlands.

Garrote, G.L., Abraham, A.G. & De Antoni, G.L. (2001) Chemical and microbiological characterisation of kefir grains. *Journal of Dairy Research*, 68, 639–652.

Ghibaudo, F., Gerbino, E., Campo Dall' Orto, V., Gómez-Zavaglia, A. (2017). Pectin-iron capsules: Novel system to stabilise and deliver lactic acid bacteria. *Journal of Functional Foods, 39*, 299-305.

Ghibaudo, F., Gerbino, E., Copello, G.J., Campo Dall' Orto, V., & Gómez-Zavaglia, A. (2019) Pectin-decorated magnetite nanoparticles as both iron delivery systems and protective matrices for probiotic bacteria. *Colloids and Surfaces B: Biointerfaces, 180*, 193-201.

Ghibaudo, F., Gerbino, E., Hugo, A.A., Simões, M. G., Alves, P., Costa, B.F.O., Campo Dall´ Orto, V., & Gómez-Zavaglia, A., Simões, P. N. (2018) Development and characterization of iron-pectin beads as a novel system for iron delivery to intestinal cells. *Colloids and Surfaces B: Biointerfaces, 170*, 538-543.

Hadzieva, J., Mladenovska, K., Crcarevska, M. S., Dodov, M. G., Dimchevska, S., Geškovski, N., Grozdanov, A., Popovski, E., Petruševski, G., Chachorovska, M., Ivanovska, T. P., Petruševska-Tozi, L., Ugarkovic, S., & Goracinova, K. (2017). *Lactobacillus casei* encapsulated in soy protein isolate and alginate microparticles prepared by spray drying. *Food Technology and Biotechnology*, *55*(2), 173-186.

Hati, A. G., Bassett, D. C., Ribe, J. M., Sikorski, P., Weitz, D. A., & Stokke, B. T. (2016). Versatile, cell and chip friendly method to gel alginate in microfluidic devices. *Lab on a Chip*, *16*(19), 3718-3727.

Heidebach, T., Först, P., & Kulozik, U. (2010). Influence of casein-based microencapsulation on freeze-drying and storage of probiotic cells. *Journal of Food Engineering*, *98*(3), 309-316.

Kabanov, A. V., Batrakova, E. V., & Alakhov, V. Y. (2002). Pluronic® block copolymers as novel polymer therapeutics for drug and gene delivery. *Journal of Control Release, 21*(2-3), 189-212.

Kalyanaraman, M., Retterer, S. T., McKnight, T. E., Ericson, M. N., Allman, S. L., Elkins, J. G., Palumbo, A. V., Keller, M., & Doktycz, M. J. (2009, March). Controlled microfluidic production of alginate beads for in situ encapsulation of microbes. In *2009 First Annual ORNL Biomedical Science & Engineering Conference* (pp. 1-4). IEEE.

Latheef, M. B. (2012). *Pulsed ultrasound-assisted solvent extraction of oil from soybeans and microalgae*. McGill University (Canadian theses). Library and Archives Canada = Bibliothèque et Archives Canada, 2012 ISBN: 0494847700, 9780494847701.

Ma, Y., Pacan, J. C., Wang, Q., Sabour, P. M., Huang, X., & Xu, Y. (2012). Enhanced alginate microspheres as means of oral delivery of bacteriophage for reducing *Staphylococcus aureus* intestinal carriage. *Food Hydrocolloids*, *26*(2), 434-440.

Marquis, M., Davy, J., Cathala, B., Fang, A., & Renard, D. (2015). Microfluidics assisted generation of innovative polysaccharide hydrogel microparticles. *Carbohydrate Polymers*, *116*, 189-199.

Mazutis, L., Vasiliauskas, R., & Weitz, D. A. (2015). Microfluidic production of alginate hydrogel particles for antibody encapsulation and release. *Macromolecular Bioscience*, *15*(12), 1641-1646.

Niu, X., & de Mello, A. J. (2012). Building droplet-based microfluidic systems for biological analysis. *Biochemical Society Transactions*, *40*(4), 615-623.

Pimentel-González, D. J., Campos-Montiel, R. G., Lobato-Calleros, C., Pedroza-Islas, R., & Vernon-Carter, E. J. (2009). Encapsulation of *Lactobacillus rhamnosus* in double emulsions formulated with sweet whey as emulsifier and survival in simulated gastrointestinal conditions. *Food Research International, 42*(2), 292-297.

Quintana, G., Gerbino, E. & Gomez-Zavaglia, A. (2018). Valorization of okara oil for the encapsulation of *Lactobacillus plantarum*. *Food Research International*, *106,* 81-89.

Quintana, G., Simões, M. G., Hugo, A., Alves, P., Ferreira, P., Gerbino, E., Simões, P. N., & Gómez-Zavaglia, A. (2017). Layer-by-layer encapsulation of *Lactobacillus delbrueckii* subsp. *bulgaricus* using block-copolymers of poly (acrylic acid) and pluronic for safe release in gastro-intestinal conditions. *Journal of Functional Foods*, *35*, 408-417.

Quintana, G., Spínola, V., Martins, G. N., Gerbino, E., Gómez-Zavaglia, A., & Castilho, P. C. (2020). Release of health-related compounds during in vitro gastro-intestinal digestion of okara and okara fermented with *Lactobacillus plantarum*. *Journal of Food Science and Technology*, *57*(3), 1061-1070.

Quitain, A. T., Oro, K., Katoh, S., & Moriyoshi, T. (2006). Recovery of oil components of okara by ethanol-modified supercritical carbon dioxide extraction. *Bioresource Technology, 97*(13), 1509-1514.

Rodríguez-Huezo, M. E., Estrada-Fernández, A. G., García-Almendárez, B. E., Ludeña-Urquizo, F., Campos-Montiel, R. G., & Pimentel-Gonzalez, D. J. (2014). Viability of *Lactobacillus plantarum* entrapped in double emulsion during Oaxaca cheese manufacture, melting and simulated intestinal conditions. *Food Science and Technology, 59,* 768-773.

Rokka, S., & Rantamäki, P. (2010). Protecting probiotic bacteria by microencapsulation: challenges for industrial applications. *European Food Research and Technology*, *231*(1), 1-12.

Sandoval-Castilla, O., Lobato-Calleros, C., García-Galindo, H. S., Alvarez-Ramírez, J., & Vernon-Carter, E. J. (2010). Textural properties of alginate–pectin beads and survivability of entrapped *Lactobacillus casei* in simulated gastrointestinal conditions and in yoghurt. *Food Research International*, *43*(1), 111-117.

Scialabba, N. E. (2014). Food wastage footprint: full-cost accounting). Food wastage footprint: Full-cost accounting. FAO. ISBN 978-92-5-108512-7. http://www.fao.org/3/a-i3991e.pdf Accessed 06.10.20.

Seifert, A., Kashi, Y., & Livney, Y. D. (2019). Delivery to the gut microbiota: A rapidly proliferating research field. *Advances in Colloid and Interface Science, 274,* art. no. 102038. doi: 10.1016/j.cis.2019.102038.

Sookkasem, A., Chatpun, S., Yuenyongsawad, S., & Wiwattanapatapee, R. (2015). Alginate beads for colon specific delivery of self-emulsifying curcumin. *Journal of Drug Delivery Science and Technology*, *29*, 159-166.

Stanojevic, S. P., Barac, M. B., Pesic, M. B., Jankovic, V. S., & Vucelic-Radovic, B. V. (2013). Bioactive proteins and energy value of okara as a byproduct in hydrothermal processing of soy milk. *Journal of Agricultural and Food Chemistry*, *61*(38), 9210-9219.

Utada, A. S., Lorenceau, E., Link, D. R., Kaplan, P. D., Stone, H. A., & Weitz, D. A. (2005). Monodisperse double emulsions generated from a microcapillary device. *Science*, *308*(5721), 537-541.

Utech, S., Prodanovic, R., Mao, A. S., Ostafe, R., Mooney, D. J., & Weitz, D. A. (2015). Microfluidic generation of monodisperse, structurally homogeneous alginate microgels for cell encapsulation and 3D cell culture. *Advanced Healthcare Materials*, *4*(11), 1628-1633.

Vinner, G. K., Vladisavljević, G. T., Clokie, M. R., & Malik, D. J. (2017). Microencapsulation of *Clostridium difficile* specific bacteriophages using microfluidic glass capillary devices for colon delivery using pH triggered release. *PLoS One*, *12*(10) *12*(10), e0186239.

Zhang, Y., Lin, J., & Zhong, O. (2015). The increased viability of probiotic *Lactobacillus salivarius* NRRL B-30514 encapsulated in emulsions with multiple lipid-protein-pectin layers. *Food Research International, 71,* 9-15.

Zinchenko, A., Devenish, S. R. A., Kintses, B., Colin, P.-Y., Fischlechner, M., Hollfelder, F. (2014). One in a million: Flow cytometric sorting of single cell-lysate assays in monodisperse picolitre double emulsion droplets for directed evolution. *Analytical Chemistry*, *86*(5), 2526-2533.

Authors contribution:

G.Q. and E.G.: experimental work (methodology, validation, formal analysis) (work with microfluidic devices, bacteria involving assays);

P.A. and P.S.: synthesis of PPP12 (methodology, formal analysis);

M.L.R. and C.F.: conceptualization, methodology (microfluidic equipment), resources, discussion of results, supervision;

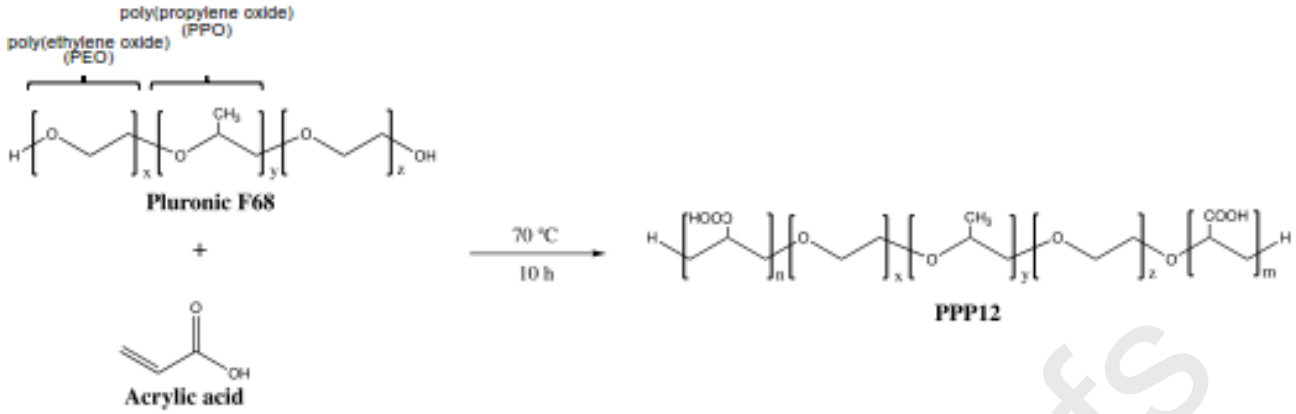
A.G.-Z.: conceptualization, resources, analysis and discussion of results, writing original draft, writing review and editing, visualization, supervision.

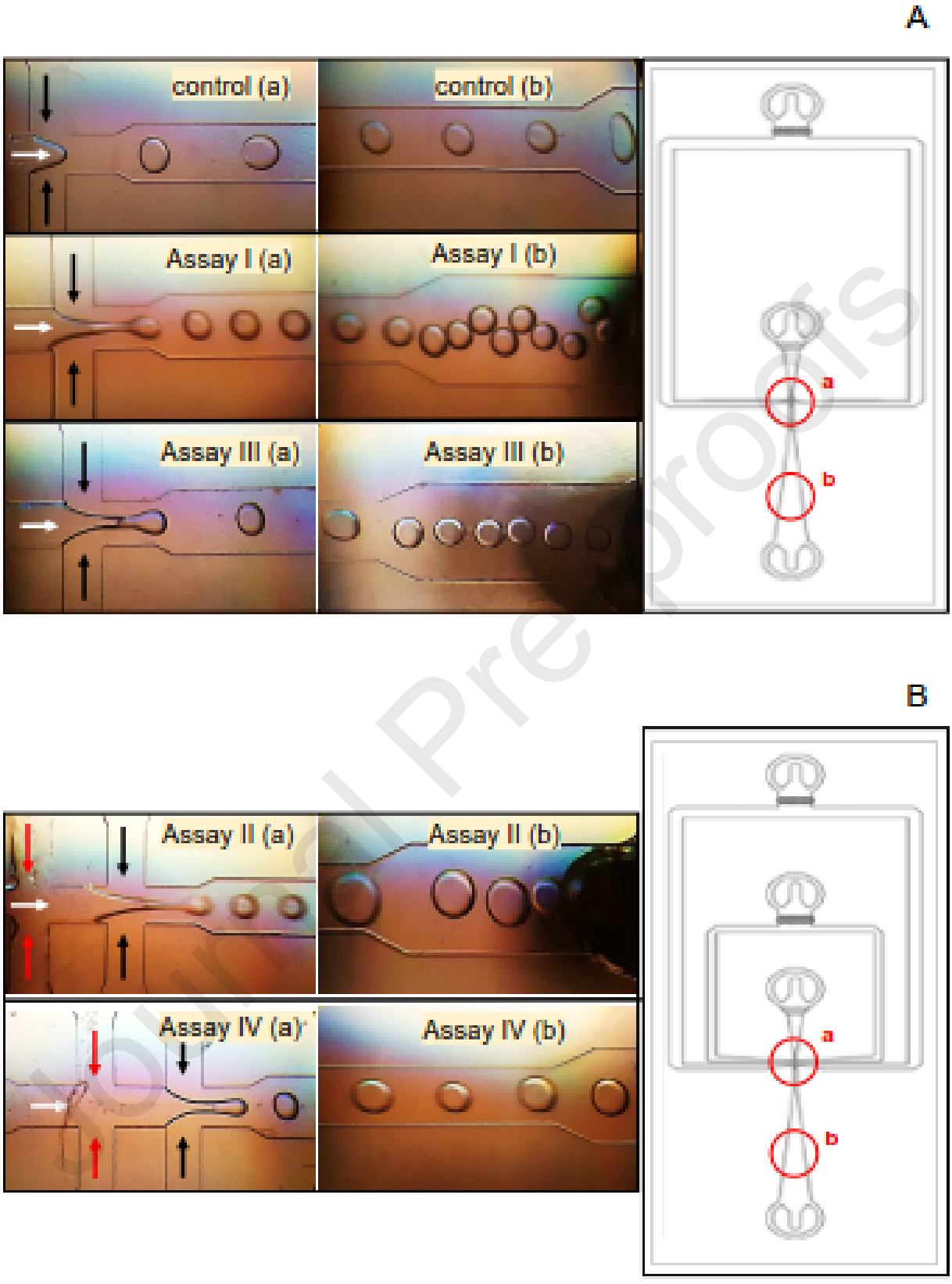
All authors have approved the final version of the manuscript.

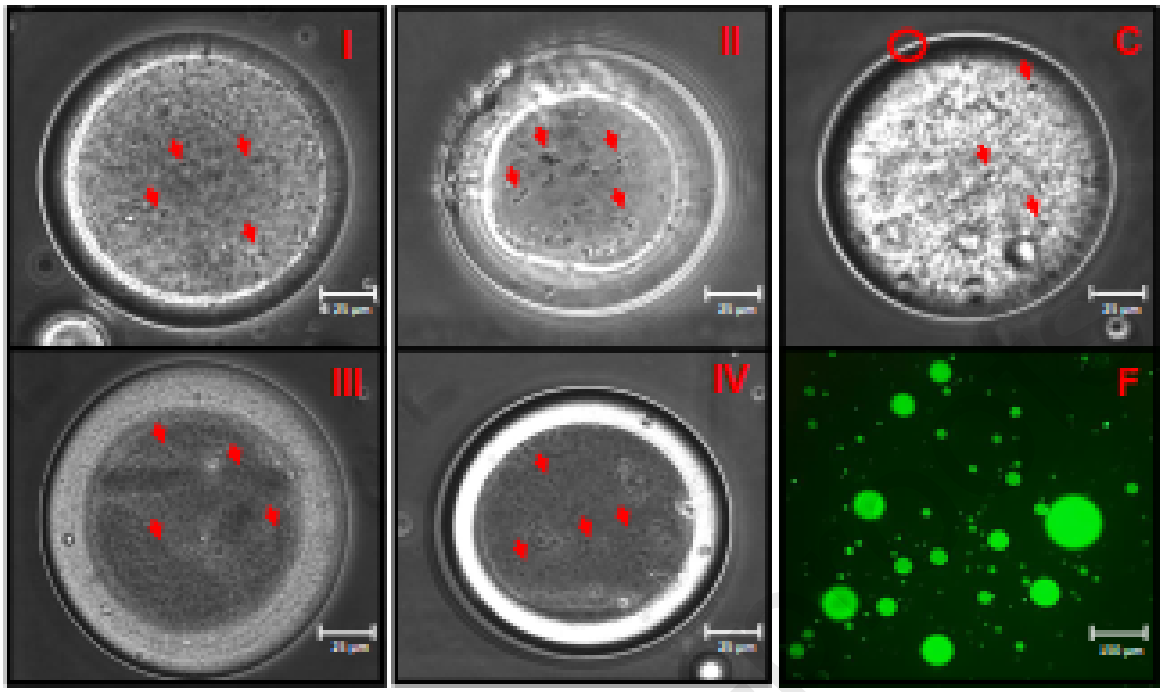
Declaration of Interests

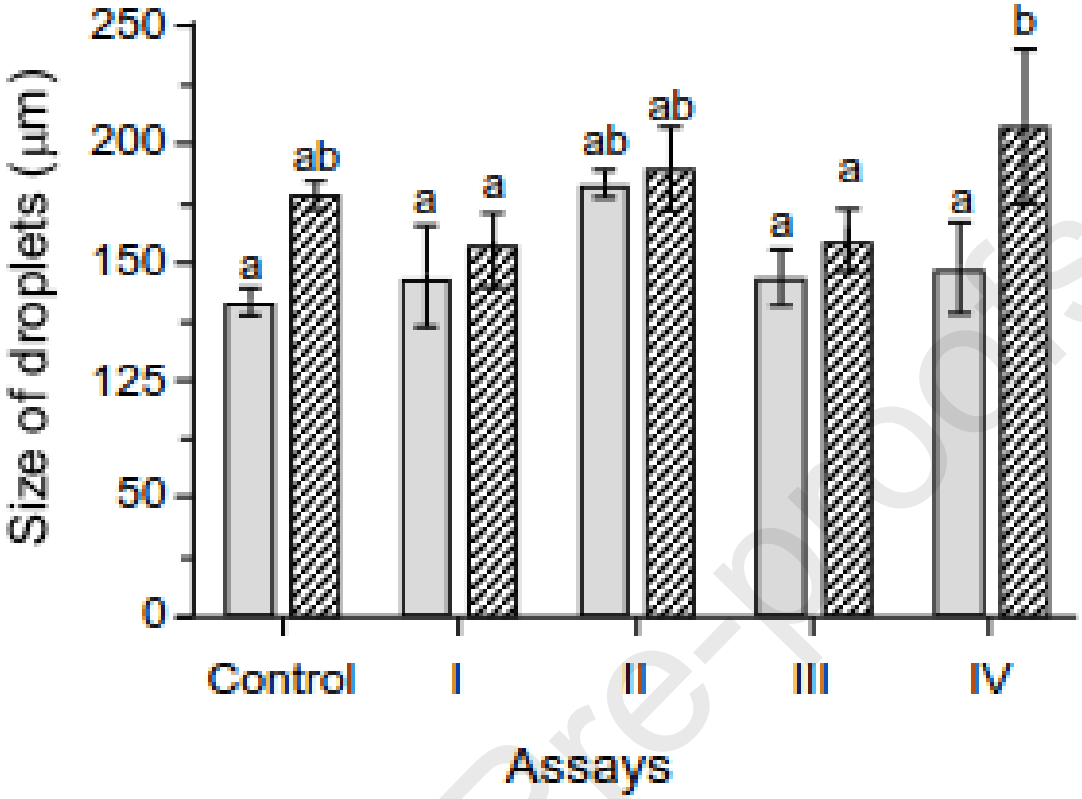
☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:









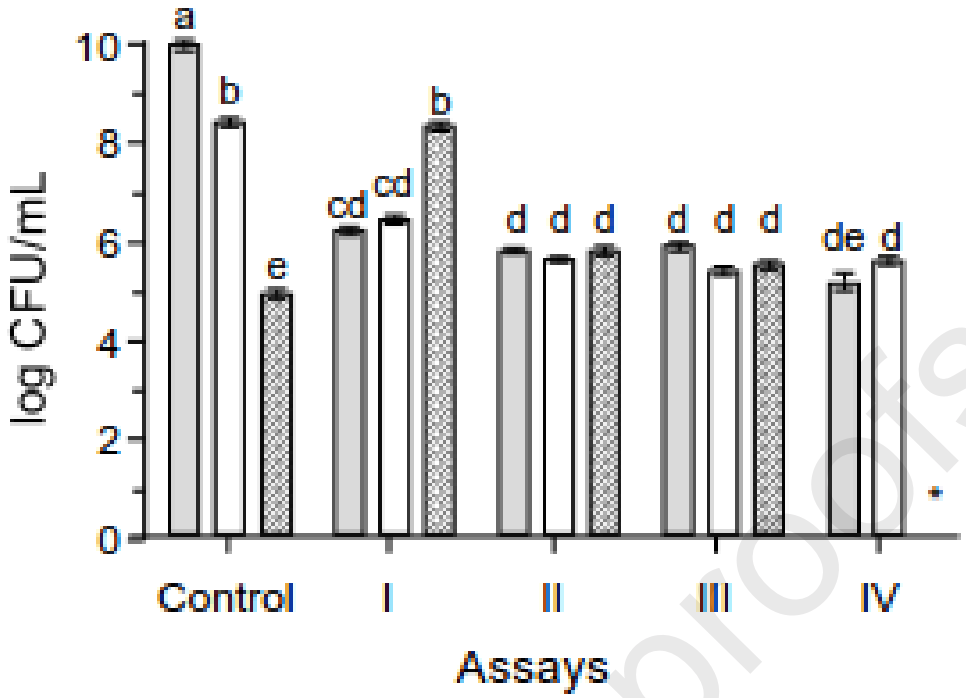


Table 3. Volumetric ratio of the dispersed and continuous phases in the emulsions for assays I, II, III and IV.

W/O Emulsions				
Assays	**Phases**	**Composition[a]**	**Flow rate (µL/h)**	**Volumetric ratio Dispersed/Continuous**
Control	Dispersed	*L. plantarum* CIDCA 83114 in water + CF[b]	120	1:8
	Continuous	Okara oil+lecithin	960	
I	Dispersed	*L. plantarum* CIDCA 83114 in PPP12[c] + CF	120	1:5
	Continuous	Okara oil+lecithin	600	
II	Dispersed 1	*L. plantarum* CIDCA 83114 in PPP12 + CF	40	1:6
	Dispersed 2	Alginate	60	
	Continuous	Okara oil+lecithin	600	
III	Dispersed	*L. plantarum* CIDCA 83114 in alginate + CF	60	1:10
	Continuous	Okara oil+lecithin	600	
IV	Dispersed 1	*L. plantarum* CIDCA 83114 in alginate + CF	40	1:6
	Dispersed 2	PPP12	60	
	Continuous	Okara oil+lecithin	600	

[a]When using lecithin, PPP12 and alginate, their concentrations were 4% w/v, 2% w/v and 1.5 w/v, respectively.

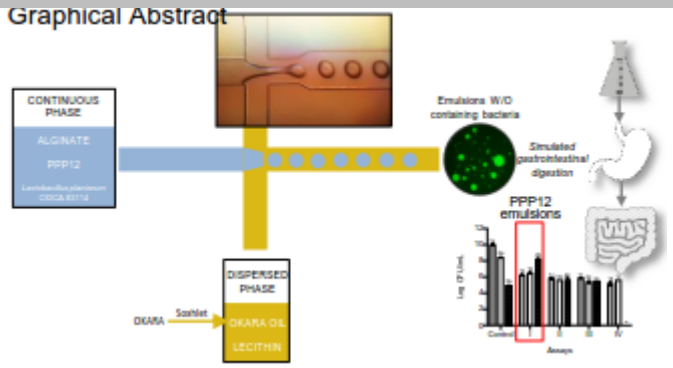
[b]CF: carboxyfluorescein.

[c] PPP12: block-copolymer of *ca.* 12 kDa constituted of pluronic® and poly(acrylic acid).

Table 2. Composition of simulated gastrointestinal juices

Stock solutions	Gastric	Duodenal	Bile
Distilled water (mL)	100	100	100
NaCl (g)	0.55	1.40	1.05
KCl (g)	0.16	0.11	0.08
$NaHCO_3$ (g)	-	0.68	1.16
$CaCl_2.H_2O$ (g)	0.08	-	-
NaH_2PO_4 (g)	0.053	-	-
KH_2PO_4 (mg)	-	16.06	-
NH_4Cl (g)	0.061	-	-
$MgCl_2$ (mg)	-	10.08	-
Urea (g)	0.02	0.02	0.05
Concentrated HCl (mL)	1.30	0.03	0.03
Adjuncts (g)	0.50 pepsin 0.60 mucin	1.80 pancreatin 0.30 lipase	2.40 Bile salts
pH	1.3 ± 0.02	8.1 ± 0.2	8.2 ± 0.2

Graphical Abstract



Highlights

► Okara oil, PPP12 and alginate were used to prepare emulsions in microfluidic devices ►*L. plantarum* was suspended in four different dispersed phases and freeze-dried ► No significant differences in bacterial viability were observed in gastric conditions ► Using PPP12 as only dispersed phase protected bacteria from gastric conditions ►At the same time, such conditions enabled the greatest bacterial release in the gut