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Germinated soybean protein hydrolysate: ionic gelation encapsulation and release under colonic conditions

Hidrolizado proteico de soya germinada: encapsulación por gelación iónica y su liberación en condiciones colónicas

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Abstract

The objective of this work was to develop a formulation and encapsulation method for the delivery of protein hydrolysate to the colon. Alginate (A) and xanthan gum (XG) were selected as wall materials. Three formulations were used to encapsulate the protein hydrolysate from germinated soybean (PHGS) by ionic gelation followed by freeze-drying. During *in vitro* digestion, the microstructure was monitored by ESEM, and the site-specific release of beads was confirmed by fermentation under colonic conditions with human inoculum. The three formulations had high encapsulation efficiencies (≥90%). All beads were probed to resist *in vitro* digestion, preserving their structure at the end of digestion. The release of protein during digestion was lower for the beads containing A+XG than for those with only A. The beads with A released 19.38 ± 1.58% of the protein, and the beads containing A+XG released 17.32 ± 0.29% for 0.3% XG and 7.19 ± 0.00% for 0.5% XG. During fermentation, the encapsulates with A+XG in the proportion of 0.5% produced 7.28 ± 0.21 μmol/mL of short chain fatty acids (SCFA) while those encapsulated with 0.3% of XG 5.43 ± 0.18 μmol/mL and those encapsulated with only alginate produced the least amount of SCFA (3.25 ± 0.08 μmol/mL). All formulations protected PHGS from conditions of the upper gastrointestinal tract and proved to be fermentable by the intestinal microbiota; thus, they can be used as controlled release systems for protein hydrolysate in the colon.

Keywords: Encapsulation, protein hydrolysate, controlled release, *in vitro* gastrointestinal digestion, *in vitro* colonic fermentation.

Resumen

El objetivo de este trabajo fue desarrollar una formulación y un método de encapsulación para liberar hidrolizado proteico en el colon. Se seleccionaron alginato (A) y goma xantana (GX) como materiales de pared. Se utilizaron tres formulaciones para encapsular hidrolizado proteico de soya germinada (HPSG) mediante gelificación iónica seguida de liofilización. Durante la digestión *in vitro*, se evaluó la microestructura de los encapsulados mediante ESEM, y la liberación de los encapsulados se confirmó mediante fermentación colónica con inóculo humano. Las tres formulaciones tuvieron eficiencias de encapsulación altas (≥90%). Todos los encapsulados probaron ser resistentes a la digestión *in vitro*, conservando su estructura. La liberación de proteína durante la digestión fue menor para los encapsulados que contenían A+GX en comparación con únicamente A. Las cápsulas con A liberaron 19.38 ± 1.58% de proteína, y las cápsulas que contenían A+GX liberaron 17.32 ± 0.29% para 0.3% GX y 7.19 ± 0.00% para 0.5% GX. Durante la fermentación, los encapsulados con A+GX en la proporción de 0.5% produjeron 7.28 ± 0.21 μmol/mL de ácidos grasos de cadena corta (AGCC) mientras que los encapsulados con 0.3% de GX 5.43 ± 0.18 μmol/mL y los encapsulados con únicamente alginato produjeron la menor cantidad de AGCC (3.25 ± 0.08 μmol/mL). Todas las formulaciones protegieron al HPSG de las condiciones del tracto gastrointestinal superior y demostraron ser fermentables por la microbiota intestinal; por tanto, pueden utilizarse como sistemas de liberación controlada de hidrolizado proteico en el colon.

Palabras clave: Encapsulación, hidrolizado proteico, liberación controlada, digestión gastrointestinal *in vitro*, fermentación colónica *in vitro*.

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1 Introduction

Soybean is an excellent protein source for preparing hydrolysates with biological activity attributed to bioactive peptides (BPs) (Singh *et al*., 2014). However, oral administration or commercial application of protein hydrolysates or BPs can be impeded by their low bioavailability, bitter taste, hygroscopicity and likelihood of interacting with food matrices. To overcome these challenges, encapsulation can be used as a delivery mechanism (Mohan *et al*., 2015).

Encapsulation is an effective strategy to conserve bioactive compounds in food matrices and control release at target sites, ensuring their efficacy (McClements, 2017). Colon target delivery systems of bioactive compounds present advantages because the colon has prolonged transit time, decreased digestive enzyme activities, and a near neutral pH and has been proven to show good bioavailability for peptides and other bioactive compounds. Colon target delivery systems also provide treatment for different local pathologies (Arévalo-Pérez *et al*., 2020). However, colon-targeted delivery systems are still in an early stage in the field of food science (Feng *et al*., 2020).

Colon target delivery systems must resist chemical and enzymatic degradation that occur in the upper gastrointestinal tract and release their active compound into the colon. The extremely low pH conditions in the stomach might accelerate the degradation of pH-sensitive compounds and proteins, while the proteolytic activity in both the stomach and the small intestine can denature bioactive compounds (Bak *et al*., 2018). The most studied colon-targeted delivery systems mainly depend on approaches such as pH-sensitive systems, time-dependent systems, colonic microbiota-activated systems and several kinds of polymers that have been studied in an effort to overcome the strong physiological variations in the upper gastrointestinal tract and focus drug release in the colon. Recently, the use of polysaccharides, including alginate, pectin, carrageenan, gum and cellulose, that cannot be degraded in the upper gastrointestinal tract but can be digested by the action of colonic microbiota, has been a promising alternative for developing a colon-targeted delivery system (Feng *et al*., 2020). However, one limitation is that swelling or premature degradation of beads in the upper gastrointestinal tract tends to adversely influence the efficiency of the colonic delivery system when hydrogel beads are designed by using a single polysaccharide. An important approach that has been popularly adapted to address this challenge is to add a second polymer (Feng *et al*., 2020). Few polymer mixtures have been studied as colon target delivery systems. Recently, the use of not so common biopolymers such as nopal mucilage with alginate has been explored (Velázquez-Gutiérrez *et al*., 2020). However, Feng *et al*. (2020) and Arévalo-Pérez *et al*. (2020) carried out thorough reviews of the polymers studied for the delivery of different bioactive compounds, and the performance of pectin, carboxymethylcellulose, polyethylene oxide, hydroxypropyl methylcellulose PLGA, polymethacrylates and alginate stands out.

Alginate is a natural polymer that can be cross-linked by the ionic gelation method using polyvalent cations to form hydrogel beads. This encapsulation method is the simplest and widest technique used in the encapsulation of bioactive compounds (McClements, 2017). However, alginate is used mainly with other polymers in the formulation of encapsulates covered with chitosan or eudragit (Bak *et al*., 2018; Rajasree *et al*., 2018; Zhang *et al*., 2017). There are few studies on the use of blends of alginate with other polymers that are not covered.

In this context, the aim of this work was to develop a simple formulation and encapsulation method for the delivery of protein hydrolysate to the colon. We produce protein hydrolysate from germinated soybean as a bioactive compound. We propose a simple mixture of two polysaccharides, alginate, and xanthan gum, as wall materials, to obtain encapsulates resistant to upper gastrointestinal tract conditions without the need for coverage and to take advantage of encapsulation through ionic gelation.

2 Materials and methods

2.1 Protein Hydrolysate from Germinated Soybean (PHGS)

Soybean seeds (*Glycine max*) were obtained from the central market in Iztapalapa, Mexico City. Germination and protein isolation were performed according to González-Montoya *et al*. (2018). The protein isolate was digested with porcine pepsin and pancreatin (González-Montoya *et al*., 2016). The hydrolysis degree of protein hydrolysate from germinated soybean (PHGS) was determined

by measuring the TCA-soluble nitrogen content as described by Segura-Campos *et al*., (2012). Additionally, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of no germinated and germinated soybean as well as protein isolate and protein hydrolysate from germinated soybean was performed under reducing conditions according to the method by Laemmli (1970). Samples were separated in 17% polyacrylamide gel under reducing conditions at 110 V in a unit (Mini-Protean 3, Bio-Rad). The gel was stained with Coomassie R-250 blue (0.1%) solution for 30 min and unstained with a methanol-acetic acid-water (10-10-80, v/v) solution. A pre stained protein standards (precision plus protein, Bio-Rad) was used as molecular weight marker.

2.2 Encapsulation of PHGS by ionic gelation followed by freeze-drying

The encapsulation of PHGS was carried out using an encapsulator (B-390, BUCHI, Switzerland) equipped with a 200-μm nozzle. All solutions were prepared in deionized water; an aqueous alginate solution (1.5% w/v) was prepared by continuously stirring the powder at 60 °C until completely dissolved. Then, PHGS was added at two core-to-wall ratios of 1:2 and 1:1. For the formulation containing xanthan gum, the aqueous alginate (1.5% w/v) solution was prepared first, xanthan gum was added (0.3% w/v and 0.5% w/v), and finally, PHGS was added at the same core-to-wall ratio (1:2 and 1:1). All the formulations were filtered and degassed at room temperature before encapsulation. The concentrations used were selected based on the viscosity of the mixtures before being encapsulated, since we observed that this is a limiting factor in the equipment used. The feeding solutions were placed in a water bath at 60 °C and forced to be extruded through a nozzle forming the hydrogel beads. The microencapsulation conditions were a flow rate of 3.5 ± 0.5 mL/min, vibration frequency of 1500 ± 200 Hz, and electrode voltage of 450 ± 50 V, these conditions were chosen based on results obtained in preliminary experiments carried out with different usage conditions of the equipment. The hydrogel beads were cross linked with a hardening solution of 10% $CaCl_2$ at 15 °C for 1 h with continuous stirring at 300 rpm. The hardened beads were collected by filtration and subsequently washed with distilled water to remove any excess of $CaCl_2$ from their surfaces and non-encapsulated PHGS. The beads were dried in a freeze-dryer (FreeZone 2.5, Labconco, Kansas, MO, USA) at -50 °C and 0.2 mbar.

2.3 Encapsulation Efficiency (EE)

The encapsulation efficiency (%) was determined by dividing the remaining PHGS in the beads by the initial amount in the feeding solutions according to the equation presented below Ec. (1). The remaining PHGS beads were taken to be the difference between the initial protein and that released into the hardening solution and wash water (Zhang *et al*., 2017). The protein was measured using a Bradford assay (Quick Start Bradford protein assay, Bio-Rad) at 595 nm. A calibration curve with the same PHGS was used as a reference.

$$\%EE = \frac{TP - P}{TP} * 100 \tag{1}$$

where TP is the total protein content in the feeding solution before the encapsulation process, and P is the protein content in the hardening solution and wash water.

2.4 In vitro Gastrointestinal Digestion of PHGS Beads

In vitro gastrointestinal digestion (oral, gastric, and intestinal phases) was simulated following the harmonized INFOGEST protocol published by Minekus *et al*. (2014) with some modification. The simulated salivary fluid (SSF), the simulated gastric fluid (SGF), and the simulated intestinal fluid (SIF) were prepared fresh from stock solutions daily. Additionally, α-amylase (10070) was added to SSF at a final concentration of 1 mg/mL, pepsin (P7000 Sigma, ≥250 units/ mg solid) from porcine gastric mucosa was added to SGF (3.2 mg/mL), and pancreatin (P1750 Sigma, 8 × USP) from porcine pancreas and bile salts (OXOID LP0055) were added to SIF at a final concentration of 5 mg/mL. For the oral phase simulation, 100 mg of beads was mixed with 5 mL of SSF (w/v). The mixture was incubated with agitation at 37 °C for 5 min. Then, SGF (pH 3) was added at a ratio of 1:1 (v/v) to each tube containing the oral mixture, and the pH was adjusted to 3 with HCl (1 N). The samples were incubated at 200 rpm at 37 °C for 2 h in a shaking water bath system (WDS20, PolyScience). Finally, SIF (pH 7) was added at a ratio of 1:1 (v/v) to each tube containing the gastric mixture, the pH was adjusted to 7 with NaOH (1 N), and the mixture was incubated with continuous agitation (200 rpm) at 37 °C for 2 h. The concentration of PHGS released as total protein was measured by a Bradford assay at 595 nm. At each interval, a sample was taken,

and the concentration of protein content in SSF, SGF and SIF was discarded through a reagent blank.

2.5 *Microstructural Analysis by Environmental Scanning Electron Microscopy (ESEM)*

The microstructural changes in PHGS beads were determined using an environmental scanning electron microscope (Evo LS10, Zeiss, Germany) with a methodology similar to that reported by Cárdenas-Pérez *et al*., (2017). The beads were centrifuged at 20 g to eliminate the simulated fluids of gastrointestinal digestion. The supernatant was decanted, and the remaining beads were mounted on aluminum stubs with double-sided carbon adhesive tape for direct observation under an electron microscope. The images were captured at 50x, 300x, and 1000x magnification and stored in TIFF format. The ESEM images of 638 × 438 pixels, in greyscale, were used to evaluate Feret's diameter with ImageJ software (National Institutes of Health, Bethesda, MD, USA). At least 300 capsules of each formulation were measured until obtaining a coefficient of variation of less than 10%.

2.6 *In vitro Colonic Fermentation of PHGS Beads*

The *in vitro* fermentation was carried out according to a modified version of the method by Saura-Calixto, Garcia-Alonso, Goni, & Bravo (2000). The anaerobic fermentation medium was prepared with tryptone, macro-micro mineral solution, and resarzurine. For the inoculum, human faecal samples were collected from five normal weight volunteers in an age range of 25 to 35 years who did not ingest antibiotics or dewormers for at least 6 months before the assay. The inoculum was mixed with fermentation medium (5 g/50 mL medium) for 15 min and filtered. The remaining beads (100 mg) from the digestion were weighed in sterile glass bottles with a rubber stopper; 8 mL of fermentation medium and 2 mL of inoculum were added. For anaerobic conditions, CO_2 was injected. The bottles were incubated with constant agitation in a 37 °C water bath with sampling at 0, 2, 4, 6, 8, 10, and 24 h in triplicate, and lactulose was used as a control. The pH was measured at each incubation time. The fermentation was stopped by adding 3 mL of NaOH (1 N) to each bottle, the content was centrifuged at 1100 g/15 min, and the supernatant was collected and stored at -70 °C for quantification of short chain fatty acids (SCFA). The precipitate was dried in an oven at 90 °C to quantify the non-fermentable residue (NFR).

2.7 *Quantification of Short Chain Fatty Acids (SCFA)*

A sample (400 μL) of each supernatant obtained in the fermentation was mixed with 100 μL of internal standard (2-methyl valeric acid, Sigma, 10987-8, in a concentration from 0.05 to 5.0 mM), 10 μL of CH2O2 to keep the sample pH constant, and 490 μL of H2O. The mixture was centrifuged at 12 300 g for 15 min at 4 °C, and the supernatant was transferred to vials for gas chromatography (Perkin-Elmer, 2 mL). The content (5 μL) of each vial was automatically injected into a gas chromatograph (Plus HP 6890, Agilent, USA) equipped with a flame ionization detector and a silica column (WCOT, CP7747, Varian, USA), with a temperature ramp from 115 °C to 250 °C at constant pressure and temperature in the injector/detector of 250/300 °C, respectively (Saura-Calixto *et al*., 2000). The SCFA were identified and quantified by comparison with reference standards and expressed as μmol/mL acetate, propionate, butyrate, and valerate.

2.8 *Statistical analysis*

Differences between bead formulations were analysed by one-way ANOVA and Tukey's post hoc test for repeated samples. To compare the total SCFA concentrations, the data were analysed by a paired one-tailed Student's t-test. Data were processed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). Values of $p \leq 0.05$ were considered statistically significant.

3 Results and discussion

3.1 *Germination and Protein Hydrolysate*

PHGS had a hydrolysis degree of 68%, the molecular weight of the protein and peptide aggregates was less than 37 kDa, and enzymatic hydrolysis principally resulted in fractions below 15 kDa. Figure 1 shows SDS-PAGE of no germinated soybean, germinated soybean for 6 days, and protein isolate and protein hydrolysate from germinated soybean. The protein profile was different between the samples.

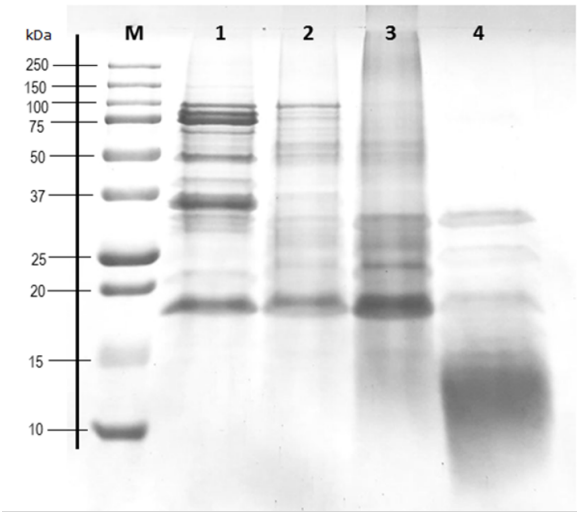

Fig. 1. SDS-PAGE of non-germinated soybean (Lane 1), soybean germinated for 6 days (Lane 2), protein isolate from germinated soybean (Lane 3), and protein hydrolysate from germinated soybean (Lane 4). Molecular weight marker (Lane M).

The germination of soybean for 6 days caused protein breakdown, mainly of β-conglycinin and glycinin fractions (Mora-Escobedo *et al*., 2009). This change in the protein profile modified the PI, which hampered the complete extraction of the protein (Figure 1, Lane 3). However, protein breakdown during germination improved the bioactivity of the hydrolysate (Mora-Escobedo *et al*., 2009). Our workgroup has previously reported (González-Montoya *et al*., 2018) the anti-inflammatory and antiproliferative effects of protein hydrolysate from germinated soybean in the 5-10 kDa fraction. This fraction showed an important action against colorectal cancer cells. Nevertheless, a high degree of hydrolysis, such as that obtained, can be metabolized and absorbed in the gastrointestinal tract which may prevent them from achieving their effect on the target site. Since peptides when are orally consumed, are subjected to acidic digestion in the stomach followed by protease action in the intestinal phase before being absorbed through the enterocytes. Biostability and bioavailability are the main factors for achieving physiological effects as the peptides need to reach their targets intact in order to exert their bioactivity (Mohan *et al*., 2015). Therefore, it is important to develop strategies for delivering PHGS in the target. We encapsulated PHGS to be delivered under colon conditions.

3.2 Encapsulation Efficiency (EE)

The encapsulation efficiency is defined as the amount of bioactive compounds trapped in the core or surface of the capsule with respect to the initial amount of bioactive compounds (Mohan *et al*., 2015). An increase in the amount of material allows for better coating and interaction with the bioactive compound. An excess of the bioactive compound causes an overload in the capsule, preventing the wall material from being able to retain them. However, no significant difference ($p \leq 0.05$) was observed due to the ratio of protein hydrolysate-wall material in the EE (Table 1). The results suggest that it is the interaction between the bead-forming components that primarily influence the EE. In addition, it has already been reported that xanthan gum can interact with alginate through the formation of hydrogen bonds, which increases the ability of the gel to retain encapsulated compounds (Pongjanyakul & Puttipipatkhachorn, 2007).

Table 1. Encapsulation efficiency of beads produced by ionic gelation and loaded with germinated soybean protein hydrolysate.

Core-to-wall ratio	Wall material mix	Encapsulation efficiency
1:1	A (1.5%)	98.28 ± 0.025[a]
	A (1.5%) + XG (0.3%)	98.15 ± 0.105[a]
	A (1.5%) + XG (0.5%)	98.36 ± 0.029[a]
2:1	A (1.5%)	98.73 ± 0.129[a]
	A (1.5%) + XG (0.3%)	98.77 ± 0.039[a]
	A (1.5%) + XG (0.5%)	98.84 ± 0.013[a]

Values with the same lower-case letter in a column are not statistically different ($p \leq 0.05$), n = 3. In this table, the wall material of beads was identified as A (alginate) and XG (xanthan gum).

Another factor that could influence the EE is the anionic character of the wall materials that allows them to interact with the amino acids that have a positive charge on the hydrolysate (Tovar-Benítez *et al*., 2016). The EE was higher than 90% for all formulations, data like those reported by Zhang *et al*. (2017), who used only sodium alginate as a wall material to encapsulate a whey protein isolate by the same method used in this work. However, the EE it is high when proteins are encapsulated in comparison with peptides or molecules with lower molecular weight as the compounds encapsulated in this work (Mohan *et al*., 2015). An advantage of using polymer blends instead of just alginate as a wall material is the increased encapsulation efficiency (Setty *et al*., 2005).

3.3 In vitro gastrointestinal digestion of beads and microstructure

Evaluation by Environmental Scanning Microscopy (ESEM)

Figures 2, 3, and 4 show the micrographs of PHGS beads made with different wall materials (A and XG) in each of the stages of the gastrointestinal simulation. It is important to note that the microstructure of the control beads did not present a spherical shape, this is the result of the drying process since there are reports that show changes in the structure due to the loss of water (Plazola-Jacinto *et al*., 2019; Ponce-Noguez *et al*., 2020). Figure 2 corresponds to beads of PHGS with only sodium alginate as a wall material. It was observed that after the salivary phase, there were no changes in the structure of the beads, perhaps due to the short incubation time. However, components of the SSF, probably α-amylase, were observed to adhere to the bead surface. Regarding the gastric phase, no changes in the structure of the beads were observed compared to the control and salivary phases, and the components of the fluids (salts and enzymes) adhered to the surface of the beads. No damage was observed on the surface, and there were no significant changes in Feret's diameter of the beads (145±7.87 μm). It has been reported that alginate chains tend to protonate and thus increase swelling resistance in an acidic pH, such as the gastric phase (Lee & Mooney, 2012). At the end of the intestinal phase, no significant change ($p \leq 0.05$) was observed in the size of the beads (151±6.92 μm). In contrast, changes in the surface of the beads were observed (Figure 2), probably due to the first stage of the swelling phenomenon that occurs when water is absorbed into the polymer network formed by sodium alginate (Tavakoli *et al*., 2019). In general, the beads were structurally resistant to temperature, changes in pH, and tested incubation time.

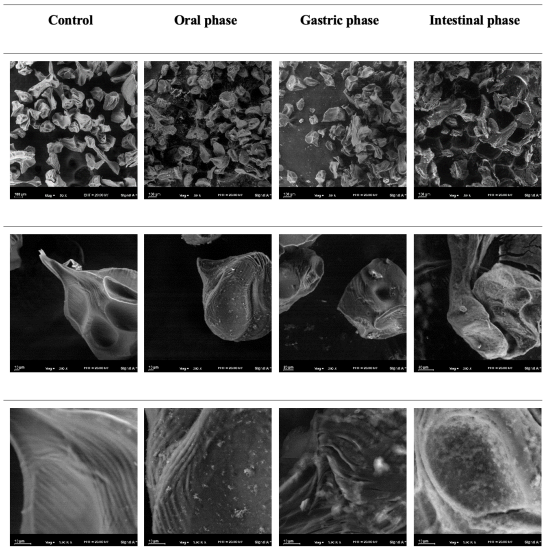

Fig. 2. ESEM images of PHGS beads with alginate (1.5%) as wall material during oral, gastric, and intestinal phases of the *in-vitro* gastrointestinal digestion at different magnifications (50x, 300x, and 1000x). Control images are undigested PHGS beads.

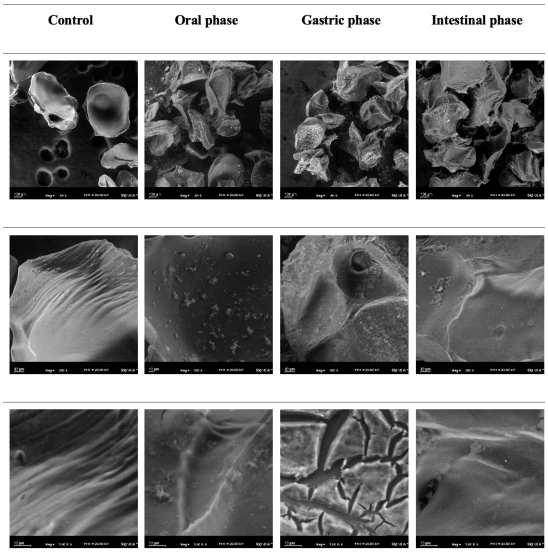

Fig. 3. ESEM images of PHGS beads with alginate (1.5%) and xanthan gum (0.3%) as wall material during oral, gastric, and intestinal phases of the *in-vitro* gastrointestinal digestion at different magnifications (50x, 300x, and 1000x). Control images are undigested PHGS beads.

The resistance of the beads obtained in this work could be closely related to the drying process used, since the structure formed during the drying process can be more compact which impedes fluid uptake (Santhanes *et al*., 2018). It has been reported that the change in the diameter of alginate capsules exposed to simulated gastrointestinal fluids is less when the capsules are lyophilized compared to dried capsules at room temperature because the fluid is absorbed into a hollow or low-density core that is formed during the drying process (Santhanes *et al*., 2018).

Figure 3 shows the morphology of PHGS beads with A and XG (0.3%) as wall materials during the gastrointestinal simulation. No changes due to salivary simulation were observed. Feret's diameter was slightly increased from 368±4.56 μm, to 377±7.61 μm, albeit not significantly, after the gastrointestinal process. In the micrographs, this is likely related to the increase in size and the swelling phenomenon at an earlier stage compared to alginate beads. It has been reported that during swelling, electrostatic repulsion is generated between the polymer chains of encapsulated xanthan gum. This is caused by an increase in the dissociated groups, resulting in a separation of the polymer chains that attract more water into the beads (Trombino *et al*., 2019).

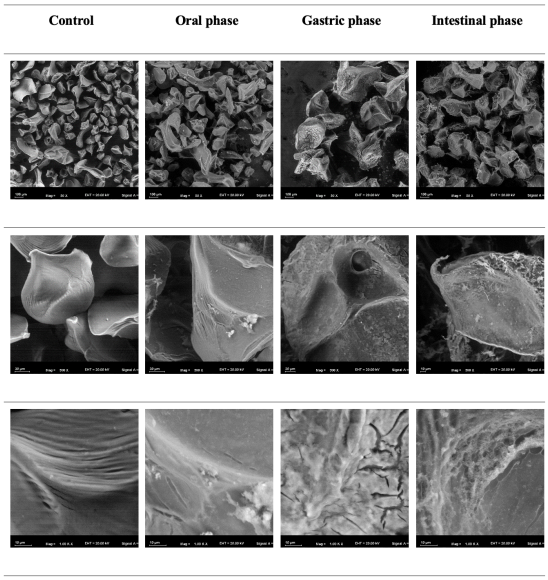

Fig. 4. ESEM images of PHGS beads with alginate (1.5%) and xanthan gum (0.5%) as wall material during oral, gastric, and intestinal phases of the *in-vitro* gastrointestinal digestion at different magnifications (50x, 300x, and 1000x). Control images are undigested PHGS beads.

There are reports indicating that the degree of swelling and water absorption is greater when there are mixtures of sodium alginate and xanthan gum versus only one alginate (Pongjanyakul & Puttipipatkhachorn, 2007). This is related to the glucuronic acid and the acetic acid esters present in the xanthan gum structure; they provide a greater polarity and better affinity for water than alginate (Torres *et al*., 2012).

After the swelling phenomenon in the gastric phase, an erosion process was observed at the end of the intestinal phase since the outermost layer of the beads detached, leaving a smooth surface similar to the undigested beads (control). Figure 4 shows the PHGS beads with sodium alginate and xanthan gum as wall materials, which were found in a larger proportion (0.5%). A similar behaviour was observed for beads with a lower percentage of xanthan gum (0.3%). No changes were made in the beads until the gastric phase, where the Feret's diameter of the beads increased due to the swelling of the more superficial layer of the beads from 153±2.48 μm to 159±4.13 μm. An erosion process was later observed in the intestinal phase. The detached layer measured was 15.87±7.29 μm; nevertheless, any of the 3 formulations tested could be used in the delivery of bioactive compounds in the lower gastrointestinal tract since they were structurally resistant to gastrointestinal simulation *in vitro*.

3.4 PHGS release from beads during in vitro gastrointestinal digestion

During the *in vitro* simulation of gastrointestinal conditions, the release of protein hydrolysate was measured for all bead formulations (Figure 5). Protein release was higher during the intestinal phase compared to the gastric for all formulations, probably due to the longer digestion time in this phase, which favors the swelling process. In addition, the solubility of alginate is higher in alkaline conditions such as those of the intestinal phase compared to the acidic pH of the gastric phase (George & Abraham, 2006). The formulation containing alginate as the wall material showed the greatest loss of protein (19.38±1.58%), although no specific changes were observed in the size and structure of the beads because of the gastrointestinal simulation. This indicates that the loss of protein may be due to the solubilization and release of the PHGS found on the surface of the beads due to the swelling process observed in Figure 2.

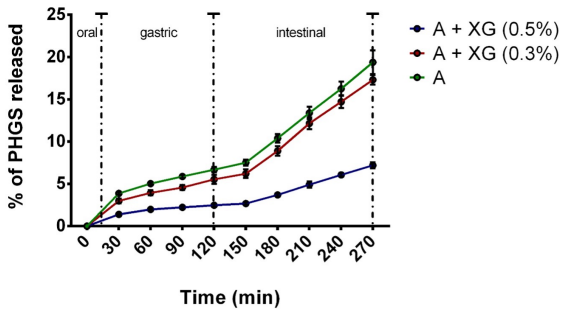

Fig. 5. PHGS release from beads during *in vitro* gastrointestinal digestion.

The beads with alginate in combination with xanthan gum in different proportions exhibited minor protein losses of 17.32±0.29% and 7.19±0.00% in the minimum and maximum xanthan gum contents, respectively. There was a significant difference ($p \leq 0.05$) between the three formulations. The beads containing the major proportion of xanthan gum (0.5%) were able to better retain the protein despite the swelling and erosion phenomena evidenced in the micrographs (Figures 3 and 4). This indicates that either PHSG is found at a greater proportion inside the beads using these formulations or xanthan gum increases the affinity for hydrolysate during the encapsulation process. In the three formulations tested, the total release of PHGS in gastrointestinal conditions was less than 20%, which indicates the potential use of this encapsulation method to deliver bioactive compounds in colon conditions.

3.5 *In vitro colonic fermentation of PHGS beads*

Once the beads were confirmed to resist gastrointestinal digestion, colonic fermentation of the three formulations was carried out to verify that the beads were used as substrates in the colon by the microbiota. The results corresponding to the change in pH due to the fermentation process as well as the quantification of NFR are shown in Figure 6.

During fermentation, the change in pH indicates that the intestinal microbiota consumes the substrate and metabolizes it. This results in the production of different compounds containing some gases, such as CO_2, H_2, CH_4, water, AGCC (acetic, propionic and butyric), and small proportions of other organic acids (isobutyric, valeric, lactic) as well as an increase in bacterial biomass (Wang *et al*., 2019). The change in pH (Figure 6a) due to bead fermentation was significantly ($p \leq 0.05$) less than that in lactulose (control), which indicates that the fermentation of the wall materials is smaller than that of the control. The fermentation kinetics that shows the change in pH due to beads fermentation showing a significant difference ($p \leq 0.05$) between the formulation with alginate and the beds containing XG. However, there is not significant difference between the two formulations with the addition of xanthan gum (0.3% and 0.5%) during the fermentation kinetics. The total change in pH, from the beginning to the end of fermentation, for the formulation with only alginate was 7.31 ± 0.03 to 7.05 ± 0.03, for the formulation with 0.3% XG from 7.15 ± 0.04 to 6.97 ± 0.02 and from 7.10 ± 0.01 to 6.96 ± 0.03 for the formulation with 0.5% XG. There is a significant difference for the change in pH of all formulations ($p \leq 0.05$). The change in pH suggesting that xanthan gum is more easily digested by the microbiota than alginate.

Lactulose, an isomer of lactose, is a synthetic and nondigestible disaccharide. It has a β-glycosidic bond that cannot be hydrolysed by enzymes from the upper part of the digestive tract (Nooshkam *et al*., 2018). This disaccharide is rapidly fermentable, so it is used as a control for colonic fermentation.

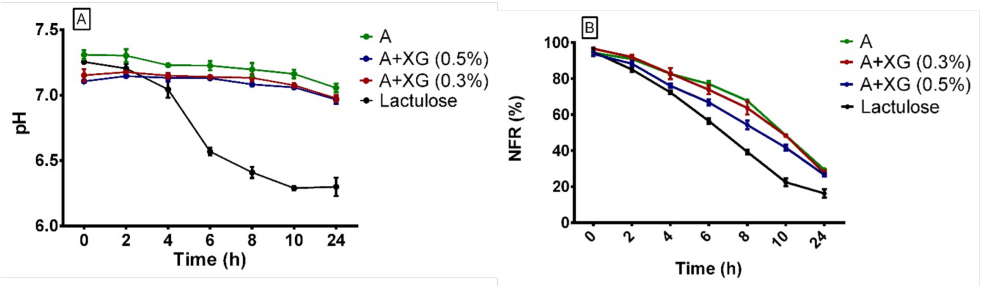

Fig. 6. Fermentation kinetics of PHGS beads. (A) pH changes during fermentation and (B) non fermentable residue (NFR). Lactulose was used as a control of fermentation, n = 3.

The NFR corresponds to the substrate not fermented by the microbiota. Figure 6b shows the quantification of NFR during fermentation. The lactulose was fermented gradually until it reaches a value of ~15% at 24 h; that is, it was almost completely fermented. In contrast, the encapsulated beads of A + XG (0.5%) presented a lower NFR during fermentation, followed by A + XG (0.3%), and finally those with only alginate as a wall material. This behaviour agrees with the change in pH during kinetics, where the beads containing xanthan gum were the most easily fermented. The difference in the chemical structure of alginate and xanthan gum is probably responsible for the difference in the fermentation rate of the beads since it has been reported that the fermentation speed depends mainly on the chemical structure of the polysaccharides (Wang *et al*., 2019). In this case, the NFR and the change in pH are two factors that show the fermentation of the encapsulates and we can relate it to the fermentation rate (Wang *et al*., 2019). The fermentation rate of the encapsulates could be related to the release of the encapsulated protein hydrolysate. There are reports in the literature indicating that sodium alginate is fermented by Bacteroides ovatus G19, which produces β-manuronan lyase and α-guluronan lyase, enzymes that can ferment alginate and its low-molecular-weight polymer derivatives, including mannuronic acid and guluronic acid (Li *et al*., 2017). Therefore, alginate has been used as a wall material in the delivery of different compounds in the colon (Arévalo-Pérez *et al*., 2020; Bak *et al*., 2018; Kaffash *et al*., 2019). The differences in the fermentation kinetics of the different beads can be used to implement controlled release systems depending on the fermentation rate of the wall material.

3.6 Short chain fatty acid production

PHGS has bioactive properties such as antioxidant and anti-inflammatory activities (González-Montoya *et al*., 2016, 2018). However, the SCFA produced by the microbiota can also have beneficial effects (Wang *et al*., 2019). In that sense, we quantified the production of the main SCFA because of the fermentation of the beads. SCFA production was different due to the composition of the beads. The PHGS beads fermented with xanthan gum at the proportions tested (0.5% and 0.3%) produced the most SCFA (Table 2). Acetic acid was obtained in greater quantities than propionic, butyric, and valeric acids (Figure 7a) a similar behavior in the production of SCFA has been reported for the fermentation of other compounds (Romero-López *et al*., 2015). Acetic acid is the main metabolite of most bacteria and plays an important role in controlling inflammation and combating pathogen invasion (Maslowski *et al*., 2009). The production of propionic acid was the lowest for all beads until 10 h and reached its maximum after 24 h (Figure 7b). On the other hand, the butyric acid production was lower than that of the control (lactulose) for all beads (Figure 7c). However, in a typical healthy microbiota, this acid is one of the minor produced (Lee, Jenner, Low, & Lee, 2006). Finally, only valeric acid was produced in a similar quantity as the control, with no significant differences ($p \leq 0.05$) for the beads containing xanthan gum. This acid, like butyric acid, also appears to be a potent histone deacetylase (HDAC) inhibitor. High levels of HDAC proteins have been implicated in a variety of disease pathologies, from cancer and colitis to cardiovascular disease and neurodegeneration (Yuille *et al*., 2018).

Table 2. Total short chain fatty acid production (μmol/mL) during *in vitro* colonic fermentation of PHSG beads.

Time (h)	A	A + XG (0.3%)	A + XG (0.5%)	Lactulose
0	0.45 ± 0.00^{a}	0.55 ± 0.01^{a}	0.40 ± 0.04^{a}	1.32 ± 0.04^{b}
2	0.52 ± 0.04^{ab}	0.73 ± 0.04^{a}	0.45 ± 0.00b	2.08 ± 0.19^{c}
4	0.65 ± 0.01^{a}	1.25 ± 0.01^{b}	1.13 ± 0.00^{bc}	3.78 ± 0.01^{d}
6	1.01 ± 0.00^{a}	1.81 ± 0.01^{b}	1.91 ± 0.02^{bc}	6.23 ± 0.03^{d}
8	1.29 ± 0.12^{a}	2.27 ± 0.03^{b}	2.54 ± 0.10^{c}	8.70 ± 0.00^{d}
10	1.42 ± 0.03^{a}	2.61 ± 0.07^{b}	2.69 ± 0.01^{bc}	10.34 ± 0.07^{d}
24	3.25 ± 0.08^{a}	5.43 ± 0.18^{b}	7.28 ± 0.21^{c}	11.51 ± 0.20^{d}

Values with the same lower-case letter in the same column are not statistically different ($p \leq 0.05$), n = 3. In this table, the wall materials of beads were identified as A (alginate) and XG (xanthan gum). Lactulose was used fermentation control.

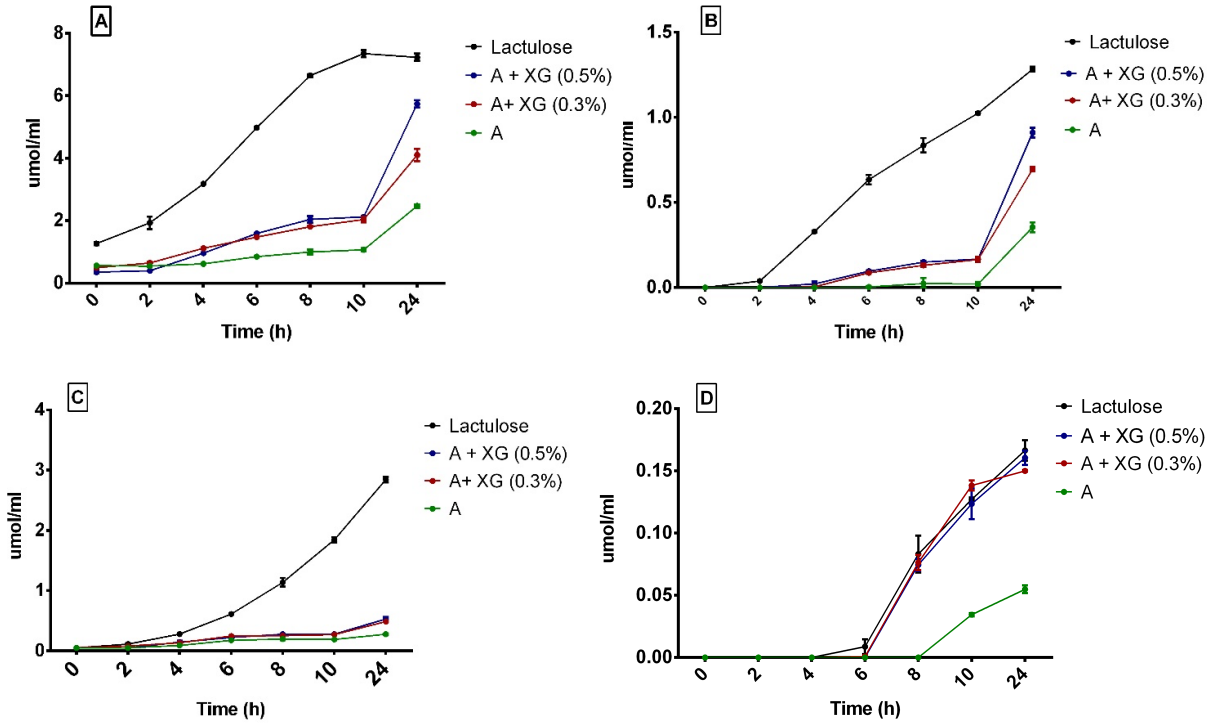

Fig. 7. Short chain fatty acid production (μmol/mL) through *in vitro* colonic fermentation of PHGS beads. (A) Acetic acid. (B) Propionic acid. (C) Butyric acid. D) Valeric acid. Lactulose was used as a control of fermentation, n = 3.

The combination of SCFA production and the effect of encapsulated PHGS could exert a beneficial effect on colon diseases; however, further in vivo studies are needed to demonstrate their effect on health status.

Conclusions

The encapsulation process by ionic gelation followed by freeze-drying and the combination of wall materials, alginate and xanthan gum, allowed us to obtain beads of protein hydrolysate from germinated soybeans with a high encapsulation efficiency. The freeze-dried structure of the beads resists the gastrointestinal digestion process with a low percentage of protein hydrolysate release under these conditions; therefore, these beads are a promising system for the delivery of bioactive compounds in the colon. The differences in the fermentation of the wall materials could be used as a regulator of the delivery rate of the compound. In addition, the production of SCFA due to fermentation of the wall material could generate a positive effect on health status. Therefore, it would be interesting to further evaluate the administration of the beads in vivo to verify the effect.

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