Journal of Food Engineering 247 (2019) 104–114

Contents lists available at ScienceDirect

Journal of Food Engineering

journal homepage: www.elsevier.com/locate/jfoodeng

Production of whey protein isolate – gellan microbeads for encapsulation and release of flaxseed bioactive compounds

Kátia Regina Kuhn[a], Fernanda Guimarães Drummond e Silva[b], Flavia Maria Netto[b], Rosiane Lopes da Cunha[a,*]

[a] Department of Food Engineering, Faculty of Food Engineering, University of Campinas (UNICAMP), 13083-862, Campinas, SP, Brazil
[b] Department of Food and Nutrition, Faculty of Food Engineering, University of Campinas (UNICAMP), 13083-862, Campinas, SP, Brazil

ARTICLE INFO

Keywords:
Extrusion
Flaxseed protein hydrolysate
Flaxseed oil
Rheology
In vitro digestion

ABSTRACT

Production of 1.5% (w/v) whey protein isolate (WPI) – 0.1, 0.3 or 0.5% (w/v) gellan gum microbeads from extrusion of the oil-in-water (O/W) emulsions into a 0.56% (w/v) calcium chloride ($CaCl_2$) solution was evaluated to encapsulate flaxseed oil (15% v/v) and protein hydrolysate (FPH) (0, 0.25 or 0.5% w/v). Microgels resistance and controlled release of oil and FPH were also investigated. Microscopic images showed few free oil droplets and a prevailing presence of gellan on the external surface of the microbeads, indicating that oil and FPH were encapsulated. Microbeads produced at higher gellan concentrations (0.3 or 0.5% w/v) showed a more regular and spherical morphology. However a significant decrease in microbeads size (from ~55 μm to ~50 μm) and an increase in the polydispersity were observed with the FPH addition, which can be a consequence of the formation of a more dense biopolymers network. FPH presence (0.25% w/v) decreased the viscosity and shear thinning behavior of microbeads suspensions (10–90% w/v), which could be partly attributed to the smaller size of particles. The microbeads suspensions were stable at different salt concentrations (0.56, 1.11 or 2.22% w/v) regarding their shape, not releasing the encapsulated oil. 1.5% (w/v) WPI – 0.3% (w/v) gellan microbeads were resistant to simulated gastric conditions, but did not resist to intestinal conditions. Our results show that these microgels are adequate to encapsulate bioactive compounds to be released in the small intestine, passing intact in the stomach, which makes the process attractive in order to maintain the bioavailability and functionality of such compounds.

1. Introduction

Encapsulation allows controlling the release and preserving the stability of bioactive compounds during processing and storage, in order to ensure their bioavailability and efficacy (Klaypradit and Huang, 2008; Nedovic et al., 2011), since health-promoting bioactive compounds are sensitive to oxygen, light, heat, and water (Đorđević et al., 2015). In the food sector, materials used for encapsulation must be food-grade and biodegradable, providing maximal protection to the encapsulated material against environmental conditions and not reacting with it (Nedovic et al., 2011).

The extrusion method followed by an ionotropic gelation is extensively used to produce microbeads (Pasukamonset et al., 2016; Perrechil et al., 2012; Piornos et al., 2017; Zhang et al., 2016), which can be used as encapsulating system of bioactive compounds. It consists in forming droplets by the use of a syringe or an atomizer nozzle into a gelling solution (ionic), where microgels are formed, hardened and then collected (Perrechil et al., 2012). One advantage of these microgels is that they can be used in hydrated media without breaking (Burey et al., 2008).

Polysaccharides are widely used as wall materials (Nedovic et al., 2011) due to their good biocompatibility, biodegradability, low toxicity, abundance in nature and low cost (Kaihara et al., 2011; Karewicz et al., 2011; Perrechil et al., 2012). Gellan gum is an anionic polysaccharide that forms transparent hydrogels in the presence of cations (better cross-linking with divalent than with monovalent cations), which are more resistant to heat and acidic media as compared to other polysaccharide hydrogels (Dahiya et al., 2017; D'Arrigo et al., 2012). Proteins are also a good coating material for the encapsulation of bioactive compounds, due to their unique functional properties such as the ability to form gels and stabilize emulsions (Chen et al., 2006). Whey proteins are effective encapsulating agents capable of forming microcapsules easily under mild conditions using different techniques, being thus commonly used in encapsulation processes (Çabuk and

* Corresponding author.
E-mail address: rosiane@unicamp.br (R.L. da Cunha).

https://doi.org/10.1016/j.jfoodeng.2018.12.002
Received 23 August 2018; Received in revised form 29 November 2018; Accepted 6 December 2018
Available online 07 December 2018

Harsa, 2015). Protein-polysaccharide mixtures have been also evaluated as wall materials (Chen and Subirade, 2006; Nag et al., 2011; Perrechil et al., 2012; Piornos et al., 2017; Tang et al., 2013) and the appropriate combination between them can improve the properties of delivery systems in terms of encapsulation efficiency, stability and bioavailability of bioactive compounds, when compared with systems obtained by using a single wall material (de Souza Simões et al., 2017).

Flaxseed (*Linum usitatissimum* L.) has been emerging as one of the key sources of bioactive ingredients in functional food products (Zhang et al., 2009). Flaxseed is rich in lipids, dietary fiber (both soluble and insoluble), proteins and lignans (Marambe et al., 2008). The oil is the major product from flaxseed and it is the richest known plant source of omega 3 (ω-3) fatty acid (α-linolenic acid, ALA) (Marambe et al., 2008), increasingly recognized for their role in reducing the risk of diseases (Liu et al., 2010; Shahidi and Miraliakbari, 2005). Flaxseed proteins are gaining prominence with respect to their health benefits (Rabetafika et al., 2011), since studies have reported that their hydrolysates have biological activities such as antihypertensive, antioxidant and anti-inflammatory properties (Doyen et al., 2014; Marambe et al., 2008; Udenigwe et al., 2009a, 2009b).

The stability of the compounds in the gastrointestinal (GI) system and their controlled release at the appropriate GI target are necessary to ensure health benefits (Champagne and Fustier, 2007). In view of this, the protection of flaxseed bioactive compounds, through encapsulation, could be an alternative in the attempt to avoid their functionality and bioavailability loss before their release. Thus, the aim of this work was to produce microbeads of whey protein and gellan gum in order to encapsulate flaxseed oil and protein hydrolysate, and evaluate the release of these compounds using *in vitro* digestibility process.

2. Material and methods

2.1. Material

The whey protein isolate (WPI) was obtained from New Zealand Milk Products (ALACEN 895, New Zealand), the deacylated gellan gum was donated by Kelco Biopolymers (San Diego, California, USA) and the flaxseed protein hydrolysate (FPH) was extracted from partially defatted flaxseed meal (Cisbra, Panambi, RS, Brazil), according to methodology from Silva et al. (2013). The hydrolysis condition chosen resulted in a FPH with the highest antioxidant capacity (pH 8.5 and enzyme:substrate ratio 1:90, w/w). The protein concentration (% w/w, wet basis), determined by the Kjeldahl analysis (AOAC, 1997), of WPI, gellan and FPH was 87.66 ± 0.91% (N x 6.38), 0.40 ± 0.00% (N x 6.25) and 70.38 ± 0.91% (N x 6.25), respectively. Flaxseed oil was purchased from Cisbra (Panambi, RS, Brazil), showing the following fatty acids composition: 6.2% C16:0, 5.3% C18:0, 20.1% C18:1, 13.7% C18:2 and 52.3% C18:3 (provided by the manufacturer). The enzymes Alcalase 2.4 L, pepsin from porcine gastric mucosa (P7000) and pancreatin from porcine pancreas (P1625), bile extract porcine (B8631) and FITC were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Reagents dimethyl sulfoxide (DMSO), pyridine and dibutyltin dilaurate were purchased from Merck KGaA (Darmstadt, Germany). All other reagents were of analytical grade.

2.2. Preparation of stock solutions

The WPI (10% w/v) and the FPH (2.5% w/v) stock solutions were prepared by dispersing the powder in deionized water using magnetic stirring during 90 and 120 min, respectively, at room temperature (25 °C). These dispersions were kept overnight at 10 °C allowing complete protein dissolution. The gellan (1% w/v) stock solution was prepared by dissolving the powder in deionized water during 120 min (25 °C), followed by heat treatment at 90 °C for 30 min under magnetic stirring and subsequent cooling to room temperature in an ice bath. Stock solutions were prepared at natural pH (WPI solution ≅ 6.5, FPH solution ≅ 8.4–8.8 and gellan solution ≅ 5.2).

2.3. Preparation of the O/W emulsions

Primary oil-in-water (O/W) emulsions were prepared at 25 °C by homogenizing the oil with the aqueous phase using two sequential homogenization methods. The first involved mixing the solutions in an Ultra Turrax model T18 homogenizer (IKA, Germany) for 4 min at 14,000 rpm, and the second method involved a homogenization at 60 MPa/5 MPa, with two passes, using a Panda 2K NS1001L double stage homogenizer (Niro Soavi, Italy). The WPI and flaxseed oil content were fixed at 3% (w/v) and 30% (v/v), respectively, and the FPH concentration varied between 0 and 1% (w/v) of the total emulsion. The WPI and the FPH stock solutions were added in the aqueous phase. Secondary O/W emulsions were prepared by mixing the primary emulsion into aqueous gellan solutions using magnetic stirring for 1 h at 25 °C. The final composition of secondary emulsions was 15% (v/v) flaxseed oil, 1.5% (w/v) WPI, 0–0.5% (w/v) FPH and 0.1–0.5% (w/v) gellan.

2.4. Production of microbeads

Secondary emulsions were extruded through an atomizer nozzle (1 mm diameter) into a 0.56% (w/v) calcium chloride ($CaCl_2$) solution (concentration determined in preliminary trials) at room temperature (25 °C) (Fig. 1). The collecting distance between the atomizer nozzle and the $CaCl_2$ solution (H) was 30 cm and the feed flow rate and the compressed air pressure were fixed at 0.268 L/h and 0.1 MPa, respectively. In order to obtain smaller particles diameter, the highest difference between the air pressure and the feed rate was used (Herrero et al., 2006; Perrechil et al., 2011; Rizk and Lefebvre, 1980), avoiding that the salt solution was splashed out of the container. The best ratio gellan/FPH to produce microbeads was investigated from the particles preparation using different concentrations of gellan (0.1, 0.3 or 0.5% w/v) and FPH (0, 0.25 or 0.5% w/v). Microbeads were maintained in the salt solution under magnetic stirring for 30 min (Chan et al., 2009; Perrechil et al., 2011), filtered using a sieve with opening of 0.037 mm and then stored at room temperature for 1 day, before their evaluation and characterization (sections 2.5, 2.6 and 2.7).

2.5. Microbeads stability

Microbeads stability in different media (water and $CaCl_2$ aqueous solutions (0.56, 1.11 or 2.22% w/v)) was monitored by particle size distribution (section 2.7.2) during 60 min, and samples were collected every 10 min. Suspensions were prepared by dispersing microbeads containing 0.3% (w/v) gellan in distilled water, directly in the

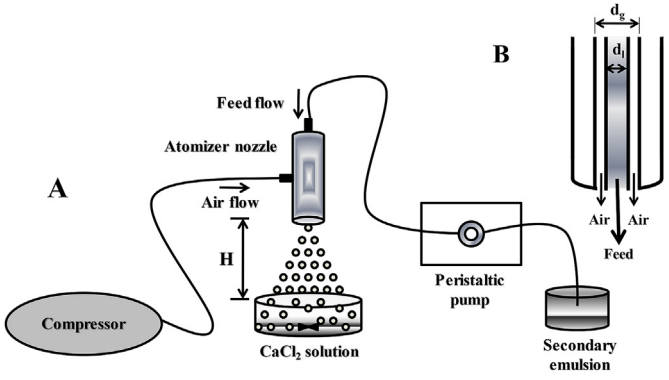
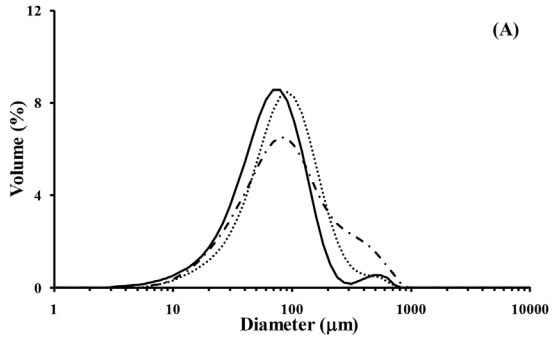
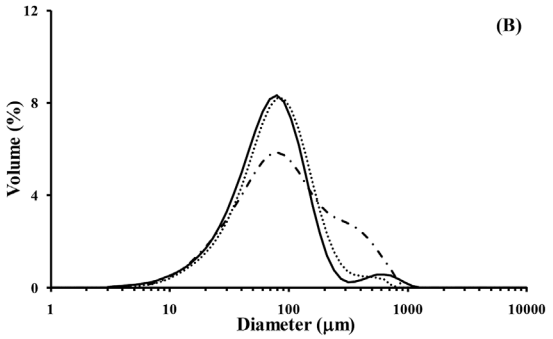
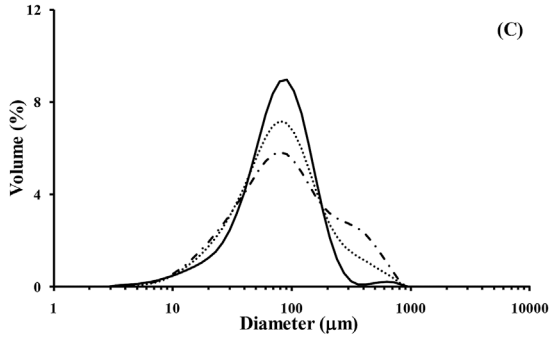


Fig. 1. A) Schematic representation of the production of microbeads by extrusion and B) atomizer nozzle. H = distance between the atomizer nozzle and the salt solution, d_l = diameter of the feed nozzle exit (1 mm) and d_g = diameter of air nozzle exit (3.08 mm).







Gellan (%)		FPH (%) 0	0.25	0.5
0.1	d_{32} (μm)	42.50 ± 0.49^{cB}	43.24 ± 1.39^{bB}	47.42 ± 0.33^{bA}
	Span	1.81 ± 0.04^{bA}	1.84 ± 0.02^{bA}	1.69 ± 0.02^{cB}
0.3	d_{32} (μm)	56.41 ± 0.43^{aA}	50.72 ± 0.26^{aB}	49.38 ± 0.41^{aC}
	Span	1.80 ± 0.04^{bB}	1.93 ± 0.04^{bB}	2.57 ± 0.09^{bA}
0.5	d_{32} (μm)	54.51 ± 0.30^{bA}	50.48 ± 0.71^{aB}	50.20 ± 0.80^{aB}
	Span	3.21 ± 0.10^{aB}	3.58 ± 0.12^{aA}	3.55 ± 0.05^{aA}

Different letters indicate significant differences ($p<0.05$). Small letters: differences in the same column. Capital letters: differences in the same line.

Fig. 2. Particle size distribution, mean particle diameter (d_{32}) and span of microbeads containing 15% (v/v) flaxseed oil, 1.5% (w/v) WPI, (A) 0, (B) 0.25 or (C) 0.5% (w/v) FPH and (▬) 0.1, (…) 0.3 or (▬ •) 0.5% (w/v) gellan.

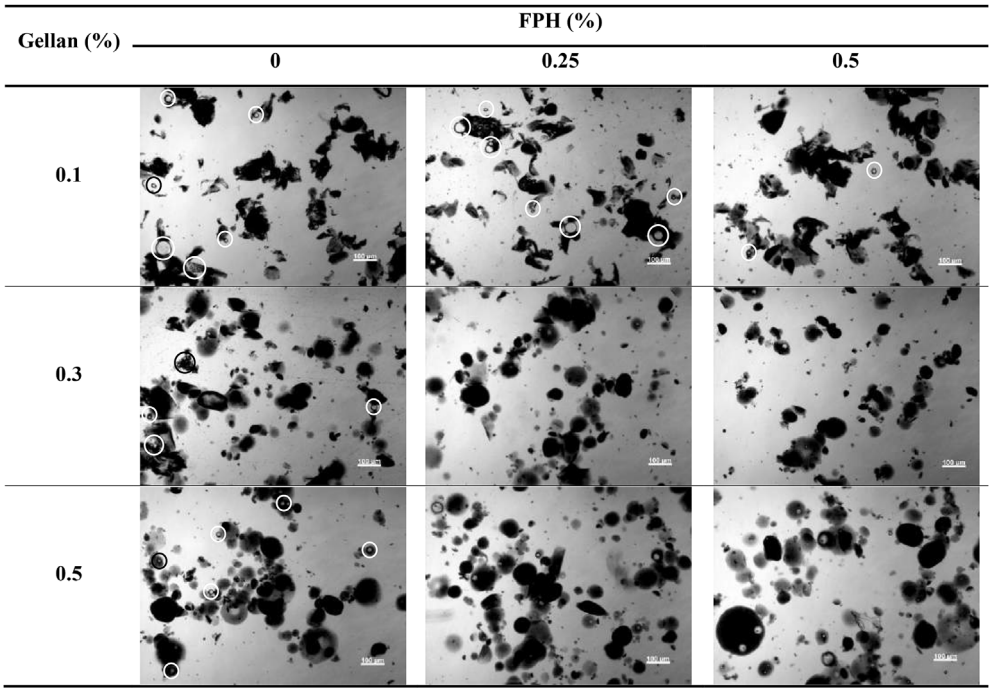


Fig. 3. Microstructure of microbeads composed by 15% (v/v) flaxseed oil, 1.5% (w/v) WPI, 0–0.5% (w/v) FPH and 0.1–0.5% (w/v) gellan. Scale bar = 100 μm.

measurement unit of the laser diffraction equipment (Mastersizer 2000; Malvern Instruments Ltd., UK). At the same time, the microstructure of microbeads suspensions in these different media was evaluated by optical microscopy (section 2.7.3).

2.6. Simulation of in vitro digestion process

Microbeads were incubated in different media simulating buccal (salivary), gastric and intestinal digestion. In the simulation of oral digestion step, microgels were mixed in phosphate buffer (0.005 M, pH 6.9, 1 M NaCl + 0.004 M $CaCl_2$) at a ratio of 1 g of sample to 4 mL of buffer (Hoebler et al., 2002). Then, gastric and intestinal digestions were simulated, following the methodology of Garrett et al. (1999). Porcine pepsin (40 mg/mL in 0.1 M HCl) was added to simulate the gastric juice, at a ratio of 0.5 g of pepsin per 100 g of sample (Miller et al., 1981), the pH was adjusted to 2 by adding 6 M HCl and then the mixture was incubated for 1 h at 37 °C under orbital stirring (100 rpm). After, in intestinal digestion, the pH of the resulting mixture was raised to 5.3 using 0.9 M sodium bicarbonate solution followed by the addition of a mixture of pancreatin and bile extract (2 mg/mL pancreatin

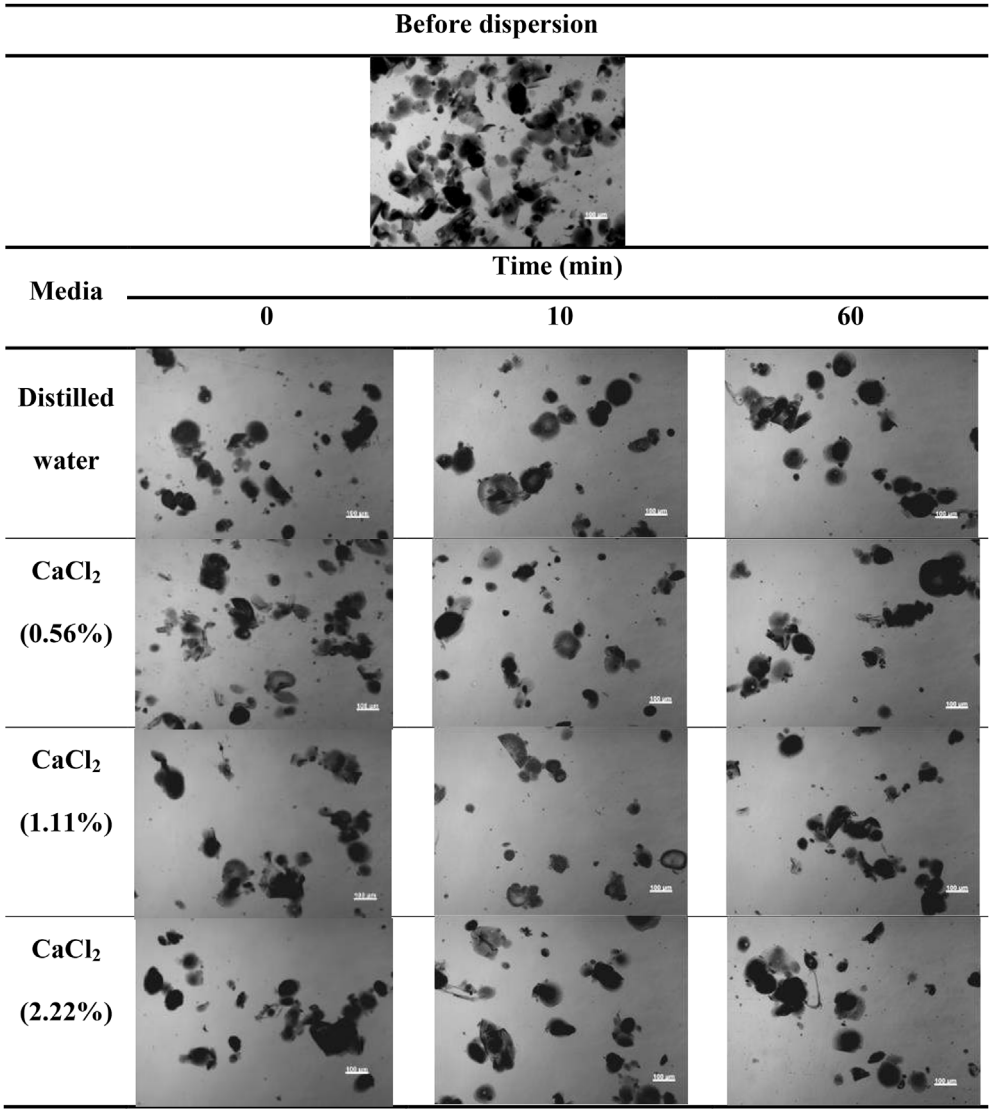


Fig. 4. Microstructure of microbeads composed by 15% (v/v) flaxseed oil, 1.5% (w/v) WPI and 0.3% (w/v) gellan in different media. Scale bar = 100 μm.

+12 mg/mL bile extract in 0.1 M sodium bicarbonate), in order to obtain final concentrations of 0.4 mg/mL pancreatin and 2.4 mg/mL bile extract. The pH of the system was adjusted to 7 with 2 M NaOH prior to its incubation at 37 °C for 2 h in an orbital shaking incubator (100 rpm) (Model TE-420, Tecnal, Brazil). The zeta potential, particle size distribution and morphology (sections 2.7.1, 2.7.2 and 2.7.3, respectively) of the particles were evaluated before, during (samples were collected every 15 or 30 min) and after the gastric and intestinal digestion steps. A control of digestive media, without sample addition, was also carried out for the zeta potential measurements.

2.7. Evaluation of microbeads

2.7.1. Zeta potential

To determine the surface electrical charge, aliquots of microbeads suspensions were diluted in Milli-Q water (~0.005% v/v) and a Zetasizer Nano-ZS (Malvern Instruments Ltd., UK) equipment was used. Measurements were performed at 25 °C in triplicate.

2.7.2. Particle size distribution

A Mastersizer 2000 (Malvern Instruments Ltd., UK) was used to determine the particle size distribution of microbeads in distilled water as dispersion medium (~0.06% v/v, refractive index of water = 1.333). The mean particles size was determined as the volume-surface diameter (d_{32}) and the dispersion index (span) was also reported, according to Eqs. (1) and (2), respectively. The particle size and span were reported as the average and corresponding standard deviation of three replicates.

$$d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2 \quad (1)$$

$$Span = (d_{90} - d_{10})/d_{50} \quad (2)$$

where n_i is the number of microbeads with diameter d_i, and d_{10}, d_{50} and d_{90} are diameters at 10, 50 and 90% of cumulative volume, respectively.

2.7.3. Optical microscopy

The morphology of microbeads was evaluated by optical microscopy. Microgels were poured onto microscope slides, carefully covered with glass cover slips and observed using a Carl Zeiss Model Axio Scope. A1 optical microscope (Zeiss, Germany), with a 10× objective lens.

2.7.4. Confocal scanning laser microscopy (CSLM)

Fluorescein-5-isothiocyanate (FITC) was used to label the gellan gum according to methodology described by Heilig et al. (2009). 20 mL DMSO and 80 μL pyridine were mixed with 1 g gellan gum and stirred

Table 1
Rheological parameters obtained from the power law model and the apparent viscosity at 50 s^{-1} (η_{50}) for the microbeads suspensions composed by 15% (v/v) flaxseed oil, 1.5% (w/v) WPI, 0 or 0.25% (w/v) FPH and 0.3% (w/v) gellan in $CaCl_2$ solution (0.56% w/v).

Suspension (% w/v)		FPH (%)	
		0	0.25
10	*n*	0.94 ± 0.00^{aB}	0.95 ± 0.01^{aA}
30		0.87 ± 0.01^{bB}	0.91 ± 0.00^{bA}
50		0.80 ± 0.00^{cB}	0.89 ± 0.01^{cA}
70		0.72 ± 0.00^{dB}	0.86 ± 0.00^{dA}
90		0.67 ± 0.01^{eB}	0.84 ± 0.01^{eA}
10	*k* (Pa.s^n)	0.002 ± 0.000^{eA}	0.002 ± 0.000^{eB}
30		0.004 ± 0.000^{dA}	0.002 ± 0.000^{dB}
50		0.008 ± 0.000^{cA}	0.004 ± 0.000^{cB}
70		0.017 ± 0.001^{bA}	0.006 ± 0.000^{bB}
90		0.028 ± 0.001^{aA}	0.007 ± 0.000^{aB}
10	η_{50} (mPa.s)	1.4 ± 0.00^{eA}	1.2 ± 0.06^{eB}
30		2.2 ± 0.00^{dA}	1.8 ± 0.00^{dB}
50		3.5 ± 0.10^{cA}	2.3 ± 0.06^{cB}
70		5.7 ± 0.15^{bA}	3.3 ± 0.06^{bB}
90		7.8 ± 0.15^{aA}	3.8 ± 0.00^{aB}

Different letters indicate significant differences ($p < 0.05$). Small letters: differences in the same column. Capital letters: differences in the same line.

for 30 min at room temperature. Then, 0.1 g FITC and 40 μL dibutyltin dilaurate were added, the mixture was incubated for 3 h in a water bath (95 °C) and after cooled down to room temperature. The resulting sample was crushed, washed with ethanol (99.5%) and dried in a vacuum oven (65 °C). To prepare the polysaccharide solution, FITC-labeled gellan was dissolved and heat treated as described in section 2.2. The proteins (WPI and FPH) were stained with a fluorescent dye Rhodamine B, which was directly added to solutions.

Emulsions and microbeads were prepared with protein and polysaccharide labeled solutions, according to sections 2.3 and 2.4, respectively. Samples were evaluated using a Zeiss LSM 780-NLO confocal on an Axio Observer Z.1 microscope (Carl Zeiss AG, Germany) with a 40× objective. Images were collected using 488 and 543 nm laser lines for excitation of FITC and Rhodamine B fluorophores, respectively, with pinholes set to 1 airy unit for each channel, 1024 × 1024 image format.

2.7.5. Rheological properties of microbeads suspensions

The rheological properties of the suspensions (10, 30, 50, 70 or 90% w/v) of microbeads (0 or 0.25% (w/v) FPH and 0.3% (w/v) gellan) in 0.56% (w/v) $CaCl_2$ solution were evaluated using a Physica MCR301 modular compact rheometer (Anton Paar, Austria). A stainless steel plate geometry (50 mm) and a 0.6 mm gap were used for the measurements. Flow curves were obtained using an *up-down-up* steps program with the shear rate varying between 0 and 300 s^{-1}. All measurements were performed in triplicate at 25 °C. The model for shear-thinning fluids (power law model) (Eq. (3)) was used to fit the flow curves.

$$\sigma = k \,.\, (\dot{\gamma})^n \tag{3}$$

where σ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (s^{-1}), k is the consistency index (Pa.s^n) and n is the flow behavior index (dimensionless).

2.8. Statistical analysis

The results were evaluated by analysis of variance (ANOVA), and significant differences ($p < 0.05$) between the treatments were evaluated by the Tukey procedure. The statistical analyses were carried out using the software STATISTICA 6.0 (Statisoft Inc., Tulsa, USA).

3. Results and discussion

3.1. Choosing the ratio gellan/FPH

Different concentrations of gellan (0.1, 0.3 or 0.5% w/v) and FPH (0, 0.25 or 0.5% w/v) were evaluated aiming to determine the appropriate ratio gellan/FPH for producing stable and smaller microgels. Fig. 2 shows that microgels containing 0.1% (w/v) gellan exhibited a bimodal distribution, which was less pronounced increasing FPH concentration (Fig. 2C). A similar behavior was observed with 0.3% (w/v) gellan, but microbeads with greater gellan concentration (0.5% w/v) showed higher polydispersity, which could be attributed to the increased solution viscosity (Chen and Subirade, 2006). Such observations were confirmed by the increase in the mean diameter (d_{32}) and span values (dispersion index) of microbeads (Fig. 2) with the increase of gellan concentration. However the effect of flaxseed hydrolysate depended on the gellan concentration. Microbeads produced with 0.1% (w/v) gellan showed a significant increase ($p < 0.05$) of the size at the highest FPH concentration (0.5% w/v), while a significant decrease was observed for microbeads containing 0.3 or 0.5% (w/v) gellan. Opposite result was found for the span values. Such size reduction and polydispersity increase of microbeads could be related to electrostatic and hydrophobic interactions between biopolymers and/or to bridges formed between the negatively charged groups of gellan and the $CaCl_2$. Both events could contribute to a more dense biopolymers network and consequently with a reduced size despite of the higher solids content.

The influence of gellan and FPH concentration on the microstructure of microbeads can be observed in Fig. 3. According to the pictures, the increase of gellan concentration led to more regular and spherical microgels, as observed by Tang et al. (2013) for alginate-whey protein microspheres produced at high polymers concentration. The hydrolysate concentration affected the morphology of microgels in a lesser extension. However, microbeads without the FPH addition or with lower gellan concentration seemed to have a greater number of free oil droplets (indicated in the Figure).

Thus, although the smaller size of microgels with 0.1% (w/v) gellan, the production of microbeads was done with 0.3% (w/v) gellan in the following steps, since at this concentration microgels were more uniform and presented less free oil droplets in solution. The hydrolysate concentration chosen was 0 or 0.25% (w/v) in order to evaluate the influence of the FPH addition on the microgels produced with 0.3% (w/v) gellan.

3.2. Evaluation of stability

Microbeads stability was evaluated in different media (water and $CaCl_2$ solutions (0.56, 1.11 or 2.22% w/v)) in order to determine their resistance to release the encapsulated oil during the storage. The mean diameter (d_{32}) of microbeads decreased from 65.25 ± 0.21 μm to 50.16 ± 0.03 μm in distilled water over time (from 0 to 60 min), which could be indicating a disaggregation of some particles agglomerates. In $CaCl_2$ solutions, the mean diameter decreased mainly in the first 10 min (from ~67 μm to ~60 μm) and then became almost stable, with no difference between the $CaCl_2$ concentrations. Such size reduction with the salts addition could be explained by the type of interaction performed with divalent cations. Bridges are formed between the negatively charged groups on biopolymers and calcium, allowing the release of free water (Chandrasekaran et al., 1988; Kuhn et al., 2010; Vilela et al., 2011), thus leading to shrinkage of the particles.

The morphology of microbeads before and after dispersion in distilled water or in $CaCl_2$ solutions was observed (Fig. 4). The oil droplets were not released from microbeads during 1 h in any medium, which may be an indication of good stability of these microgels during the storage. In general, it was not observed difference between the microstructures of microbeads dispersed in distilled water or in $CaCl_2$ solutions, nor with respect to time of dispersion. Then, according to these

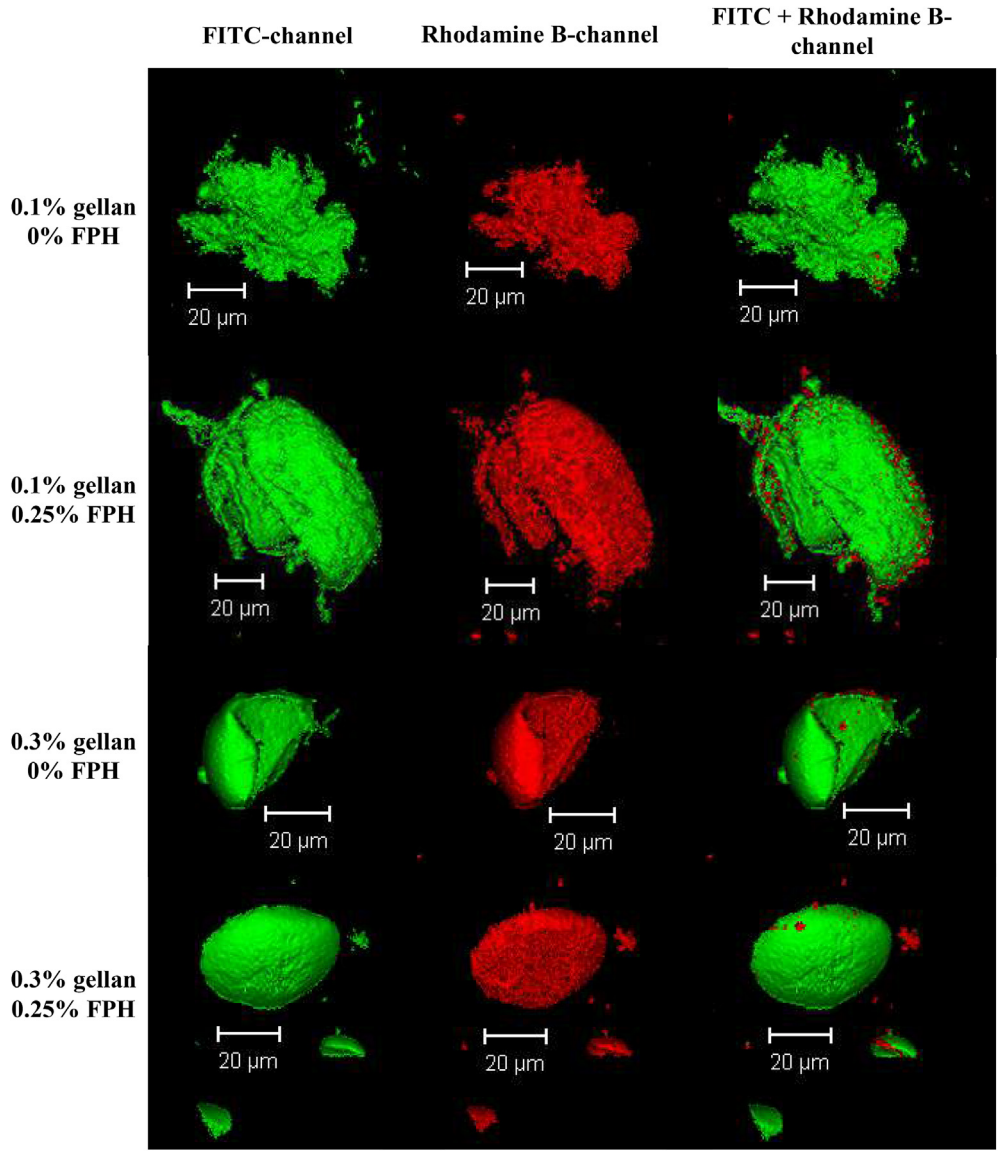


Fig. 5. CSLM micrographs of microbeads composed by 15% (v/v) flaxseed oil, 1.5% (w/v) WPI, 0 or 0.25% (w/v) FPH and 0.1 or 0.3% (w/v) gellan. Scale bar = 20 μm.

results, the lowest salt concentration (0.56% (w/v) $CaCl_2$) was chosen as dispersion medium of microgels to be evaluated from rheological measurements.

3.3. Rheological properties

The flow curves were fitted to power law model ($R^2 > 0.996$) and the flow behavior index (n), the consistency index (k), as well as the apparent viscosity at 50 s^{-1} (η_{50}) are shown in Table 1. The microbeads suspensions in $CaCl_2$ solution (0.56% w/v) showed shear thinning behavior, regardless of the suspensions concentration. Apparent viscosity was greater in the microbeads suspensions without the hydrolysate addition than of microbeads with 0.25% (w/v) FPH, which was more noticeable at larger particles concentration (above 50% w/v). This behavior became more evident as the microbeads concentration in suspension was increased and could be at least partly explained by the lowest particles size with the FPH addition (Fig. 2). An increase of the consistency index (k) was also observed with increasing suspensions concentration, mainly for microbeads without the FPH addition, which also showed a flow behavior index (n) lower ($p < 0.05$) than microgels with hydrolysate.

The rheological behavior of suspensions at low volume fraction is determined by the continuous phase since there are no interactions between the suspended particles (Sato and Cunha, 2012). However, at higher solids concentration there is an increase of interactions of weak intensity, which are broken with increasing shear rate, leading to re-aggregation and shear shinning. The apparent viscosity (η_{50}) showed a significant increase ($p < 0.05$) with the increase of microbeads concentration. However, the viscosity values were very low (maximum was 0.0078 Pa s), which could be indicating that the addition of microbeads would exert little influence on the rheological behavior of food products.

3.4. Microbeads microstructure

The microstructure of microbeads with 0 or 0.25% (w/v) FPH and 0.1 or 0.3% (w/v) gellan was characterized by CSLM (Fig. 5). As observed in optical microscopy (section 3.1), microgels composed by 0.3% (w/v) gellan showed a more regular shape. In these micrographs, proteins (WPI and FPH) are represented in red and the green areas indicate the presence of gellan gum. Thus, it can be verified that there was a predominance of polysaccharide at the external surface of microbeads,

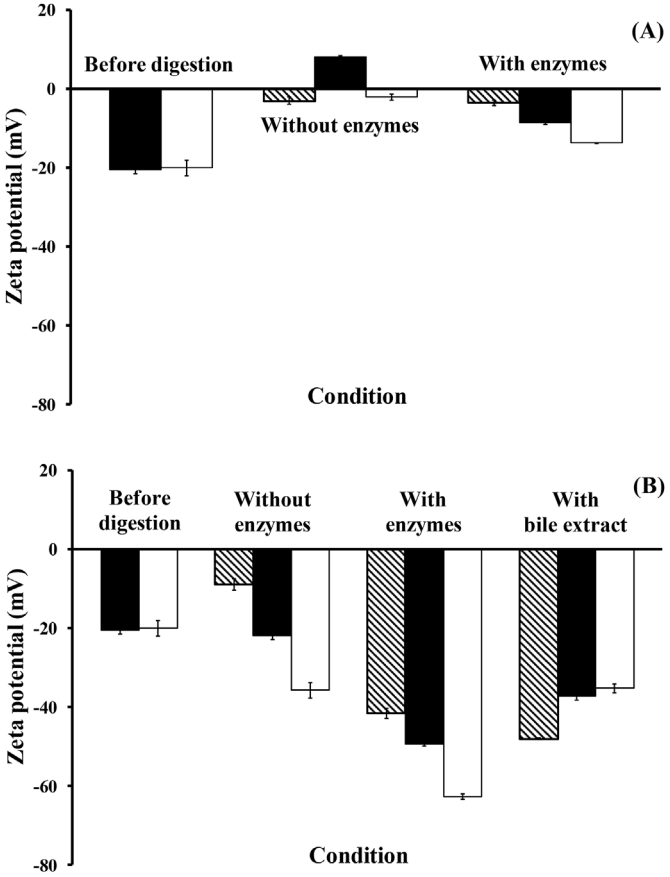


Fig. 6. Zeta potential (mV) of microbeads composed by 15% (v/v) flaxseed oil, 1.5% (w/v) WPI, (■) 0 or (□) 0.25% (w/v) FPH and 0.3% (w/v) gellan, before digestion and after the (A) gastric and (B) intestinal media without and with enzymes addition and with bile extract addition. (▨) Control without microbeads addition.

regardless of gellan concentration and FPH addition, which confirms that the hydrolysate was encapsulated.

Due to the amphiphilic character of proteins and the polar character of gellan, it is believed that the WPI and the FPH were onto the oil/water interface, whereas the gellan migrated to the external surface, coating the particles.

3.5. *In vitro digestion*

The evaluation of the resistance of microbeads and release of encapsulated compounds was performed by simulating *in vitro* human digestive process in three different media: without enzymes addition, with enzymes addition (pepsin, pancreatin and bile extract) and with only bile extract addition. The results were presented according to the methodology used, zeta potential (section 3.5.1), microscopy and particle size distribution (section 3.5.2).

3.5.1. *Zeta potential*

Fig. 6 shows the zeta potential of microgels before digestion and after the gastric and intestinal steps without and with enzymes addition (pepsin, pancreatin and bile extract) and with only bile extract addition. Microbeads were negatively charged before digestion (≅ −20 mV) due to the anionic character of gellan gum (pK_a = 3.5) (Picone et al., 2017). The zeta potential determined for the gastric and intestinal media without sample and enzymes addition was around −3 and −9 mV, respectively (Fig. 6A and B). After microbeads addition, the zeta potential of microgels even showed small values (closed to neutral charge) in gastric step (Fig. 6A), but after intestinal medium (Fig. 6B) were observed values around −22 and −35.50 mV without and with the FPH addition, respectively.

Microbeads in gastric medium with enzymes addition (Fig. 6A) showed a higher charge density (around −9 and −13.50 mV with 0 and 0.25% (w/v) FPH, respectively) than the control (−3.5 mV). And, in intestinal medium (Fig. 6B), they showed an even greater increase in zeta potential with values around −49.50 and −62.50 mV without and with the FPH addition, respectively, compared to −41.50 mV observed for the control. According to Picone et al. (2017), the increase in the net negative charge observed in the presence of pancreatin could be an indicative of the breakdown of the gellan network despite the lack of a specific enzyme for polysaccharide hydrolysis.

In intestinal medium without sample and with only bile extract addition (Fig. 6B), the zeta potential value was around −48 mV, similar to that found with enzymes addition and quite different from the medium without enzymes, suggesting a great influence of bile extract on the surface electrical charge. However, microbeads (0 and 0.25% (w/v) FPH) showed zeta potential values around −35 mV with bile extract addition, which could be indicating an interaction between gellan and bile salts.

The decrease in the magnitude of the negative charge observed for microbeads after the simulation of gastric digestion could be related to the low pH and high ionic strength of the medium, which may have led to protonation of ionizable groups and electrostatic screening (Zhang et al., 2015a, 2015b, 2016, 2017). On the other hand, the high negative zeta potential values observed after the intestinal digestion step could be result of the presence of anionic particles, such as micelles, vesicles, calcium salts, undigested lipids and proteins, which may have come from the original emulsions (e.g., peptides or free fatty acids) or from the gastrointestinal fluids (e.g., bile salts) (Zhang et al., 2015a, 2015b, 2016).

3.5.2. *Optical microscopy and particle size distribution*

The effect of the digestive process conditions on the resistance of microbeads was also evaluated (Fig. 7). Morphology of microbeads did not change after digestion steps without enzymes addition, and free oil droplets were not observed. A similar monomodal particle size distribution was observed for microbeads without and with the FPH addition (0.25% w/v) before digestion and after the gastric digestion step in the absence of enzymes. However, in intestinal step, an agglomeration of microbeads was observed, mainly for microgels without the FPH addition. Microbeads with 0% (w/v) FPH showed a higher polydispersity, while microgels with the FPH addition (0.25% w/v) showed a monomodal distribution, which is in agreement with the results of zeta potential (Fig. 6B). Higher absolute value of zeta potential for microbeads with the hydrolysate addition indicated that the higher electrostatic repulsion between negatively charged groups (pH > pI) of WPI and FPH prevented the agglomeration of microgels.

The gastric digestion step with enzymes addition also did not alter the morphology and the monomodal size distribution of microgels when compared to the microbeads before digestion (0 and 0.25% (w/v) FPH) (Fig. 7). On the other hand, in the simulation of intestinal digestion, from 15 min a large amount of O/W emulsion droplets without gel covering were observed in both systems, indicating that the presence of enzymes destabilized the gelled layer with release of the emulsion. According to Vilela et al. (2015), the disruption of the gellan gel network could be explained by the sequestration of the cations responsible to maintain the gel network by ionized bile acids. Nevertheless, it can be observed that some particles remained almost unchanged, which was corroborated by the bimodal particle size distribution, showing that despite the size of microgels has been substantially reduced and O/W emulsion droplets without gel covering were dispersed in the medium (first peak), a population with large particle size (second peak) could still be observed. Vilela et al. (2015) also did not observe significant change in the size distribution for microbeads of gellan (1% w/w) produced with gelation induced by $CaCl_2$

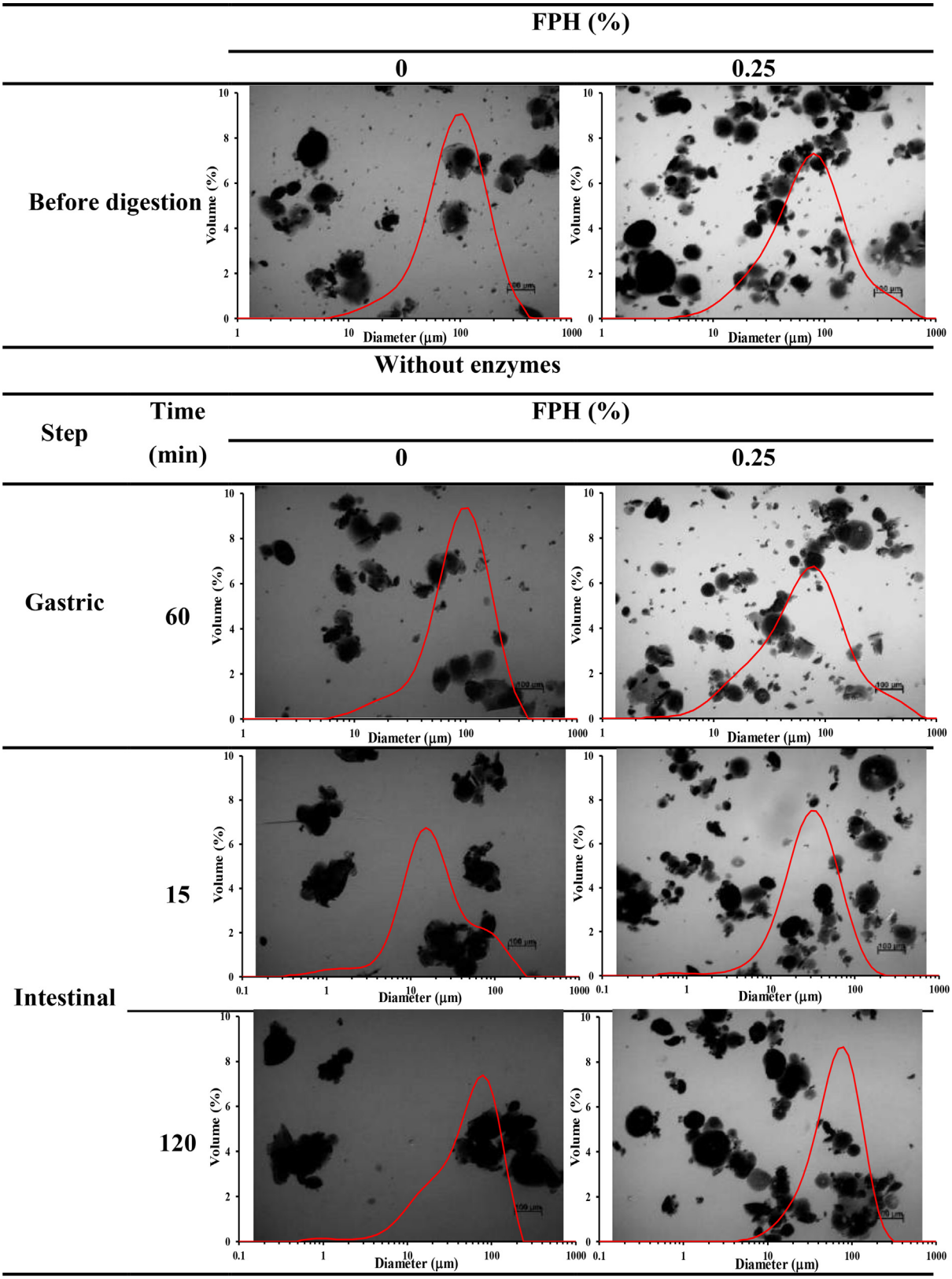


Fig. 7. Microstructure and particle size distribution of microbeads composed by 15% (v/v) flaxseed oil, 1.5% (w/v) WPI, 0 or 0.25% (w/v) FPH and 0.3% (w/v) gellan, before and after the gastric and intestinal steps, without and with enzymes addition and with bile extract addition. Scale bar = 100 μm.

(1.1% w/v) or KCl (2.28% w/v) after the gastric digestion step, and also found a physical fragmentation after the intestinal digestion.

Microgels (0 and 0.25% (w/v) FPH) remained almost intact after the digestion with only bile extract addition (Fig. 7). A bimodal size distribution was observed for microbeads without the FPH addition, while microgels with the hydrolysate addition (0.25% w/v) showed a monomodal distribution. The two peaks observed without the FPH addition seemed to correspond mainly to the smaller particles (first

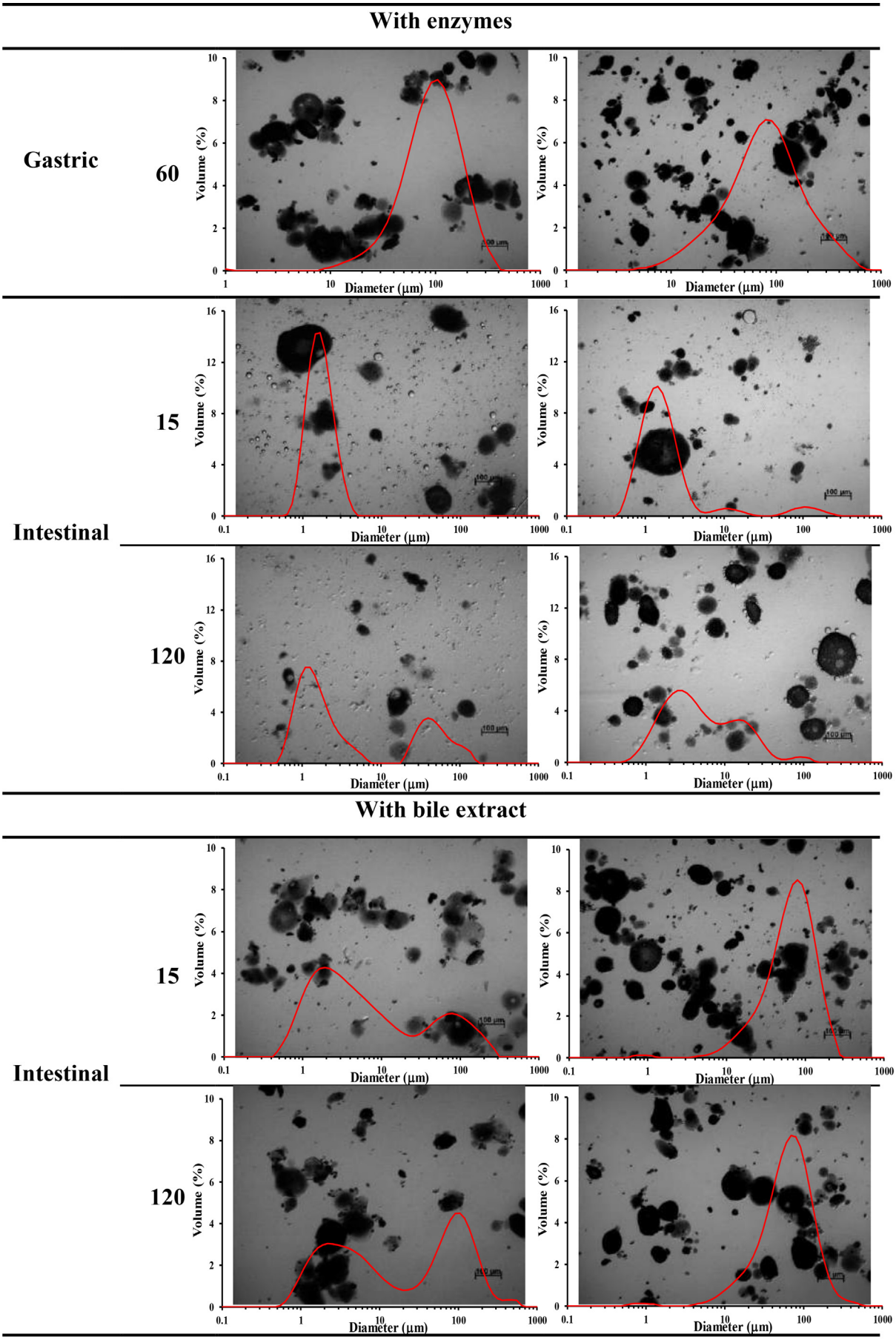


Fig. 7. (*continued*)

peak) and to the remaining microbeads (second peak) over the digestion time. On the other hand, the FPH presence seems to have assisted in stabilizing the emulsions and in the flaxseed oil encapsulation, since only one peak similar to the non-digested microbeads was observed, and it did not change significantly ($p > 0.05$) over time.

These results indicate that microbeads of WPI and gellan show resistance against the conditions found in the human stomach (gastric digestion), but they are partially disintegrated in intestinal digestion step with enzymes addition. Pepsin is known to preferentially attack peptide bonds involving hydrophobic aromatic amino acids (Beaulieu et al., 2002). In its native structure, the major protein of whey, β-lactoglobulin (β-Lg), is known to be resistant to peptic digestibility (Beaulieu et al., 2002; Morr and Ha, 1993; Reddy et al., 1988), since its amino acid hydrophobic lateral chains are located on the core (Beaulieu et al., 2002; Bertrand and Turgeon, 2007). Furthermore, the gellan gum is not easily degraded by the action of enzymes (Nag et al., 2011) and is resistant to acidic environment (Nag et al., 2011; Sun and Griffiths, 2000), which could explain the resistance to gastric medium of WPI – gellan microbeads. The degradation by pancreatin (intestinal digestion) would be attributed to the combined effect of the proteases, mainly trypsin, chymotrypsin, and elastase, which catalyze the hydrolysis of the peptide bonds, but with different specificities (Beaulieu et al., 2002). At low concentrations, bile salts can decrease the interfacial tension, facilitating the emulsification of oil droplets, and leading to an increase in the available surface area for pancreatic lipase activity (Bauer et al., 2005; Malaki Nik et al., 2011).

Beaulieu et al. (2002) observed that whey protein beads produced using an emulsification/cold gelation method, exhibited resistance to the hydrolytic action of pepsin (after 30 min), but they were totally degraded in pancreatic medium (within 6 h). According to the authors, after the gastric incubation, the beads showed a very slight degradation, suggesting that they are gastroresistant and therefore, they can be used as matrix to protect fat-soluble bioactive molecules that are sensible to stomach pH (i.e., retinol) (Beaulieu et al., 2002). Pure alginate and pure whey protein microspheres did not allow satisfactory protection of bioactive compounds in gastric fluids, but the mixture of alginate and WPI produced microspheres capable of delaying riboflavin release in the stomach and allow complete release in the small intestine, which could be indicating that they are potentially useful as oral delivery vehicles for bioactive compounds in the food and nutraceuticals industry (Chen and Subirade, 2006), as well as the systems WPI – gellan gum or WPI − FPH – gellan gum. The bioactive compounds release, such as the highly lipophilic bioactives, in the small intestine is desirable in order to increase their bioavailability, which is normally low because of their limited solubility in gastrointestinal fluids (Zhang et al., 2015c).

4. Conclusions

The results showed that it was possible to produce WPI – gellan microbeads to encapsulate flaxseed bioactive compounds using a simple process. Amphiphilic character of proteins and polar of gellan led to the migration of WPI and FPH to the oil/water interface and of gellan to the external surface, coating microgels. At higher gellan concentrations, the hydrolysate addition led to a significant size reduction and to a polydispersity increase of microbeads, maybe due to interactions between biopolymers and/or to bridges between gellan and $CaCl_2$. Microgels were resistant to gastric conditions, since that showed no change in size distribution and morphology, but they were disintegrated under intestinal conditions in the presence of enzymes, releasing the encapsulated flaxseed oil, although some microbeads remained almost intact during the evaluated time. After the digestion with only bile extract addition, microgels also did not undergo major changes. Thus, microbeads of WPI and gellan gum produced from extrusion of the O/W emulsions into a $CaCl_2$ solution could be used as a vehicle for the encapsulation and protection of flaxseed oil and protein hydrolysate during the digestion process and for their release in the small intestine.

Acknowledgments

The authors are grateful to CNPq (140287/2009-4), CAPES and FAPESP (EMU 09/54137-1) for their financial support and to Professor Valdemiro Carlos Sgarbieri (DEPAN/UNICAMP) for the donation of whey protein isolate. The authors also thank the access to equipment and assistance provided by the National Institute of Science and Technology on Photonics Applied to Cell Biology (INFABIC) at the University of Campinas; INFABIC is co-funded by FAPESP (08/57906-3) and CNPq (573913/2008-0).

References

AOAC, 1997. Official Methods of Analysis of AOAC International, sixteenth ed. Association of Official Analytical Chemists, Gaithersburg, USA.
Bauer, E., Jakob, S., Mosenthin, R., 2005. Principles of physiology of lipid digestion. Asian-Australas. J. Anim. Sci. 18 (2), 282–295.
Beaulieu, L., Savoie, L., Paquin, P., Subirade, M., 2002. Elaboration and characterization of whey protein beads by an emulsification/cold gelation process: application for the protection of retinol. Biomacromolecules 3 (2), 239–248.
Bertrand, M.-E., Turgeon, S.L., 2007. Improved gelling properties of whey protein isolate by addition of xanthan gum. Food Hydrocolloids 21 (2), 159–166.
Burey, P., Bhandari, B.R., Howes, T., Gidley, M.J., 2008. Hydrocolloid gel particles: formation, characterization, and application. Crit. Rev. Food Sci. Nutr. 48 (5), 361–377.
Çabuk, B., Harsa, Ş.T., 2015. Improved viability of *Lactobacillus acidophilus* NRRL-B 4495 during freeze-drying in whey protein–pullulan microcapsules. J. Microencapsul. 32 (3), 300–307.
Champagne, C.P., Fustier, P., 2007. Microencapsulation for the improved delivery of bioactive compounds into foods. Curr. Opin. Biotechnol. 18 (2), 184–190.
Chan, E.-S., Lee, B.-B., Ravindra, P., Poncelet, D., 2009. Prediction models for shape and size of ca-alginate macrobeads produced through extrusion-dripping method. J. Colloid Interface Sci. 338 (1), 63–72.
Chandrasekaran, R., Puigjaner, L.C., Joyce, K.L., Arnott, S., 1988. Cation interactions in gellan: an X-ray study of the potassium salt. Carbohydr. Res. 181, 23–40.
Chen, L., Subirade, M., 2006. Alginate–whey protein granular microspheres as oral delivery vehicles for bioactive compounds. Biomaterials 27 (26), 4646–4654.
Chen, L., Remondetto, G.E., Subirade, M., 2006. Food protein-based materials as nutraceutical delivery systems. Trends Food Sci. Technol. 17 (5), 272–283.
D'Arrigo, G., Di Meo, C., Gaucci, E., Chichiarelli, S., Coviello, T., Capitani, D., Alhaique, F., Matricardi, P., 2012. Self-assembled gellan-based nanohydrogels as a tool for prednisolone delivery. Soft Matter 8, 11557–11564.
Dahiya, S., Rani, R., Kumar, S., Dhingra, D., Dilbaghi, N., 2017. Chitosan–gellan gum bipolymeric nanohydrogels – a potential nanocarrier for the delivery of epigallocatechin gallate. BioNanoScience 7 (3), 508–520.
De Souza Simões, L., Madalena, D.A., Pinheiro, A.C., Teixeira, J.A., Vicente, A.A., Ramos, Ó.L., 2017. Micro- and nano bio-based delivery systems for food applications: *In vitro* behavior. Adv. Colloid Interface Sci. 243, 23–45.
Doyen, A., Udenigwe, C.C., Mitchell, P.L., Marette, A., Aluko, R.E., Bazinet, L., 2014. Antidiabetic and antihypertensive activities of two flaxseed protein hydrolysate fractions revealed following their simultaneous separation by electrodialysis with ultrafiltration membranes. Food Chem. 145, 66–76.
Garrett, D.A., Failla, M.L., Sarama, R.J., 1999. Development of an in vitro digestion method to assess carotenoid bioavailability from meals. J. Agric. Food Chem. 47 (10), 4301–4309.
Heilig, A., Göggerle, A., Hinrichs, J., 2009. Multiphase visualisation of fat containing β-lactoglobulin–κ-carrageenan gels by confocal scanning laser microscopy, using a novel dye, V03-01136, for fat staining. LWT – Food Science and Technology 42 (2), 646–653.
Herrero, E.P., Martín Del Valle, E.M., Galán, M.A., 2006. Development of a new technology for the production of microcapsules based in atomization processes. Chem. Eng. J. 117 (2), 137–142.
Hoebler, C., Lecannu, G., Belleville, C., Devaux, M.-F., Popineau, Y., Barry, J.-L., 2002. Development of an *in vitro* system simulating bucco-gastric digestion to assess the physical and chemical changes of food. Int. J. Food Sci. Nutr. 53 (5), 389–402.
Kaihara, S., Suzuki, Y., Fujimoto, K., 2011. In *situ* synthesis of polysaccharide nanoparticles via polyion complex of carboxymethyl cellulose and chitosan. Colloids Surfaces B Biointerfaces 85 (2), 343–348.
Karewicz, A., Łęgowik, J., Nowakowska, M., 2011. New bilayer-coated microbead system for controlled release of 5-aminosalicylic acid. Polym. Bull. 66 (3), 433–443.
Klaypradit, W., Huang, Y.-W., 2008. Fish oil encapsulation with chitosan using ultrasonic atomizer. LWT – Food Science and Technology 41 (6), 1133–1139.
Kuhn, K.R., Cavallieri, A.L.F., Cunha, R.L., 2010. Cold-set whey protein gels induced by calcium or sodium salt addition. Int. J. Food Sci. Technol. 45 (2), 348–357.
Liu, S., Low, N.H., Nickerson, M.T., 2010. Entrapment of flaxseed oil within gelatin-gum Arabic capsules. JAOCS (J. Am. Oil Chem. Soc.) 87 (7), 809–815.
Malaki Nik, A., Wright, A.J., Corredig, M., 2011. Impact of interfacial composition on emulsion digestion and rate of lipid hydrolysis using different *in vitro* digestion models. Colloids Surfaces B Biointerfaces 83 (2), 321–330.

Marambe, P.W., M L, H.K., Shand, P.J., Wanasundara, J.P.D., 2008. An in-vitro investigation of selected biological activities of hydrolysed flaxseed (*Linum usitatissimum* L.) proteins. JAOCS (J. Am. Oil Chem. Soc.) 85 (12), 1155–1164.
Miller, D.D., Schricker, B.R., Rasmussen, R.R., Van Campen, D., 1981. An in vitro method for estimation of iron availability from meals. Am. J. Clin. Nutr. 34 (10), 2248–2256.
Morr, C.V., Ha, E.Y.W., 1993. Whey protein concentrates and isolates: processing and functional properties. Crit. Rev. Food Sci. Nutr. 33 (6), 431–476.
Nag, A., Han, K.-S., Singh, H., 2011. Microencapsulation of probiotic bacteria using pH-induced gelation of sodium caseinate and gellan gum. Int. Dairy J. 21 (4), 247–253.
Nedovic, V., Kalusevic, A., Manojlovic, V., Levic, S., Bugarski, B., 2011. An overview of encapsulation technologies for food applications. Procedia Food Science 1, 1806–1815.
Pasukamonset, P., Kwon, O., Adisakwattana, S., 2016. Alginate-based encapsulation of polyphenols form *Clitoria ternatea* petal flower extract enhances stability and biological activity under simulated gastrointestinal conditions. Food Hydrocolloids 61, 772–779.
Perrechil, F.A., Sato, A.C.K., Cunha, R.L., 2011. κ-Carrageenan–sodium caseinate microgel production by atomization: critical analysis of the experimental procedure. J. Food Eng. 104 (1), 123–133.
Perrechil, F.A., Vilela, J.A.P., Guerreiro, L.M.R., Cunha, R.L., 2012. Development of Na-CN—κ-carrageenan microbeads for the encapsulation of lipophilic compounds. Food Biophys. 7 (3), 264–275.
Picone, C.S.F., Bueno, A.C., Michelon, M., Cunha, R.L., 2017. Development of a probiotic delivery system based on gelation of water-in-oil emulsions. LWT – Food Science and Technology 86, 62–68.
Piornos, J.A., Burgos-Díaz, C., Morales, E., Rubilar, M., Acevedo, F., 2017. Highly efficient encapsulation of linseed oil into alginate/lupin protein beads: optimization of the emulsion formulation. Food Hydrocolloids 63, 139–148.
Rabetafika, H.N., Remoortel, V.V., Danthine, S., Paquot, M., Blecker, C., 2011. Flaxseed proteins: food uses and health benefits. Int. J. Food Sci. Technol. 46 (2), 221–228.
Reddy, I.M., Kella, N.K.D., Kinsella, J.E., 1988. Structural and conformational basis of the resistance of β-Lactoglobulin to peptic and chymotryptic digestion. J. Agric. Food Chem. 36 (4), 737–741.
Rizk, N.K., Lefebvre, A.H., 1980. The influence of liquid film thickness on airblast atomization. J. Eng. Power 102 (7), 706–710.
Sato, A.C.K., Cunha, R.L., 2012. Influence of dispersing media and particle characteristics on rheological behavior of noncolloidal suspensions. J. Dispersion Sci. Technol. 33 (3), 437–446.
Shahidi, F., Miraliakbari, H., 2005. Omega-3 fatty acids in health and disease: Part 2—health effects of omega-3 fatty acids in autoimmune diseases, mental health, and gene expression. J. Med. Food 8 (2), 133–148.
Silva, F.G.D., O'Callagahan, Y., O'Brien, N.M., Netto, F.M., 2013. Antioxidant capacity of flaxseed products: the effect of in vitro digestion. Plant Foods Hum. Nutr. 68 (1), 24–30.
Sun, W., Griffiths, M.W., 2000. Survival of bifidobacteria in yogurt and simulated gastric juice following immobilization in gellan–xanthan beads. Int. J. Food Microbiol. 61 (1), 17–25.
Tang, Z., Huang, X., Baxi, S., Chambers, J.R., Sabour, P.M., Wang, Q., 2013. Whey protein improves survival and release characteristics of bacteriophage Felix O1 encapsulated in alginate microspheres. Food Res. Int. 52 (2), 460–466.
Udenigwe, C.C., Lin, Y.-S., Hou, W.-C., Aluko, R.E., 2009a. Kinetics of the inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. J. Funct. Foods 1 (2), 199–207.
Udenigwe, C.C., Lu, Y.-L., Han, C.-H., Hou, W.-C., Aluko, R.E., 2009b. Flaxseed protein-derived peptide fractions: antioxidant properties and inhibition of lipopolysaccharide-induced nitric oxide production in murine macrophages. Food Chem. 116 (1), 277–284.
Vilela, J.A.P., Cavallieri, A.L.F., Cunha, R.L., 2011. The influence of gelation rate on the physical properties/structure of salt-induced gels of soy protein isolate–gellan gum. Food Hydrocolloids 25 (7), 1710–1718.
Vilela, J.A.P., Perrechil, F.A., Picone, C.S.F., Sato, A.C.K., Cunha, R.L., 2015. Preparation, characterization and *in vitro* digestibility of gellan and chitosan–gellan microgels. Carbohydr. Polym. 117, 54–62.
Zhang, W., Xu, S., Wang, Z., Yang, R., Lu, R., 2009. Demucilaging and dehulling flaxseed with a wet process. LWT – Food Science and Technology 42 (6), 1193–1198.
Zhang, R., Zhang, Z., Zhang, H., Decker, E.A., McClements, D.J., 2015a. Influence of emulsifier type on gastrointestinal fate of oil-in-water emulsions containing anionic dietary fiber (pectin). Food Hydrocolloids 45, 175–185.
Zhang, R., Zhang, Z., Zhang, H., Decker, E.A., McClements, D.J., 2015b. Influence of lipid type on gastrointestinal fate of oil-in-water emulsions: in vitro digestion study. Food Res. Int. 75, 71–78.
Zhang, Z., Zhang, R., Chen, L., Tong, Q., McClements, D.J., 2015c. Designing hydrogel particles for controlled or targeted release of lipophilic bioactive agents in the gastrointestinal tract. Eur. Polym. J. 72, 698–716.
Zhang, Z., Zhang, R., McClements, D.J., 2016. Encapsulation of β–carotene in alginate-based hydrogel beads: impact on physicochemical stability and bioaccessibility. Food Hydrocolloids 61, 1–10.
Zhang, Z., Zhang, R., McClements, D.J., 2017. Control of protein digestion under simulated gastrointestinal conditions using biopolymer microgels. Food Res. Int. 100 (Part 2), 86–94.
Đorđević, V., Balanč, B., Belščak-Cvitanović, A., Lević, S., Trifković, K., Kalušević, A., Kostić, I., Komes, D., Bugarski, B., Nedović, V., 2015. Trends in encapsulation technologies for delivery of food bioactive compounds. Food Eng. Rev. 7 (4), 452–490.