Suitability of β-lactoglobulin micro- and nanostructures for loading and release of bioactive compounds

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Abstract

β-lactoglobulin (β-Lg) has the ability to form three-dimensional networks when heated above denaturation temperature (ca. 76 °C), since it undergoes conformational changes followed by subsequent protein-protein interactions, which allows designing stable micro- and nanostructures with affinity to bind to a wide range of molecules. In this sense, β-Lg micro (with particle size from 200 to 300 nm) and nano (with particle size ≤ 100 nm) structures were developed as a delivery system for the controlled release of hydrophilic and hydrophobic model compounds. Several concentrations of bioactive compounds were incorporated into β-Lg micro- and nanostructures and their association efficiency (*AE*) and loading capacity (*LC*) were determined. β-Lg structures were characterized in terms of structural properties, morphology, binding mechanisms, conformational changes and secondary structure. The impact of several conditions (e.g., pH, thermal processing, ionic strength and storage temperature) on the stability of β-Lg structures was also investigated. The release profile of bioactive compounds from β-Lg structures was determined *in vitro* using two food simulants with different hydrophobicities under different temperature conditions (at 4 °C and 25 °C). Data recorded showed that β-Lg nanostructures had the highest *AE* and *LC* comparing with β-Lg microstructures, for both bioactive compounds tested. β-Lg micro- and nanostructures with or without association of bioactive compounds showed to be stable under acidic (pH 2 to 3), neutral (pH 6) or alkaline (pH 10) conditions, thermal treatments up to 70 °C and during storage for 50 and 90 days at 25 °C and 4 °C, maintaining their particle size, PDI and surface charge ($p > 0.05$). The release kinetics of bioactive compounds from micro- and nanostructures fitted well the Linear Superimposition Model, being the relaxation the main release mechanism. Both compounds showed an initial burst effect followed by a slow release. All these findings provide new insights on which conditions the β-Lg micro- and nanostructures are more stable, and therefore more suitable to act as potential delivery systems for hydrophilic and hydrophobic bioactive compounds.

Keywords: β-Lactoglobulin; Micro- and Nano structures; Delivery systems; Hydrophilic compounds; Hydrophobic compounds; Food-grade; Food simulant

1. Introduction

The emerging demand for safer, nutritious and high-quality functional foods as a way to address specific human health requirements has gained recently the attention from food industry players and scientific community for developing new sustainable ways to encapsulate, protect and deliver bioactive compounds in food products (Devi et al., 2017; Sedaghat Doost et al., 2018).

β-lactoglobulin (β-Lg), the main protein fraction of whey proteins, is an interesting matrix for designing delivery systems for controlled release of bioactive compounds, due to its bio-based nature, Generally Recognized As Safe (GRAS) designation, high nutritional value, biological and functional (e.g., emulsification) properties, as well as, to its gelation and high binding capacity (Madalena et al., 2016). The conformational state of native β-Lg becomes unfolded when induced by thermal heating, thus resulting in the exposure of initially buried hydrophobic aminoacid residues with subsequent self-aggregation of protein molecules through physical (electrostatic and hydrophobic) and chemical (disulphide) interactions (Delahaije et al., 2015). β-Lg structural characteristics abovementioned allows it to perform well as delivery system for various hydrophilic and hydrophobic bioactive compounds (Aprodu et al., 2017).

Even though previous researches demonstrated that β-Lg has affinity to bind to a wide range of molecules with distinct physiochemical properties (e.g., solubility in water, molecular weight, and structure), its ability to act as delivery system is still limited and there are current challenges related with binding to bioactive compounds that need to be, firstly, well understood, and then overcome. For instance, Berino et al. (2019) investigated the interaction between β-Lg with vitamin D_3 (at several concentrations) and the stability of the complex formed to protect vitamin D_3 from degradation by light, oxygen and pressure. The authors found limitations in the binding of vitamin D_3 to the hydrophobic sites in the calyx and to the hydrophobic surface patches of β-Lg with conformational changes recorded in the protein secondary structure. In another study, Guo et al. (2017) aimed at masking caffeine's bitter taste and unpleasant aftertaste by using β-Lg nanoparticles as delivery systems. The authors obtained low encapsulation efficiencies (i.e., 13.5 %). In fact, the delivery systems should display high encapsulation efficiencies of bioactive compounds and protect them against degradation during production and over storage periods. However, information regarding the best conditions to design and process β-Lg delivery systems, about their performance as encapsulating agents, stability in several environmental conditions

(e.g., pH values, ionic strength, temperature) and over time, ability to bind bioactive compounds and behaviour in food matrices is very scarce or inexistent. A few studies are available regarding the use of other proteins as encapsulating agents for bioactive compounds with emphasis to one conducted by Bourbon et al., (2016). These authors developed lactoferrin-glycomacropeptide nanohydrogels by thermal gelation to encapsulate curcumin (as model lipophilic compound) and caffeine (as model hydrophilic compound). This study showed that such nanohydrogels successfully bound both bioactive compounds, enhanced their antimicrobial activity and promoted their controlled release.

Taking into account the limited information available regarding the use of proteins as bio-based delivery systems, and in particular of β-Lg, it is considered of utmost importance to understand how different sizes of β-Lg structures, below and above 100 nm, may affect their physicochemical properties. According to Recommendation 2011/696/EU of the European Commission of 18 October 2011 (Commission, 2011a), and to recent literature reviews (Jafari, 2017; Mcclements, 2017; Singh, 2016) a nanomaterial means "*A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm"*. This definition aims to inform the consumers and proceed as a guideline to different regulatory sectors. Therefore, in this study, β-Lg structures at nano- (with diameters ≤ 100 nm) and at micro- (with diameters between 200 and 300 nm) scales were used to understand how its size may impact the encapsulation, protection and controlled release of bioactive compounds. Due to reduced size, the micro-, and even more, the nanostructures may be able to improve the solubility, bioavailability, and protect the bioactive compounds from degradation when compared with macrostructures formed from the same protein (Monteiro et al., 2016; Simões et al., 2020). This behaviour can be resulted from a high surface/volume ratio, but also from different effects of physical and chemical interactions imparting by β-Lg nano- (with diameters ≤ 100 nm) and micro- (with diameters between 200 and 300 nm) structures, which may have a significant influence on their performance as potential delivery systems (Cerqueira et al., 2014).

In this context, β-Lg nano- (with diameters ≤ 100 nm) and micro- (with diameters between 200 – 300 nm) structures were proposed here as potential delivery systems to encapsulate, protect and

controlled release of bioactive compounds (i.e., riboflavin and quercetin) with different water solubilities. Riboflavin is a vitamin essential to human growth (Beztsinna et al., 2016), however this compound cannot be naturally synthetized by the human body, thus must be obtained from external sources (Gironés-Vilaplana et al., 2017). Nonetheless, its use in food products is still limited by several issues such as its high degradability when exposed to light and low solubility in water (Ashoori & Saedisomeolia, 2014). For all these reasons riboflavin was studied as hydrophilic model compound. Quercetin attracted the interest of the scientific community due to its anticancer, antioxidant and antiviral activities (Haghi et al., 2017). However, its use in food products is still limited due to its hydrophobic nature (i.e., poor aqueous solubility), low oral bioavailability (i.e., it is highly degraded through the gastrointestinal tract), and stability (Aditya et al., 2014). Therefore, quercetin was investigated as hydrophobic model compound. Hence, this research aims at deepening the understanding of the binding mechanisms of β-Lg micro- and nanostructures to riboflavin (as model hydrophilic compound) and quercetin (as model hydrophobic compound) and assess their potential as encapsulating agents. Also, the impact of various environmental conditions on the physicochemical stability of loaded β-Lg micro- and nanostructures, and the release profile of such model compounds from β-Lg micro- and nanostructures when they are added into food simulants, will be assessed.

2. Materials and methods

2.1. Feedstocks and chemicals

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): quercetin (≥ 95%, HPLC), riboflavin (≥ 98%), sodium azide, ANS (8-Anilino-1-napththalenesulfonic), and sodium phosphate tribasic dodecahydrate 98 %. Whey Protein Isolate (WPI) powder (Lacprodan DI-9212), kindly supplied by Arla Foods Ingredients (Viby, Denmark), has in a total protein content of 91 % (in dry weight), moisture content of ca. 6 % and vestiges of lactose (max. 0.5 %) and fat (max. 0.2 %), according to the information provided by the supplier. Sodium hydroxide and phosphoric acid were obtained from Merck (Merck KGaA, Darmstadt, Germany). Hydrochloric acid and monosodium phosphate were purchased from Panreac (Barcelona, Spain), whereas sodium phosphate dibasic, acetonitrile (HPLC grade) and methanol were obtained from Chem-Lab (Zedelgem, Belgium). Dimethyl sulfoxide was obtained from Fisher Scientific, while Ethanol

was purchased from JMGS (Portugal). All other chemicals used in this study were reagents of analytical grade.

2.2. Development of β–Lg micro- and nanostructures

β-Lg micro- and nanostructures were prepared according to the procedure previously optimized (Simões, Araújo, Vicente, & Ramos, 2020). For this purpose, β-Lg powder at 5 and 15 mg mL^{-1} was used to develop nano- and microstructures, respectively, being dissolved in sodium phosphate buffer at pH 6 containing 0.02 % of sodium azide (used as preservative to avoid microbial growth). The pH was adjusted with 0.5 mol L^{-1} of H_3PO_4 and/or 1 mol L^{-1} of NaOH, as necessary. Then, solutions were stirred continuously during 120 min at 400 rpm at room temperature (ca. 25 °C). The β-Lg solutions were kept overnight at a temperature of 4 °C to ensure full protein rehydration. Afterwards, the samples were filtered through a 0.2 µm cellulose acetate membrane syringe filter (VWR International, USA) to remove any protein aggregates or impurities. Subsequently, β-Lg solutions were placed into cylindrical screw-capped glass tubes (100 mm total length and diameter of 20 mm) and submitted to thermal treatment at 80 ºC for 15 min (conditions previously optimized) then cooled in ice for 10 min.

2.3. Association efficiency and loading capacity

After β-Lg solutions thermal treatment, a given volume of each bioactive compound stock solution was added to the resulting β-Lg solutions, and then were cooled in ice for 10 min to form β-Lg micro- and nanostructures.

2.3.1. Riboflavin – hydrophilic bioactive model compound

Riboflavin, as a hydrophilic bioactive model compound, was dissolved in 0.1 mol L^{-1} Na_3PO_4, being the resulting solutions protected from light with aluminium foil (Zhang et al., 2016) to obtain β-Lg solutions with final concentrations of riboflavin ranging from 0.007 to 0.105 mg mL^{-1}.

In order to separate the free riboflavin in supernatant, 0.5 mL of β-Lg/riboflavin micro- and nanostructure solutions were placed into Amicon® Ultra-0.5 centrifugal filter units with molecular weight cut-off 3 kDa (Merck Millipore, Ireland) and centrifuged (Model 5415 R, Eppendorf) at 16,000 *g* during 20 min at 4 °C. The filtrate with unbound riboflavin was determined spectrophotometrically at 437 nm (Azevedo et al., 2014) using the calibration curve

$y = 7.9841x + 0.056$ with R^2 = 0.99 (being *y* and *x* the absorbance and riboflavin concentration, respectively). These results were used to calculate the association efficiency (*AE*) according to the Equation (1). The concentrate with the β-Lg/riboflavin micro- and nanostructure retained in Amicon® was dried at 55 °C during 24 h in a ventilated oven and the loading capacity (*LC*) was determined by mass difference according to the Equation (2).

$$AE\ (\%) = \frac{C_{Bioactive\ compound\ total} - C_{Bioactive\ compound\ free}}{C_{Bioactive\ compound\ total}} \times\ 100 \qquad \text{Equation (1)}$$

$$LC\ (\%) = \frac{C_{Bioactive\ compound\ total} - C_{Bioactive\ compound\ free}}{W_{structure}} \times\ 100 \qquad \text{Equation (2)}$$

where, $C_{Bioactive\ compound\ total}$ is the concentration of bioactive compound in solution; $C_{Bioactive\ compound\ free}$ is the concentration of free bioactive compound separated from β-Lg structures; and $W_{structure}$ is the β-Lg structure weight.

2.3.2. Quercetin – hydrophobic bioactive model compound

The addition of quercetin to an ethanol solution (used as solvent) was limited due to the protein sensitivity to ethanol (Pace et al., 2004). Distinct quercetin concentrations were previously dissolved in ethanol in order to achieve quercetin concentrations ranging from 0.01 to 0.08 mg mL^{-1}. β-Lg/quercetin micro- and nanostructures (2 mL) were placed into tube samples and subsequently centrifuged (Model 5415 R, Eppendorf) at 14,000 *g* for 10 min at 4 °C, to separate the unbound quercetin (Madalena et al., 2016). The free quercetin in the supernatant was evaluated by high-performance liquid chromatography (HPLC) with fluorescence detection. The HPLC system used consisted of a Varian Prostar 210 pump, a Varian Prostar 410 autosampler, and a Jasco FP-920 detector. The column was a C18 reversed-phase YMC-Pack ODS (4.6 × 250 mm i.d; 5 µm particle size). The mobile phase was composed of acetonitrile and distilled water (40:60, v/v), and was pumped at a flow rate of 1.0 mL min^{-1}. The wavelengths used for quercetin detection were excitation = 355 nm and emission = 525 nm (Komori et al., 2007). The quantification was performed using a calibration curve prepared with standards at concentrations between 0.01 to 0.1 mg mL^{-1}. These results were used to assess the *AE* by Equation (1). The β-

Lg/quercetin micro- and nanostructures were dried at 55 °C during 24 h in a ventilated oven and the *LC* was determined using the Equation (2).

2.4. Stability under various environmental conditions

The stability of β-Lg micro- and nanostructures in the absence and presence of bioactive model compounds under various environmental conditions (i.e., pH, ionic strength, thermal processing and storage temperature) was evaluated in terms of their particle size, polydispersity index (PDI) and surface charge (S) by dynamic light scattering (DLS) instrument.

2.4.1. pH stability

The effect of pH was assessed by ranging pH between 2 and 10 using 0.1 mol L^{-1} NaOH and/or 0.1 mol L^{-1} H_3PO_4, as necessary.

2.4.2. Ionic strength stability

The influence of ionic strength was evaluated on β-Lg micro- and nanostructures in the absence and presence of bioactive model compounds by adding distinct NaCl concentrations (0 - 200 mmol L^{-1}) (Bourbon et al., 2015; Tokle et al., 2013).

2.4.3. Temperature stability

Thermal stability was assessed by DLS, using the temperature range from 20 to 80 °C, with increments of 5 °C, with 60 s of equilibration before each measurement (Martins et al., 2016).

2.4.4. Storage temperature stability

Samples were stored at temperatures of 4 °C (to simulate refrigeration conditions) and 25 °C (to simulate room temperature conditions) over 90 days, and at an appropriate interval of time, particle size, PDI and surface charge (S) were measured (Monteiro et al., 2016).

2.5. Physicochemical characterization

2.5.1. Particle size, polydispersity index and surface charge

Freshly prepared β-Lg structures were used to measure particle size, polydispersity index (PDI) and surface charge (S) using a DLS instrument (Zetasizer Nano ZS, Malvern Instruments, UK) equipped with He-Ne laser at a wavelength of 633 nm. β-Lg samples (1.5 mL) were poured into disposable sizing cuvettes with a path length of 10 mm for particle size and PDI analyses, which carried out with detection angle of 173 ° (Bourbon et al., 2016). Surface charge of samples was performed by ζ – potential measurements into a folded capillary cell. All samples were equilibrated at 25 °C before measurements were performed. The results are given as mean ± standard deviation of nine measures obtained.

2.5.2. Extrinsic fluorescence

The extrinsic fluorescence was carried out by fluorescence spectroscopy (Jasco FP6200, Tokyo, Japan) using 8-anilinonaphtalene-1-sulfonic acid ammonium salt (ANS) (Sigma-Aldrich, St. Louis, EUA) to assess the hydrophobic sites of β-Lg micro- and nanostructures with/without bioactive model compound associated.

For this propose, an ANS stock solution at 1.36 mmol L^{-1} was prepared in methanol in the absence of light, before analyses were performed. Subsequently, 20 µL of ANS solution were added into β-Lg samples (4 mL), in appropriated dilution, and incubated at 25 °C during 10 min (Wang et al., 2013). Fluorescence measurements of resulting solutions were performed using a 10 mm path length quartz cuvette with excitation wavelength at 370 nm, and emission spectra were recorded from 400 to 600 nm at room temperature (ca. 25 °C). Data results of extrinsic fluorescence peaks were given in arbitrary units and analysed by ORIGIN 9.0 program (Multiple Peak Fit).

2.5.3. Circular dichroism spectra

Circular dichroism (CD) spectra were recorded using a Jasco J-1500 spectropolarimeter (Jasco International Co, Japan) to evaluate the β-Lg secondary structure content and the effect of binding of bioactive model compounds. β-Lg samples, in appropriate dilution, were poured into a quartz cell with a path length of 1.0 mm (Hellma Analytics, Germany). Far-UV spectra ranging from 190 to 260 nm wavelength was performed under nitrogen atmosphere with data pitch of 1.0 nm, accumulation of 3 scans at a scan speed of 50 nm min^{-1} at 25 ºC (Bourbon et al., 2016). The CD spectra were presented as the average of three measurements.

2.5.4. Fourier- transform infrared spectroscopy

The binding of bioactive model compounds to β-Lg micro- and nanostructures was evaluated by Fourier-transform infrared (FTIR) spectra (model ABB FTLA2000, Canada), according to the procedure adopted by (Martins et al., 2016) with a few modifications. Samples (2 mg) were prior lyophilized, and then compressed into thin potassium bromide (KBr) (200 mg) pellets and finally analysed in a wavenumber range of 4000 to 500 cm^{-1}. Each spectrum was performed by averaging 16 scans with a spectral resolution of 8 cm^{-1}. Data analysis was performed using ORIGIN 9 software.

2.6. Transmission electronic microscopy

Transmission electron microscopy (TEM) imaging of β-Lg micro- and nanostructures associated with bioactive model compounds was performed by negative staining method using a Zeiss EM 902A (Thornwood, N.Y., USA) microscope at accelerating voltages of 50 and 80 kV. A drop of sample dispersion was deposited onto a carbon support film mounted on a TEM copper grid (Quantifoil, Germany) and excess of the solution was removed after 2 min using a filter paper and the grid let for air drying. The samples were then negatively stained with uranyl acetate (2 % w/w) (Merck, Germany) during 15 s. The grid was finally air dried at room temperature before introducing it in the electron microscope. This methodology was performed according to procedures usually adopted by our research group (Bourbon et al., 2015; Monteiro et al., 2016; Pinheiro et al., 2015).

2.7. *In vitro* release profile

The *in vitro* release profiles of bioactive model compounds were evaluated by dialysis over 72 h (Azevedo et al., 2014; Bourbon et al., 2016). These experiments used two food simulant solutions, composed by 10 and 50 % of ethanol to simulate hydrophilic and hydrophobic food matrices, respectively, according with the Commission Regulation (EU) No 10/2011 of 14 January 2011 (Commission, 2011b), and were conducted at 4 °C (to mimic refrigerated temperatures) and at 25 °C (to mimic room temperature).

Samples (5 mL) were placed inside of a dialysis membrane with cut-off 10 kDa (Spectra/Por, Spectrum Laboratories, USA) and then placed into a reactor with 50 mL of food simulant solutions under continuous magnetic stirring at 200 rpm. At appropriate time intervals, 0.3 mL of supernatant was taken and replaced by the same quantity of fresh food simulant solution to maintain the volume constant. The quantity of riboflavin and quercetin released from β-Lg micro- and nanostructures were evaluated by spectrophotometry for riboflavin and by HPLC for quercetin. These experiments were performed at least in triplicate.

2.7.1. *In vitro* release kinetics

The release mechanisms of bioactive model compounds from β-Lg micro- and nanostructures were assessed by the kinetic model proposed by Berens and Hopfenberg (1978), which considers both Fickian and Case II transport effects as evidenced in Equation (3):

$$M_t = M_{t,F} + M_{t,R} \quad \text{Equation (3)}$$

where, M_t is the total mass released from polymeric β-Lg micro- and nanostructures at time *t*; $M_{t,F}$ and $M_{t,R}$ are the contributions of the Fickian and biopolymer relaxation effects, respectively. The Fickian transport is described by Equation (4), as follows:

$$M_{t,F} = M_{\infty,F}\left[1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left(-n^2 k_F t\right)\right] \quad \text{Equation (4)}$$

where, $M_{\infty,F}$ is the bioactive compound released at equilibrium and k_F is the Fickian diffusion rate constant. Eq. (4) can be simplified by applying the first term of the Taylor series (Pinheiro et al., 2012), as presented in Equation (5):

$$M_{t,F} = M_F\left[1 - \frac{6}{\pi^2}\exp\left(k_F t\right)\right] \quad \text{Equation (5)}$$

Regarding biopolymer relaxation, it is driven by biopolymer swelling capacity and then associated to the dissipation of stress induced by the penetrant entrance. This behaviour can be characterized as a distribution of relaxation times, each assuming a first order-type kinetic equation (Berens & Hopfenberg, 1978), as follows Equation (6):

$$M_{t,R} = \sum_i M_{\infty,Ri}[1 - \exp(-k_{Ri}t)\,] \quad \text{Equation (6)}$$

where, $M_{\infty,Ri}$ is the contribution of the relaxation processes for bioactive compound release and k_{Ri} is the relaxation i^{th} rate constant. Commonly, only one main biopolymer relaxation influences the relaxation transport (i.e. $i = 1$) (Bourbon et al., 2016).

Thereby, the linear superimposition model (LSM) for bioactive model compounds released from β-Lg micro- and nanostructures can be described by Equation (7):

$$\frac{M_t}{M_\infty} = X\left[1 - \frac{6}{\pi^2}\exp\left(-k_F t\right)\right] + (1 - X)[1 - \exp\left(-k_R t\right)] \quad \text{Equation (7)}$$

where X is the fraction of bioactive compounds released by Fickian transport.

Experimental data were fitted with Eq. (7) (LSM) to evaluate the transport mechanism involved in riboflavin and quercetin release from β-Lg micro- and nanostructures at hydrophilic and hydrophobic food simulants at 4 and 25 °C.

2.8. Statistical analyses

The whole experiment was run at least in triplicate and was expressed as average ± standard deviation. The results were statistically analysed with one-way ANOVA followed by *post hoc* Tukey's tests using $p \leq 0.05$ as the level of significance.

Equations (4) and (7) were fitted to experimental data using a non-linear regression. The least square function minimization was done by the Leveberg-Marquardt algorithm. The quality of regression curves was assessed by the determination coefficient (R^2) and the squared root mean square error (*RMSE*). All statistical analyses were performed using the package of Statistica software version 10.0.228.8 (StatSoft Inc, USA.).

3. Results and discussion

3.1. Bioactive model compounds association

According to the available literature, β-Lg has the capability to associate both hydrophilic and hydrophobic bioactive compounds (Aprodu et al., 2017; Xu et al., 2019; Zagury et al., 2019). The different physicochemical properties of bioactive compounds (e.g., molecular weight, water solubility and chemical structure) can affect the behaviour of β-Lg structures. Therefore, a range of concentrations of each model bioactive compound associated to β-Lg micro- and nanostructures were investigated in terms of association efficiency (*AE*), loading capacity (*LC*), particle size, polydispersity (PDI) and surface charge (S). *AE* provides information about the bioactive compound fraction successfully entrapped within structures (Mirpoor et al., 2017), whereas *LC* is related to the maximum amount of bioactive model compound that can be loaded into a β-Lg structure (Zou et al., 2016).

Table 1 shows the results obtained for β-Lg micro- and nanostructures associated to riboflavin, used as hydrophilic model compound.

«Table 1 to be placed here»

The *AE* (Table 1) of β-Lg/riboflavin microstructures ranged between 31.9 and 64.4 % ($p \leq 0.05$), as riboflavin concentration increased. In turns, β-Lg/riboflavin nanostructures achieved the highest *AE* of 67.9 % at 0.014 mg mL^{-1} of riboflavin ($p > 0.05$). Above this riboflavin concentration, the *AE* did not significantly change, thus suggesting that this is the maximum concentration that such β-Lg nanostructures can associate. In a study conducted by Guo et al., (2017), it was shown that β-Lg nanoparticles were able to bind caffeine (used as hydrophilic bioactive compounds) with an *AE* of 13.54 ± 3.3 % for a caffeine:β-Lg ratio of 50:1. In our work, β-Lg/nanostructures demonstrated a *AE* 4.8 times higher for a lower riboflavin:β-Lg ratio (i.e., 21:1).

On the other hand, the *LC* increased as riboflavin concentration increases, independently of β-Lg structure scale, as depicted in Table 1. *LC* values achieved a maximum of 1.80 ± 0.16 % and 1.13 ± 0.03 % ($p \leq 0.05$), for nano- and microstructures, respectively. This result is somehow expected, taking into consideration the higher surface area-to-volume ratio of nanoscale materials, which can bind higher concentrations of bioactive compounds (Jafari & McClements, 2017). Azevedo et al. (2014) reported a similar behaviour for alginate/chitosan nanoparticles, which presented *AE* of 55.9 ± 5.6 % and a *LC* of 2.2 ± 0.6 % for riboflavin. In another study, Shpigelman et al. (2012) developed β-Lg nanosystems by thermal gelation at 70 °C for 20 min at pH 6.8 for encapsulation of (-)-epigallocatechin-3-gallate. These authors obtained nanosystems with particles sizes lower than 50 nm, which displayed similar *AE* (i.e., 58.5 ± 2.1 %) and *LC* (1.16 ± 0.04 %) values.

In terms of particle sizes, it is possible to see (Table 1) that β-Lg/riboflavin nanostructures remained with monomodal particle size distribution ($p > 0.05$), thus suggesting that association of riboflavin did not significantly affect ($p > 0.05$) its overall size. On the other hand, for β-Lg/riboflavin microstructures it was observed a statistically significant increase of particles' diameter ($p \leq 0.05$) as the concentration of riboflavin increased. This behaviour may be attributed to structural rearrangement of β-Lg microstructures after riboflavin association. For both β-Lg

micro- and nanostructures the PDI values were relatively low (i.e., below 0.26), suggesting a relatively homogenous β-Lg structure and a narrow size distribution. Regarding surface charge (S), β-Lg/riboflavin micro- and nanostructures displayed relatively constant values ranging from -16.5 to -20.1 mV, thus indicating that electrostatic repulsion forces among the particles are enough to prevent aggregation.

The effect of quercetin concentration on β-Lg micro- and nanostructures was also investigated, and the results are presented in Table 2.

«Table 2 to be placed here»

Concerning the results presented in Table 2, the *AE* of β-Lg/quercetin micro- and nanostructures ranged from 84.5 ± 3.2 % to 98.0 ± 0.9 %. These *AE* values agree with those found in the literature. For example, Mirpoor et al. (2017) reported that nanostructures prepared with β-Lg and sodium alginate showed *AE* higher than 93 %; Li et al. (2019) showed that zein particles with soluble soybean polysaccharide coating displayed *AE* of 82.5 %; and Wijaya et al. (2019) showed that whey protein isolate-low methoxyl pectin electrostatic complexes had values of *AE* of 97 %. However, these structures required both protein and polysaccharide polymers associated to form a stable delivery system.

The *AE* obtained in this study for quercetin is slightly higher than that obtained for riboflavin (Table 1 and Table 2). This difference can be due to the hydrophobic/hydrophilic amino acids located in a balanced way along the β-Lg polypeptide chains (Ramos et al., 2014). During thermal gelation (thermal treatment at 80 °C for 15 min), the hydrophobic binding sites initially buried inside the β-Lg structure, become exposed and available to bind, thus enhancing the potential to bind to hydrophobic bioactive compounds through hydrophobic interactions. On the other hand, in terms of *LC*, it is possible to observe that both β-Lg micro- and nanostructures displayed a lower capacity to load quercetin (i.e., up 0.61 %) then riboflavin (i.e., up to 1.80 %). This difference could be attributed to the molecular weight, water solubility and chemical structure of the bioactive model compounds employed.

Overall, the β-Lg nanostructures showed higher *LC* values ($p \leq 0.05$) for riboflavin (Table 1) and quercetin (Table 2) in comparison with β-Lg microstructures. This may be related to the higher

surface area-to-volume ratio of nanoscale structures, which may be able to bind higher levels of bioactive compounds (Jafari & McClements, 2017).

In terms of particle sizes, it is possible to observe from Table 2 that β-Lg microstructures significantly increased ($p \leq 0.05$) the particle sizes (monomodal distribution) as the quercetin concentration increases. The change in particle size values of β-Lg microstructures as affected by quercetin concentration may be related to two possible phenomena that may be occurring: protein-polyphenol interaction; and/or surface charge-modulated structural changes, for instance, flexibility, specific surface (Mirpoor et al., 2017).

Nonetheless, it was found that the association of both bioactive model compounds did not affect significantly ($p > 0.05$) the β-Lg nanostructures' particle sizes, which have a monomodal distribution with relatively low PDI values (PDI < 0.26). Further, the incorporation of quercetin into β-Lg micro- and nanostructures gradually shifted the surface charge as the concentration of quercetin increased up to 0.06 mg mL^{-1}, thus suggesting conformational rearrangements of β-Lg structures with the quercetin association. However, for quercetin concentration of 0.08 mg mL^{-1}, the values of surface charge for β-Lg/quercetin micro- and nanostructures become similar to those exhibited by β-Lg structures without quercetin. This behaviour may indicate that higher quercetin concentrations (≥ 0.08 mg mL^{-1}) may be able to promote conformational rearrangements on β-Lg structures with the establishment of stable electrostatic interactions capable of preventing aggregation (Liu et al., 2018).

Based on the best results obtained for the *AE* and *LC* and taking into consideration the physicochemical properties evaluated by DLS and discussed above, and in order to minimize the number of experimental tests to be performed, the concentration of 0.105 mg mL^{-1} of riboflavin and 0.08 mg mL^{-1} of quercetin were selected to associate to β-Lg micro- and nanostructures in the subsequent assays.

3.2. Morphology

The shape and size of β-Lg micro- and nanostructures associated with riboflavin and quercetin bioactive compounds are shown in Figure 1.

«Figure 1 to be placed here»

Figure 1 shows images of β-Lg micro- (Figures 1A and 1B) and nano (Figures 1C and 1D) structures. Measurements from images of β-Lg/riboflavin microstructures (Figure 1A) and β-Lg/quercetin microstructures (Figure 1B) revealed an average particle size of ca. 152.0 and 142.3 nm, respectively. DLS results demonstrated higher particle sizes than those obtained by TEM. This difference between both techniques may be due to a drying effect caused by samples preparation for TEM visualization, which may lead to the reduction of particle size. This phenomenon has been equally reported by other authors (Bourbon et al., 2016; Zhang et al., 2018). β-Lg/riboflavin nanostructures (Figure 1C) and β-Lg/quercetin nanostructures (Figure 1D) showed particle sizes around 75.6 and 76.1 nm, respectively, which corroborates the results obtained by DLS. These results are in agreement with those reported by Liu et al. (2018), which showed homogenous β-Lg/astaxanthin nanoparticles characterized by a spherical and uniform shape, with particle sizes below 100 nm (i.e. ca. 40 nm).

3.3. Binding properties

3.3.1. Extrinsic fluorescence

The accessibility and conformational changes of protein hydrophobic regions were detected through the binding of the fluorescence hydrophobic probe ANS (Singh et al., 2019). According to the literature, β-Lg exhibit two different binding ANS sites, being located internally (i.e. in the hydrophobic protein core), which contain disulphide bridges; and at externally (i.e. close to the hydrophobic patch on the protein surface), being responsible for nonspecific ANS interaction (Aprodu et al., 2017; Collini et al., 2009). The ANS interactions with β-Lg micro- and nanostructures, in the absence or presence of bioactive compounds, are depicted in Figure 2.

«Figure 2 to be placed here»

Extrinsic fluorescence spectra (Figure 2) showed a maximum peak at a wavelength of 470 nm, which corresponds to β-Lg hydrophobic sites. The binding of riboflavin and quercetin with β-Lg micro- and nanostructures led to a loss of signal intensity, thus suggesting that some interactions occurred at the protein hydrophobic regions, competing with ANS binding (Zhang et al., 2018).

These results are in accordance with the observations reported by Bourbon et al. (2016), which obtained a decrease of peak intensity in lactoferrin-glycomacropeptide nanohydrogels with the encapsulation of curcumin and caffeine (Bourbon et al., 2016).

However, two different behaviours were observed among β-Lg micro- and nanostructures. While at microscale the binding of quercetin promoted a decrease of the peak intensity, when compared with riboflavin, at nanoscale it was observed a similar fluorescence intensity for both bioactive model compounds. This difference may be related with the fact that for β-Lg/riboflavin microstructures the *AE* reached its maximum (64.4 ± 0.8 %) for the highest concentration of riboflavin tested (i.e., 0.105 mg mL^{-1}), so most probably there were still sites available for ANS binding, whereas for β-Lg/riboflavin nanostructures the maximum *AE* was attained for a riboflavin concentration of 0.014 mg mL^{-1}, which may suggest that all binding sites were occupied by riboflavin, and thus not available to bind to ANS.

Another possible explanation for this observation is that riboflavin has affinity to bind initially to β-Lg through hydrogen and electrostatic interactions, and consequently enable that some hydrophobic patches are available to binding with ANS (Li et al., 2019). However, besides hydrogen and electrostatic interactions and due to the high surface area-volume ratio, β-Lg/riboflavin nanostructures also have the capability to block hydrophobic patches available on the protein surface. This could also be the explanation for the higher *LC* obtained for β-Lg/riboflavin nanostructures (Table 1) when compared with β-Lg/riboflavin microstructures for the same concentration of the hydrophilic bioactive compound.

3.3.2. Circular dichroism

A Far-UV CD spectra (190-260 nm) were used to analyse protein structural transitions due to the fact that β-Lg interactions with small ligands may lead to relevant changes in the β-Lg secondary structure (Liang et al., 2008). β-Lg secondary structure have a characteristic CD spectrum according to their major elements, i.e., an α-helix (positive band at 190 nm and negative peaks at 208 and 220 nm), an β-sheet (negative peak in the 215 nm region) and a random coil (positive peak at 215 nm and negative band near 200 nm). Thus, it is crucial to investigate the impact of riboflavin and quercetin on the secondary structures of β-Lg in micro- and nanostructures (Essemine et al., 2011; Gomaa et al., 2016).

«Figure 3 to be placed here»

Far UV-CD spectra (Figure 3) of β-Lg microstructures showed a negative peak with a minimum at 218 nm, which reveals the presence of β-sheet structures (characteristic at 215 nm) and the presence of α-helix conformation due to the band near 220 nm. β-Lg nanostructures displayed a conformational profile with an anti-parallel β-structure with a broad negative minimum at 214 nm. The CD spectra of β-Lg/quercetin micro- and nanostructures were superposed with the corresponding structures of isolated β-Lg. This finding indicates that interaction between quercetin and β-Lg did not cause changes in the protein secondary structure; however, this evidence should be confirmed by other complementary techniques. On the other hand, for far-UV spectra of β-Lg/riboflavin micro- and nanostructures, it was observed a slight red shift (from 202 to 200 nm for micro- and from 201 to 200 nm for nanostructures), thus suggesting that riboflavin chromophoric groups were exposed to the hydrophilic environment.

3.3.3. Fourier-transform infrared spectroscopy

The FTIR spectra of β-Lg/riboflavin (Figure 4A) and β-Lg/quercetin (Figure 4B) micro- and nanostructures were evaluated in terms of their protein secondary structure and main functional groups present.

«Figure 4 to be placed here»

Protein conformational changes in the secondary structure are related with amide I band ranging between 1700 to 1600 cm^{-1} and amide II band around 1530 cm^{-1} (Xu et al., 2019). As shown in Figure 4A (a, d) for the β-Lg microstructures and in Figure 4B (a, d) for the β-Lg nanostructures, both amide I (mainly C=O stretch) and amide II (C–N stretch coupled with N–H bending mode) increased in intensity with the association of riboflavin – Figure 4A (b, e) or quercetin – Figure 4B (b, e), independently of the structures' scale used. These changes have been reported in the literature as conformational changes in the β-sheet structure, thus suggesting that the association of both bioactive model compounds led to relevant perturbations on the secondary

structure of the protein. The association of such bioactive compounds may also cause some disturbances in the tertiary structure of the protein, resulting from rearrangement of hydrophobic interactions, hydrogen and ionic bonds (Ramos et al., 2014).

FTIR spectra of free riboflavin presented an absorption band between 3571 and 2974 cm^{-1} attributed to -OH stretch, and peaks at 1731 and 1542 cm^{-1} corresponding to the carbonyl stretching vibration of the amide group and to $-CH_3$ stretching, respectively, as depicted in Figure 4A (c). Besides, the peak assigned at 848 cm^{-1} can be ascribed as out-of-plane N-H wagging vibration from the pyrimidine ring (Orsuwan et al., 2019; Ye et al., 2019). In addition, C-O stretching appeared in two different absorption bands at 1072 and 1014 cm^{-1} (Mansour et al., 2014).

In Figure 4A, comparing β-Lg/microstructures (a) to β-Lg/riboflavin microstructures (b), it is possible to observe a band shift from 1654 to 1643 cm^{-1} (C=O stretching) and from 1527 to 1539 cm^{-1} ($-CH_3$ stretching), promoted by the association of riboflavin. The intensities of C-O stretching (present at 1161 and 1068 cm^{-1}) and of out-of-plane N-H wagging vibration (present at 864 cm^{-1}), characteristic of β-Lg structure, increased in β-Lg/riboflavin microstructures.

For β-Lg/riboflavin nanostructures (e), it was also observed a shift in stretching vibration of C=O (from 1531 to 1542 cm^{-1}) and in $-CH_3$ stretching (from 1650 to 1647 cm^{-1}), as well as an increase in the intensity of peaks at 1157 and 1080 cm^{-1} (mainly C-O stretch) and in the region from 3571 to 2974 cm^{-1}, which may be attributed to the establishment of hydrogen bonds. Riboflavin has a hydrophobic extremity (due the presence of an aromatic ring), which enables binding to β-Lg through hydrophobic bonds; and a hydrophilic group (due to the ribitol chain and pyrimidine ring), which allows the interaction to β-Lg by hydrogen and electrostatic bonding (Morrison et al., 2013; Zhang et al., 2016). These findings were consistent with our β-Lg/riboflavin micro- and nanostructure *LC* results.

FTIR spectra of free quercetin – Figure 4B (c) exhibited bands in 3394 cm^{-1} region, which is attributed to O-H stretching vibration of hydroxyl group and aromatic groups (Li et al., 2019), C=O stretching (at 1658 cm^{-1}), C=C stretching (at 1515 cm^{-1}), C-O aromatic stretching (at 1315 cm^{-1}) and an aromatic bending and stretching (at 810 cm^{-1}) (Ma et al., 2018; Patel et al., 2012).

As shown in Figure 4B (b), FTIR spectra of β-Lg/quercetin microstructures revealed characteristic bands of free quercetin with a peak intensity increase at 1650 cm^{-1} (C=O stretch), 1164 cm^{-1}

(aromatic bending and stretching) and at 856 cm^{-1} (aromatic bending and stretching). The peak in free quercetin at 3394 cm^{-1} related to O-H stretching vibration of the hydroxyl group, was shifted from 3278 cm^{-1} in β-Lg microstructures to 3290 cm^{-1} in β-Lg/quercetin microstructures. The broader peak of free quercetin suggests that hydrogen bonding was one of the main interactions involved in the binding to β-Lg microstructure. This evidence is in agreement with Shpigelman et al. (2012) findings that indicate that polyphenols may interact with protein partially by hydrogen bonds.

The differences on the FTIR spectra of β-Lg nanostructures – Figure 4B (d) when compared with β-Lg/quercetin nanostructures – Figure 4B (e) are mainly due to the progressive increase of peaks at 1647 cm^{-1}, corresponding to C=O stretch, at 1307 cm^{-1} due to C-O aromatic stretching, and at 933 cm^{-1} attributed to aromatic bending and stretching. Moreover, the band in the region of 3394 cm^{-1} associated to the presence of hydroxyl group and aromatic groups was not obtained for β-Lg/quercetin nanostructures, thus suggesting that hydrogen bonding was not the main binding interactions occurring between quercetin and β-Lg structures at the nanoscale.

3.4. β-Lg structures stability under various environmental conditions

The effect of several environmental conditions (e.g., pH, temperature, ionic strength, and storage temperature) on β-Lg micro- and nanostructures associated with bioactive compounds was assessed to provide information about the food product characteristics that facilitate their incorporation.

3.4.1. pH stability

The effect of pH was evaluated by adjusting aqueous solutions of β-Lg micro- and nanostructures (data were available in the Supplementary Material, Table S.1 and Table S.2, respectively) structures to various pH values in order to assess its impact on their particle size, PDI and surface charge (S). Taking in consideration the β-Lg isoelectric point (pI), which is near pH = 4.8 (Ramos et al., 2012), the zeta potential of aqueous solutions of β-Lg micro- and nanostructures shifted from positive values, at pH 2, towards negative values, at pH 10 (Table S.1 and Table S.2). At pH values near the pI of β-Lg, aggregates with large mean particle sizes (i.e., > 5 μm) and white appearance were obtained, independently of the structures' scale. This result is somehow

577 expected since at pI the electrostatic repulsions become weak and proteins tend to aggregate
578 and/or flocculate, forming structures with larger sizes (Ramos et al., 2013).
579 Chen et al. (2014) evaluated the pH stability, from 3 to 7, of zein/β-Lg nanoparticles encapsulating
580 tagerentin (i.e., low water solubility compound), showing aggregation phenomena for such
581 nanoparticles complex for pH values between 4.5 and 5.5, and high stability for pH above 5.5 or
582 below 4.5.
583 The particle diameter of β-Lg micro- and nanostructures with/without bioactive model compounds
584 remained constant ($p > 0.05$) (Table S.1 and Table S.2) with low PDI values (i.e., below 0.25) for
585 pH range between 2 and 3 (Table S.1 and Table S.2). According to the literature, β-Lg structure
586 has stability and remains mostly intact in acid conditions (Liang & Subirade, 2012; Shafaei et al.,
587 2017). For pH conditions between 6 and 10, particle size and PDI are stable, which may be
588 attributed to the formation of strong covalent or hydrophobic bonds among β-Lg molecules and
589 bioactive model compounds during thermal gelation (Bengoechea et al., 2011).
590 These results suggest that β-Lg micro- and nanostructures may be suitable carriers for riboflavin
591 and quercetin into soft drinks (with pH values ranging from 2 to 3) and into some dairy products
592 (with pH values between 6 to 9), and also effective delivery systems upon oral intake since they
593 resist to the acid environment, which is characteristic of the human stomach.
594
595 **3.4.2. Ionic strength stability**
596 The impact of ionic strength on properties of β-Lg micro- and nanostructures was evaluated in
597 order to estimate their behaviour in aqueous commercial products composed by distinct types
598 and amounts of electrolytes, as well as within the human gastrointestinal tract (Chen et al., 2014).
599 The ionic strength study was carried out by solubilizing β-Lg micro- and nanostructures in various
600 concentrations of sodium chloride (NaCl), in order to evaluate the ionic strength effect on particle
601 size and PDI (data were present in the Supplementary Material, Table S.3 and Table S.4,
602 respectively). Results demonstrated that β-Lg structures displayed particle sizes in the nanoscale
603 range (i.e., 104.3 ± 5.9 nm) when added to NaCl until 100 mmol L^{-1}, and remained homogenous
604 (i.e., PDI < 0.22) at this scale-range. The association of riboflavin was able to maintain β-Lg
605 structures homogeneous and within the nanoscale at NaCl concentrations up to 200 mmol L^{-1};
606 PDI values were ranging from 0.21 ± 0.01 to 0.22 ± 0.01 and particles sizes were ranging from

77.4 ± 8.2 nm to 73.9 ± 6.9 nm (p > 0.05). On the other hand, the addition of NaCl at concentrations ≤ 25 mmol L^{-1} did not impact the stability of β-Lg nanostructures showing a particle size in the nano range (i.e., 77.9 ± 9.6 nm). However, for progressively higher concentrations of NaCl, the particle sizes of β-Lg/quercetin nanostructures increased significantly (p ≤ 0.05) above 100 nm, and structures became more heterogeneous. According to Bourbon et al. (2015), the presence of NaCl may impacts the size and PDI of protein structures by two different mechanisms: i) by promoting the establishment of attractive interactions, for instance, van der Waals or hydrophobic, which dominate the repulsive interactions; or ii) by modifying the organization of structural water molecules, which changes the strength of hydrophobic interactions between nonpolar groups.

Regarding the impact of ionic strength, the addition of NaCl at concentrations ≤ 200 mmol L^{-1} did not impact the stability (i.e., particle size and PDI) of β-Lg/quercetin microstructures, however higher concentrations of NaCl increased significantly (p ≤ 0.05) its particle size (383.8 ± 26.6 nm) and PDI (0.28 ± 0.03) values. This result may indicate that at higher (i.e., micro-) scale range the Na^+ and Cl^- ions may have a lower impact on β-Lg microstructures, since as the surface-to-volume ratio is lower than that in nanostructures, β-Lg is less subjected to the action of NaCl, and thus a higher concentration of salt is needed to neutralize β-Lg's charge and to reduce electrostatic repulsion (Hu & McClements, 2014).

These results suggest that β-Lg/riboflavin nanostructures, and β-Lg/riboflavin and β-Lg/quercetin microstructures are suitable delivery systems for a large variety of food systems since they are stable at a relatively broad range of salt concentrations. Additionally, they also should be stable in the gastrointestinal tract during digestion when considering this parameter, since the normal ionic strength in the stomach is 100 mmol L^{-1} and in the small intestine is 140 mmol L^{-1} (McClements & Li, 2010; Pinheiro et al., 2017).

3.4.3. Thermal stability

Heating is a common processing treatment in the food industry, therefore it is crucial to evaluate the thermal stability of delivery systems at a temperature range of at least 20 to 80 °C. Therefore, the effect of temperature on β-Lg micro- and nanostructures (data were available in the Supplementary Material, Table S.5 and Table S.6, respectively) properties was investigated at

637 those conditions. The particle size of β-Lg micro- and nanostructures in the absence and
638 presence of bioactive compounds did not change ($p > 0.05$) for temperatures below 70 °C and
639 slightly increased ($p \leq 0.05$), when subjected to temperatures between 70 and 80 °C. The PDI
640 values of β-Lg structures were below 0.25, independently of temperature, indicating a good
641 homogeneity of such protein structures within a narrow size distribution. This temperature stability
642 may be due to some changes in the conformational state (Bourbon et al., 2015) or reorganization
643 of β-Lg molecules, without compromising its functionality (Hu & McClements, 2014). These results
644 are in the same line of those presented in a previous study conducted by Yi et al. (2016), which
645 showed thermal stability of α-lactalbumin/curcumin nano-complexes for temperatures ranging
646 from 30 to 90 °C, with a slight increase in particle diameter observed for temperatures above 70
647 °C.
648
649 **3.4.4. Storage**
650 Temperature is an important condition to preserve the effective quality and benefits of bioactive
651 compounds, thus contributing to physiological wellbeing (Kang et al., 2019; Ramos et al., 2017).
652 Therefore, a storage stability study was performed to assess the effect of temperature at 4 °C (to
653 simulate refrigerated conditions) and at 25 °C (to simulate room temperature conditions) over 90
654 days of storage. β-Lg microstructures (data were present in Supplementary Material, Table S.7)
655 without association of bioactive model compounds maintained their stability in terms of particle
656 size (from 202.5 ± 5.8 nm to 202.0 ± 11.1 nm), PDI (from 0.18 ± 0.02 to 0.17 ± 0.01) and surface
657 charge (from -22.2 ± 1.9 mV to -21.4 ± 1.2 mV) at the beginning and after 90 days of storage,
658 respectively, at 4 °C; while at 25 °C it was possible to observe the formation of precipitates over
659 50 days of storage, indicating loss of colloidal stability.
660 β-Lg nanostructures (data were available in Supplementary Material, Table S.8) without the
661 association of bioactive model compounds were able to maintain their properties without
662 significant changes ($p > 0.05$) in terms of particle size, PDI and surface charge over the storage
663 period, independently of the temperature tested, thus suggesting that β-Lg nanostructures were
664 stable and able to retain their structure. These nanostructures displayed higher storage stability
665 when compared with the performance exhibited by α-lactalbumin-lysozyme nanoparticles (with
666 sizes of 61.0 ± 2.3 nm), tested by Monteiro et al. (2016). In that case, α-lactalbumin-lysozyme

667 nanoparticles were able to maintain their stability over 90 days at 4 °C but only for 30 days at 25
668 °C. This difference may be related to the different protein characteristics or to the weak
669 intermolecular interactions established between α-lactalbumin-lysozyme.
670 In terms of stability of β-Lg/quercetin nanostructures, precipitates were formed over 2 days of
671 storage, independently of the storage temperature tested. As mentioned before, quercetin use is
672 limited by its poor water solubility (Ni et al., 2017). When ethanolic solutions (used in the
673 solubilization of quercetin) were added into β-Lg aqueous systems, large precipitates were
674 formed, displaying an atypical needle-like morphology, which is extremely unstable and leads to
675 sedimentation. Despite of this, the precipitates' shape may be controlled by increasing protein
676 concentration, thus leading to a decrease in the appearance of needle-shaped crystals (Patel et
677 al., 2012).
678 Regarding the stability of β-Lg/riboflavin microstructures (Table S.7), they were stable over the
679 90 days at both temperatures tested. The association of riboflavin was even able to prevent the
680 formation of precipitates observed in β-Lg microstructures isolated after 50 days of storage. This
681 evidence may be due to possible changes promoted by riboflavin at the β-Lg surface, possibly
682 the establishment of strong interactions that may have prevented the electrostatic attraction
683 between protein molecules. The magnitude of surface charge for β-Lg microstructures with and
684 without bioactive model compounds (Table S.7) association did not change significantly ($p > 0.05$)
685 during the storage time, being around – 20.0 mV.
686 β-Lg/riboflavin nanostructures (Table S.8) demonstrated a slight change in particle size ranging
687 from 70.8 ± 2.3 nm to 78.0 ± 4.7 nm with PDI value lower than 0.25, over 80 days of storage at 4
688 °C. However, over 90 days, the particle sizes and PDI increased more pronouncedly, reaching
689 84.3 ± 8.7 nm and 0.30 ± 0.01, respectively. These results are in line with those reported by
690 Martins et al. (2016), which showed that lactoferrin/iron nanoparticles were stable in terms of
691 particle size and PDI during 76 days at 4 °C.
692 At 25 °C, the stability of β-Lg/riboflavin nanostructures decreased significantly to 22 days
693 (particles sizes of 67.8 ± 1.9 nm and PDI of 0.25 ± 0.01), when compared with those exhibited
694 over 90 days (particles sizes of 70.5 ± 1.6 nm, but PDI values of 0.40 ± 0.10), which may indicate
695 that dissociation or aggregation phenomena occurred after 22 days. This evidence indicates that

refrigeration could improve the storage stability of β-Lg micro- and nanostructures loaded with riboflavin and quercetin.

β-Lg/riboflavin micro- and nanostructures and β-Lg/quercetin microstructures showed the best performance in terms of stability for the tests performed above, and for that reason the behaviour of such structures was evaluated into food simulants.

3.5. *In vitro* release kinetics

Release profiles were used to ensure that a delivery system will act according to the designed goal. These profiles are expected to estimate the behaviour of β-Lg micro- and nanostructures into food products. The release profile of riboflavin and quercetin bioactive compounds from β-Lg micro- and nanostructures added into food simulants (i.e., 10 % of ethanol simulating hydrophilic matrices and 50 % of ethanol simulating hydrophobic matrices) were assessed at 4 °C (to simulate refrigerated conditions) and at 25 °C (to simulate room temperatures). The release kinetics of bioactive model compounds from β-Lg micro- and nanostructures were monitored during 72 h. The amount (%) of bioactive compound released as a function of time is plotted in Figure 5.

«Figure 5 to be placed here»

Figure 5 shows a typical release profile, where the initial 8 h comprises a burst release stage associated to the faster release of unbound bioactive compounds, followed by a controlled release phase attributed to the behaviour of bioactive compounds bounded to β-Lg micro- and nanostructures or to β-Lg conformational changes under contact with food simulants (Heydari et al., 2018). These release profiles are in agreement with other release studies that use proteins as the main encapsulating agent (Liu et al., 2018; Luo et al., 2011; Wang et al., 2018). O'Neill et al. (2015) reported a release of 42 % of riboflavin from whey protein hydrogels within 2 h in a water matrix at 20 °C. The authors concluded that this delivery system was not suitable for food products with high moisture content stored at room temperature conditions. Kumari et al. (2010) developed poly-D,L-lactide nanoparticles for quercetin delivery and reported a rapid burst,

725 releasing 40-45 % of quercetin within 30 min in 0.1 mol L^{-1} of phosphate-buffered saline (PBS)
726 buffer solution.
727 In our study, riboflavin and quercetin were rapidly released from β-Lg micro- and nanostructures
728 when added into a hydrophobic (50 % ethanol) food simulant at 25 °C. This result may be related
729 to the fact that ethanol present at high concentrations (i.e., 50 %) in contact with β-Lg may affect
730 the stability of this protein, by promoting irreversible changes in the protein's secondary structure
731 with consequent protein disintegration (Yoshizawa et al., 2014).
732 β-Lg/riboflavin micro- (Figure 5.A) and nanostructures (Figure 5.B) showed a similar release
733 profile when tested in both hydrophilic and hydrophobic food simulants at 4 °C and 25 °C.
734 Commonly, the "responsiveness" is related to the interactions established between bio-based
735 materials used as carriers and the surrounding environment (Musyanovych & Landfester, 2014);
736 however, it is essential to determine the mechanism involved in this process, in order to allow the
737 controlled release of bioactive compounds at the micro- and nanoscale.
738 As shown in Figure 5.C, under hydrophilic food simulant conditions, β-Lg microstructures
739 practically did not release quercetin at 4 °C, over 72 h. This could be due to the hydrophobic
740 character of quercetin that did not diffuse to the hydrophilic food simulant; thus, it was not possible
741 to describe the release profile of quercetin in these conditions. These results are in agreement
742 with those published by Bourbon et al. (2016), which showed that curcumin (hydrophobic
743 compound) was not released from lactoferrin-glycomacropeptide nanohydrogels due the low
744 solubility of curcumin at pH 7.3 in phosphate buffer solution, used as food simulant.
745 The release kinetics results of both bioactive compounds (Figure 5) were fitted to a mathematical
746 model – Linear Superimposition Model (LSM) (Equation 7) – to find the most adequate release
747 mechanism model. The plots of β-Lg/riboflavin micro- (data were present in Supplementary
748 Material, Figure S.1), β-Lg/riboflavin nanostructures (data were available in Supplementary
749 Material, Figure S.2) and β-Lg/quercetin microstructures (data were present in Supplementary
750 Material, Figure S.3) fitted well with the LSM. Table 3 shows the fitting parameters of LSM to
751 experimental data of riboflavin and quercetin released from β-Lg micro-and nanostructures.
752
753 *«Table 3 to be placed here»*
754

755 Results from Table 3 demonstrate that LSM fitted well to data obtained with a R^2 > 0.98, thus
756 indicating that this model was suitable to describe the release mechanisms of riboflavin and
757 quercetin from β-Lg micro- and nanostructures. The model suggests that the transport
758 mechanisms involved can be described by Fick's diffusion and Case II transport (biopolymer
759 relaxation), with one main relaxation of the β-Lg micro- and nanostructures.
760 Table 3 shows that release kinetics of bioactive compounds can be affected by both food
761 simulants (i.e., with hydrophilic and hydrophobic nature) and temperature (i.e., 4 and 25 °C). The
762 relaxation rate constant (K_R) increased with temperature, independently of the food simulant
763 used, which may be attributed to the input of energy into the system that promotes a more relaxed
764 and weaker protein structure, thus increasing the diffusion of the bioactive compound.
765 In the hydrophilic food simulant (10 % ethanol) the amount of riboflavin released from β-Lg micro-
766 and nanostructures was higher than that obtained for the hydrophobic counterparts. On the
767 contrary, the quercetin release into hydrophobic food simulant at 25 °C was higher than that
768 obtained in the hydrophilic environment. These findings might be due the relaxation rate constant
769 value (K_R) and related with the hydrophilic and hydrophobic nature of riboflavin and quercetin,
770 respectively, and with their affinity to diffuse to hydrophilic and hydrophobic food simulants,
771 respectively.
772 It was found that β-Lg/bioactive compound micro- and nanostructures exhibited a similar
773 relaxation rate constant for both food simulants at 4 °C. However, in the simulant at room
774 temperature (i.e., 25 °C) the β-Lg nanostructures showed faster release kinetics, confirmed by
775 the high K_F obtained, as compared to that of β-Lg microstructures. This behaviour may be related
776 with the higher exposure area as a result of the higher surface-to-volume ratio, characteristic of
777 nanoscale materials, which in association with the effect of temperature (25 °C), has enhanced
778 the protein relaxation and thus an increased riboflavin release.
779 For most conditions tested, the Fickian (K_F) rate constant was higher than the relaxation (K_R) rate
780 constant, which may be indicating that bioactive compounds release was quicker by Fick's
781 diffusion instead of relaxation mechanism (Martins et al., 2016).
782 The bioactive compound fraction released by Fickian transport (X value), defined as $\frac{M_{\infty F}}{M_t}$, allows
783 determining the governing bioactive compound release mechanism. If X > 0.5 the prevailing
784 bioactive compound release mechanism is Fickian diffusion, while if X < 0.5 relaxation is the main

785 mechanism of release (Pinheiro et al., 2012). Therefore, this implies that the release of both
786 bioactive model compounds from β-Lg micro- and nanostructures is mainly governed by the
787 relaxation mechanism.
788 Wichchukit et al. (2013) reported that riboflavin release from whey protein beads in a beverage
789 model food exhibited a transport mechanism governed by Case II, which resulted in slow release
790 rates during product shelf life and until its consumption. Yan et al. (2018) investigated the
791 controlled release of quercetin-loaded chitosan nanoparticle into 50 % ethanol and water-oil
792 (50:50) food simulants at 25 °C and reported an initial burst followed by sustained slow release.
793 These authors described the quercetin release profile as a Fick's curve; however, they did not
794 apply a mathematical model to prove this behaviour.
795 Table 4 shows β-Lg micro- and nanostructure properties (i.e., particle size, PDI and S) analysed
796 before and after bioactive compounds (i.e., riboflavin and quercetin) release, and bioactive
797 compounds final concentrations in β-Lg structures.
798
799 *«Table 4 to be placed here»*
800
801 Data presented in Table 4 show that β-Lg micro- and nanostructures increased their particle size
802 ($p \leq 0.05$) during the release period, for all conditions tested. This behaviour is typical when the
803 release mechanism is governed by biopolymer relaxation and when structures present a swelling
804 behaviour in response to chemical (e.g., pH, solvent and ionic strength) and/or physical (e.g.,
805 temperature, light) stimuli (Simões et al., 2017). The swelling capacity of β-Lg micro- and
806 nanostructures may be related with the presence of hydrophilic moieties (e.g., hydroxyl, amino,
807 or carboxyl groups) in their structure (Ramos et al., 2017). This hypothesis is supported by FTIR
808 results, which showed the presence of such hydrophilic groups in β-Lg's structure. Bourbon et al.
809 (2016) also demonstrated that lactoferrin-glycomacropeptide nanohydrogels increased their
810 particle size with the release or curcumin due to the swelling behaviour of nanohydrogels, which
811 is a typical case of release mechanisms dominated by biopolymer relaxation (Bourbon et al.,
812 2016). Nonetheless, the authors obtained small sized nanohydrogel particles when Fick's
813 diffusion was the main release mechanism.

In terms of surface charge, it was possible to observe that β-Lg/bioactive compounds maintained their surface charge constant, with zeta potential values of ca. -20 mV, during release in hydrophilic food simulant conditions, which indicates a relatively stable system. For β-Lg/quercetin microstructures, when they were added to a hydrophobic simulant (in this particular case with a higher content of ethanol), β-Lg loses its structural stability, as demonstrated by the decrease of the surface charge (Table 4). This effect can be explained by the fact that ethanol molecules affect the repulsive interactions responsible for β-Lg stability (Yoshizawa et al., 2014). On the other hand, when β-Lg/quercetin microstructures were added to a hydrophilic food simulant (i.e., 10 % ethanol) at 4 °C it was possible to observe that ca. half of the initial concentration of quercetin was released to the hydrophilic food simulant. Moreover, it was possible to see that temperature had an effect on the release of both bioactive model compounds, as mentioned above. Thus, the hydrophobic characteristics of quercetin and slow release results in hydrophilic food simulants at 25 °C, suggest that the combination of a hydrophilic food simulant with the temperature at 4 °C influences the β-Lg structure relaxation and thus a reduction of quercetin release.

An effective delivery system should be able to release the bioactive compounds associated to it, in a controlled way and for prolonged periods, to the surrounding environment (Wichchukit et al., 2013). β-Lg micro- and nanostructures were able to keep at least 5 % of the initial concentration of riboflavin, while β-Lg microstructures were able to maintain ca. 2.5 % of the initial concentration of quercetin, both over 72 h, independently of the food simulant and temperature used.

4. Conclusions

β-Lg micro- and nanostructures were able to bind bioactive compounds with different physicochemical properties (i.e., water solubility). β-Lg nanostructures maintained their physicochemical properties (i.e., size, PDI and surface charge) independently of the concentrations of both bioactive model compounds added. Furthermore, β-Lg nanostructures showed a higher association efficiency and loading capacity when compared with β-Lg microstructures, for the same concentration of hydrophilic (riboflavin) or hydrophobic (quercetin) bioactive compound used. These structures exhibited stability under acidic (pH 2 to 3) and neutral (pH 6 to 10) pH conditions, at relatively high ionic strength (NaCl concentrations up to 100 mmol

L^{-1}) and thermal processing (until 70 °C) conditions. β-Lg/riboflavin micro- and nanostructures showed to be stable over 90 days of storage, while β-Lg/quercetin microstructures were stable during 50 days of storage, being susceptible to aggregation and precipitation after that period. The release for both bioactive model compounds from β-Lg micro- and nanostructures demonstrated that the LSM model was the most suitable to describe the release kinetics, which is mainly governed by a relaxation mechanism. Additionally, the refrigerated temperature of 4 °C allowed a slow release of both bioactive model compounds, when compared with the results at room temperature (25 °C), independently of the food simulant used.

Our findings improve the knowledge about the potential applicability of β-Lg micro- and nanostructures for delivery and controlled release of hydrophilic and hydrophobic bioactive compounds into food systems with different hydrophilic natures. Nevertheless, further investigations regarding the evaluation of bioaccessibility and bioavailability of these bioactive model compounds upon ingestion are needed before their possible application into real food products.

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