Food Research International 197 (2024) 115179

Contents lists available at ScienceDirect

Food Research International

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

Influence of bile salts on the gastrointestinal digestion of agar-casein hybrid systems and the nanoassembly of their digestion products

Laura Díaz-Piñero [a], Cynthia Fontes-Candia [a,b], Estefanía Rodríguez-Dobreva [a], Isidra Recio [a], Marta Martínez-Sanz [a,*]

[a] Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM, CEI UAM + CSIC), Nicolás Cabrera, 9, 28049 Madrid, Spain
[b] University Institute of Food Engineering—FoodUPV, Universitat Politècnica de València (UPV), Camino de Vera s/n, 46022 Valencia, Spain

ARTICLE INFO

Keywords:
In vitro digestion
Hydrogels
Bile salts
X-ray scattering
Nanostructure

ABSTRACT

This manuscript reports on the effect of different bile salts concentration on the gastrointestinal digestion of casein and casein-agar hybrid systems and evaluates the effect on the nanostructural assembly of the digestion products through the application of advanced small angle X-ray scattering analysis (SAXS).

The results showed that bile salts promote the hydrolysis of micellar casein upon *in vitro* gastrointestinal digestions. It is noteworthy that in the presence of agar, bile salts interact with the polysaccharide, leading to a reduction in their proteolytic activity. While structured agar-casein hydrogels were shown to be able to limit the proteolysis during the gastric phase, increasing concentrations of bile salts promoted the diffusion of casein from the gel network during the intestinal phase, hence leading to higher degree of hydrolysis. The released digestion products were seen to interact with bile salts, forming ordered lamellar/micellar nanostructures. In particular, the presence of solubilized agar in the digesta was seen to promote the formation of these nanostructures. These nanostructures were formed at 10 mM bile salts for the hydrogels, and across all concentrations for agar-casein blends. Thus, this work demonstrates the great relevance of bile salts in the nanostructural assembly of protein digestion products, which is expected to have a great relevance in bioavailability and metabolic responses induced by protein-rich foods.

1. Introduction

Proteins are one of the most important macronutrients in human diets, not only because of their significant physiological relevance but also due to their pronounced satiating effect. The nutritional quality and metabolic impact of food proteins depend on the mechanism of gastrointestinal digestion, the type of digestion products generated, their interaction with the digestion medium components (e.g. bile salts), and their intestinal transport process. During gastrointestinal digestion, proteins are subjected to a complex combination of chemical, enzymatic, and mechanical processes to break down their structure and facilitate the release and absorption of free amino acids and peptides. Many factors such as the protein size, charge, sequence, tertiary structure, folding, aggregation and solubility, amongst others, are essential to determine the hydrolysis mechanism of food proteins. These factors can be modified by industrial technological processes, thus having an impact on protein digestibility and nutritional quality. Furthermore, the composition and structure of the whole food matrix are highly relevant to the protein digestion process. In line with this, composition and structure of food/nutraceutical products can be deliberately modified to modulate the degree of protein hydrolysis along the gastrointestinal tract. For instance, encapsulation strategies have demonstrated a great potential to delay protein digestion (Alavi et al., 2018; Li et al., 2010; McClements, 2017; Zhang et al., 2017). The interactions established between proteins and polysaccharides have been exploited to develop gel-like hybrid structures capable of protecting a labile protein, such as casein, from the hydrolysis during the gastric phase (Fontes-Candia et al., 2022; Selvasekaran & Chidambaram, 2021). Consequently, larger peptides were released after the intestinal digestion phase. The presence of these larger peptides in the intestine has been hypothesized to induce a greater secretion of anorexigenic hormones such as glucagon-like peptide 1 (GLP-1) (Santos-Hernández et al., 2020), thus being a promising strategy to control food intake, body weight and glucose levels (Gribble & Reimann, 2019).

* Corresponding author.
E-mail address: marta.martinez@csic.es (M. Martínez-Sanz).

https://doi.org/10.1016/j.foodres.2024.115179
Received 5 July 2024; Received in revised form 12 September 2024; Accepted 29 September 2024
Available online 2 October 2024

Apart from the intrinsic characteristics of the ingested food product, the physiological conditions play a very important role on the gastrointestinal digestion of proteins. Intermolecular interactions may take place between the components released upon digestion and some of the elements that compose the physiological digestion medium, such as phospholipids and bile salts, being determinant in the digestion mechanism and the absorption of nutrients. Bile salts are amphipathic molecules derived from cholesterol which act as biosurfactants, having key roles in the digestion, solubilization and transport of lipids (e.g. cholesterol) and liposoluble vitamins (Maldonado-Valderrama et al., 2011). They act also as signaling molecules which modulate proliferation, gene expression and lipid and glucose metabolism (Chiang, 2013). It is important to highlight that the composition and concentration of bile salts exhibit a large inter- and intra-individual variability in intestinal fluids. This variability is crucial, as it can impact all physiological processes involving bile salts (Clarysse et al., 2009; Cremers et al., 2014; Riethorst et al., 2016).While bile salts have been reported to enhance the transport of hydrophilic drugs across different biological barriers through a well-known mechanism (Moghimipour et al., 2015), their effect on protein digestion has not been studied in detail (López-Fandiño et al., 2010; MacIerzanka et al., 2009). Few recent studies have reported an increased proteolysis degree in a gastric resistant protein, such β-lactoglobulin, at higher bile salts concentrations (Dulko et al., 2021; Gass et al., 2007). This behaviour was related with a change in the protein structure induced by bile salts, although the exact mechanism is still unknown (Cremers et al., 2014). On the other hand, the effect of bile salts on the digestion of more labile proteins such as casein, which is highly hydrolyzed in the gastric phase, has not been described to date. Caseins compose 80 % of the total protein in bovine milk (Carter et al., 2021), presenting a less ordered and more flexible structure than globular whey proteins. As a result, caseins are highly susceptible hydrolysis by digestive enzymes during the gastrointestinal digestion process, producing mainly low molecular weight peptides and free amino acids (Miralles et al., 2021).

In a previous study, we evaluated the type of nanostructures formed after the gastrointestinal digestion of micellar casein and casein-polysaccharide hybrid structures by means of advanced characterization tools, such as small angle X-ray scattering (SAXS) (Fontes-Candia et al., 2023). Our results suggested that the interaction between bile salts and the peptides released upon hydrolysis of casein is determinant to the type of nanostructures formed (e.g. mixed micelles/lamellae or vesicles). Furthermore, the presence of sulphated polysaccharides was also seen to affect the nanostructural assembly of the digestion products, either by inducing changes in the degree of casein hydrolysis or by competitive interactions with bile salts. Based on these interesting findings, in the present work we explored the effect of bile salts on the gastrointestinal digestion of casein and casein-agar hybrid hydrogels designed aimed at limiting the degree of proteolysis. To that end, simulated *in vitro* digestions were performed and the nano-/microstructure, molecular structure and molecular weight distribution of the obtained digestion products were characterized. These findings extend the information about the role of bile salts in protein digestion and open up the possibility of developing innovative food products with high nutritional value.

2. Materials and methods

2.1. Materials

Commercial agar (PRONAGAR) (containing 80 % agar), in the form of powder, was donated by Hispanagar (Burgos, Spain). Casein powder (PRODIET 85B) in the form of micellar casein (protein content of 79.5 %) was supplied by Ingredia (Arras, France). All the reagents were analytical grade and were obtained from Sigma-Aldrich (Spain).

2.2. Preparation of agar-casein hydrogels

Agar-casein gel-like structures in the form of hydrogels were produced following the procedure described by (Fontes-Candia et al., 2022). Briefly, agar and casein aqueous solutions were prepared independently at a fixed concentration of 2 % (w/v). The agar and casein powders were dispersed in hot water (90 °C for the agar and 40 °C for the casein) during 30 min. The required volume of casein solution was added to the agar solution, adjusting the agar:casein ratio at 25:75 (v/v), and the blend was homogenized with a magnetic stirrer. About 0.4 mL of agar-casein blends were transferred into cylindrical silicon molds (7 mm diameter, 10 mm height), cooled to room temperature and stored at 4 °C overnight to induce gelation. These hydrogels were coded as HG-A25. Hydrogels without casein (100 % agar) were prepared as polysaccharide controls (coded as HG-A100). Micellar casein (coded as CAS) and control agar-casein blend solutions (coded as B-A25) obtained by mixing the agar and casein powders (without the gelling step), were also prepared to evaluate the effect of the physical structures formed upon gelation.

2.3. In-vitro simulated gastrointestinal digestions

The prepared hydrogels, agar-casein blends and micellar casein were submitted to simulated gastrointestinal digestions according to the INFOGEST protocol (Brodkorb et al., 2019) with minor modifications, as previously described (Fontes-Candia, et al., 2022). Briefly, the amount of sample required to provide 75 mg of casein was added to a polypropylene tube, 5 mL of human salivary fluid (salivary amylase concentration 35–50 U/mL) was incorporated, and the sample was incubated for 2 min at 37 °C in an orbital shaker-incubator. Then, 10 mL of simulated gastric fluid (SGF) and pepsin from porcine gastric mucosa (final pepsin concentration 2000 U/mL of gastric digest, Sigma-Aldrich, St Louis, MO, USA) were added and pH was adjusted to 3.0. The sample was incubated at 37 °C for 2 h with continuous agitation using an orbital shaker at 120 rpm. At this point, the intestinal phase was performed by mixing 20 mL of simulated intestinal fluid (SIF) containing pancreatin from porcine pancreas (100 U trypsin activity per mL of final mixture, Sigma-Aldrich) and porcine bile extract (Sigma-Aldrich) and adjusting pH to 7.0. The bile salts concentration was set to 0 mM, 2.5 mM and 10 mM to evaluate their effect on digestion. The mixture was kept in a shaking incubator at 37 °C for 120 min. Gastric and intestinal digests from the hydrogels were separated into two phases by decanting while the blends were centrifugated at 5000 × *g* for 20 min (Centrifuge 5804R, Eppendorf): non-digested or partially-digested material (solid phase) and digested soluble compounds (liquid phase). The gastric phase was stopped by increasing pH up to 7, while the intestinal phase was stopped by heating at 85 °C for 15 min using a water bath. The obtained fractions were characterized after each digestion step resulting in the following samples: gastric solid (GSol), gastric liquid (GLiq), intestinal solid (ISol) and intestinal liquid (ILiq). Digestions were performed in triplicate by using individual tubes for gastric and gastrointestinal digestion. After digestion, samples were freeze-dried and stored at − 20 °C until further analysis.

2.4. Nitrogen content distribution

The weight of freeze-dried digests (solid and liquid fractions) was registered and protein content in each fraction was measured by elemental analysis. Elemental analysis was performed using the Dumas method in a LECO CHNS-932 analyser (Thermo Fisher, USA) (Association of Official Analytical Chemists (AOAC., 1969). A nitrogen-to-protein factor of 6.25 was applied to calculate the protein content for all the samples. Further analyses were performed on a protein basis according to the results.

2.5. Polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were dissolved at 1 mg of protein/mL, except for casein, which was prepared at 0.8 mg of protein/mL, in sample buffer (Tris-HCl 0.05 M, pH 6.8, SDS 1.6 % w:v, glycerol 8 % v:v, β-mercaptoethanol 2 % and bromophenol blue indicator 0.002 % w:v (Merck, Darmstadt, Germany)), heated at 95 °C for 5 min and loaded on 12 % Bis-Tris polyacrylamide gels (Criterion XT, Bio-Rad, Hercules, CA, USA). Precision Plus Protein Unstained standard was used. Separations were run at 100 V for 5 min, then 150 V, using XT MES running buffer (Bio-Rad, Hercules, CA, USA). Coomasie Blue Biosafe from Bio-Rad was used to dye the gel.

2.6. Confocal laser scanning microscopy (CLSM)

The microstructure of agar-casein hydrogels and micellar casein before and after gastrointestinal digestion was evaluated by means of CLSM using a LSM900 confocal laser scanning microscope coupled to a Vertical Microscope Axio Imager 2 (Zeiss, Germany) with the ZEN Blue 3.4 Software. In the case of the hydrogels and their digests, the solids were cut into sections using a scalpel and Fast Green solution (0.1 % wt. in water) was added to dye proteins. Samples were placed onto a microscopy glass slide and covered with a glass cover slip. A lens with a magnification of 20 and a numerical aperture (NA) of 0.7 and HeNe laser light were used throughout the study. Fluorescence emission was detected at 647 nm. Images were processed using the ImageJ-win64 Fiji Software.

2.7. Transmission electron microscopy (TEM)

Gastric and intestinal liquid fractions (2 uL drop) were dried on a carbon coated grid (200 mesh). TEM was performed using a Transmission Electron Microscope JEM1400 Flash (Jeol, Japan) at an accelerating voltage of 120 keV. Particle sizes were determined by image analysis using the ImageJ-win64 Fiji Software.

2.8. Zeta potential

The Zeta potential (ξ) of the digests was measured in the liquid fractions using a Nano ZS Dynamic Light Scattering Analyzer (Malvern, England). For this purpose, 5 mL of sample were taken and added into a polycarbonate capillary cell with two gold-laminated electrodes (Malvern Instruments, England). Employing the Electrophoretic Light Scattering (ELS) technique, the equipment provides three measurements in a time interval of 10 min. Zetasizer Software was used to obtain the average values.

2.9. Small angle X-ray scattering (SAXS)

SAXS experiments were carried out in the Non Crystalline Diffraction beamline, BL-11, at ALBA synchrotron light source (https://www.albasynchrotron.es). The freeze-dried liquid fractions obtained after the gastrointestinal digestions were placed into 2 mm quartz capillaries (Hilgenberg GmbH, Germany), rehydrated with excess water and sealed before being analysed. The energy of the incident photons was 12.4 KeV or equivalently a wavelength, λ, of 1 Å. The SAXS scattering patterns were collected by means of a photon counting detector, Pilatus 1 M, with an active area of 168.7 x 179.4 mm^2, an effective pixel size of 172 x 172 μm^2 and a dynamic range of 20 bits. The sample-to-detector distance was set to 6570 mm, resulting in a q range with a maximum value of q = 0.2 $Å^{-1}$. An exposure time of 10 s was selected based on preliminary trials. The data reduction was treated by pyFAI python code (ESRF) (Kieffer & Wright, 2013), modified by ALBA beamline staff, to do on-line azimuthal integrations from a previously calibrated file. The calibration files were created from a silver behenate (AgBh) standard. The intensity profiles were then represented as a function of q using the IRENA macro suite (Ilavsky & Jemian, 2009) within the Igor software package (Wavemetrics, Lake Oswego, Oregon).

2.10. Fourier Transform Infrared Spectroscopy (FT-IR)

Digestion products obtained after the gastric and the intestinal phase were analyzed by means of FT-IR in attenuated total reflectance (ATR) mode using a Thermo Nicolet Nexus (GMI, USA) equipment. The spectra were taken at 4 cm^{-1} resolutions in a wavelength range between 400–4000 cm^{-1} and averaging a minimum of 32 scans.

2.11. Statistical analysis

All data have been represented as the average ± standard deviation. Different letters ($p \leq 0.05$), as well as asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$), show significant differences in graphs. Analysis of variance (ANOVA) followed by a Tukey-test was performed using GraphPad Prism version 8.0.2 for Windows, GraphPad Software, Boston, Massachusetts USA (www.graphpad.com).

3. Results and discussion

3.1. Mass balance

Agar-casein hybrid hydrogels and the unstructured agar-casein blends, as well as micellar casein, were digested by applying the Infogest *in vitro* gastrointestinal digestion protocol. After the gastric and intestinal phases, the digests from the hydrogels and the agar-casein blends were heterogeneous, being composed of two distinct fractions: (i) a liquid phase composed of the digested soluble material and (ii) a solid phase containing non-digested or partially-digested and insoluble compounds. This is not surprising, since agar is a dietary fibre, i.e. this polysaccharide is hardly hydrolysed by human digestive enzymes (less than 10 % is assimilated within the intestinal tract) (Cummings & Englyst, 1995; Lovegrove et al., 2017; Sudha et al., 2014). The consistency of the solid phase was clearly different in the two types of agar-casein systems: the hydrogels structure was preserved after digestion, while solid precipitates were obtained after centrifugation of the digests from the blends. It should be noted that in the case of the pure casein digests, only a small pellet was obtained after centrifugation, which represented less than 1 % of the total mass. Hence, the casein digests were considered as a single liquid phase.

After each digestion phase, the mass of the digests was recorded and the corresponding weight fraction for the solid and liquid phases were determined. The results, shown in Figure S1, evidenced clear differences in the dry weight distribution behaviour of the hydrogels and the blends, as well as an effect of the bile salts concentration used during the intestinal phase. Regarding the mass distribution in the hydrogels (Figure S1), the solid phase after the gastric digestion represented approximately 55 % of the total mass. Considering that the hydrogel was estimated to account for ca. 70 % of the total dry weight, it seems that part of the casein was released during the digestion process. As a reference, control hydrogels containing only agar as the structuring material, were also prepared and digested. In that case, the stronger gel network structure formed by the polysaccharide remained almost intact during the digestion process, increasing the weight fraction corresponding to the solid phase from the 55 % found in the casein-polysaccharide hydrogels to 64 % of the total weight in the pure agar hydrogels. After the intestinal phase, the weight fraction corresponding to the solid phase of the casein-agar hydrogels decreased down to 17–26 %, which is close to the theoretical values of 15 % (0 mM bile salts), 14 % (2.5 mM bile salts) and 11 % (10 mM bile salts). This indicates that although part of the casein initially presented in the hydrogel structures was hydrolysed and diffused towards the liquid medium, a certain amount of the digestion fluid and enzymes were held within the structure of the non-digested hydrogels. The impact of bile salts during the

intestinal digestion was reflected on an increase in the weight fraction corresponding to the liquid phase when increasing their concentration in the digestion medium. In the case of the agar-casein blends (Figure S1B) the solid fraction accounted for less than 20 % of the total weight after the gastric digestion. This value is close to the theoretical weight percentage of agar in the gastric digests (19 %), hence suggesting that most of the protein was solubilized in this case. In the intestinal phase, this percentage was further reduced, indicating that part of the agar was also solubilized, and a trend was observed in which a higher concentration of bile salts correlated with a decrease in the solid fraction. The significantly different behaviour observed for the hydrogels and the blends, evidences that not only the presence of polysaccharides, but also their physical state could be highly relevant to modulate the digestion of proteins.

The protein content distribution in the liquid and solid fractions after the gastric and gastrointestinal digestions were also determined and results are shown in Fig. 1. After the gastric phase, ca. 50 % of the total protein was retained within the solid fraction from the hybrid hydrogels (Fig. 1A). This protein could correspond to both the casein and the digestive enzymes retained within the gel structures. In fact, in the case of the pure agar hydrogel (HG-A100), the protein content in the solid accounted for ca. 27 %. This protein content is indicative of the approximate amount of digestive enzymes held within the hydrogel structures. Assuming that the behaviour of the agar-casein hydrogel is similar to the pure agar hydrogel (which is not true, since the greater amount of polysaccharide gives rise to stronger hydrogels, with greater sorption capacity), at least 23 % of the protein retained within the solid fraction of the hybrid hydrogels must be arising from casein which did not diffuse towards the liquid medium. This might be explained by a lower degree of proteolysis of the casein in the hybrid hydrogels during the gastric phase, as demonstrated in a previous study (Fontes-Candia et al., 2022). At the end of the intestinal phase, the percentage of protein in the solid fraction dropped down to ca. 15–22 % of the total protein, indicating that during the intestinal phase a significant amount of protein diffused towards the liquid medium. Interestingly, a decreasing trend in the protein percentage within the solid fraction was noted as the concentration of bile salts increased. As a reference, the pure agar hydrogels (digested with 10 mM bile salts) retained around 15 % of the protein within the solid fraction. This amount is very similar to the protein content found in the solid fraction from the hybrid hydrogels digested at the highest concentration of bile salts, suggesting that a relatively high proportion of the casein was solubilized in that case. Thus, higher bile salt concentrations promoted a greater diffusion of the casein towards the liquid. With regards to the casein-agar blends, less than 5 % of the protein remained within the solid fraction after the gastric phase (Fig. 1B), while at the end of the intestinal phase, an increase in the protein contents was observed with respect to the gastric phase for all the samples, regardless of the bile salts concentrations. This might be originated by the greater amount of protein in the systems due to the addition of pancreatin which remained in the solid fraction after centrifugation, as previously reported (Ménard et al., 2023).

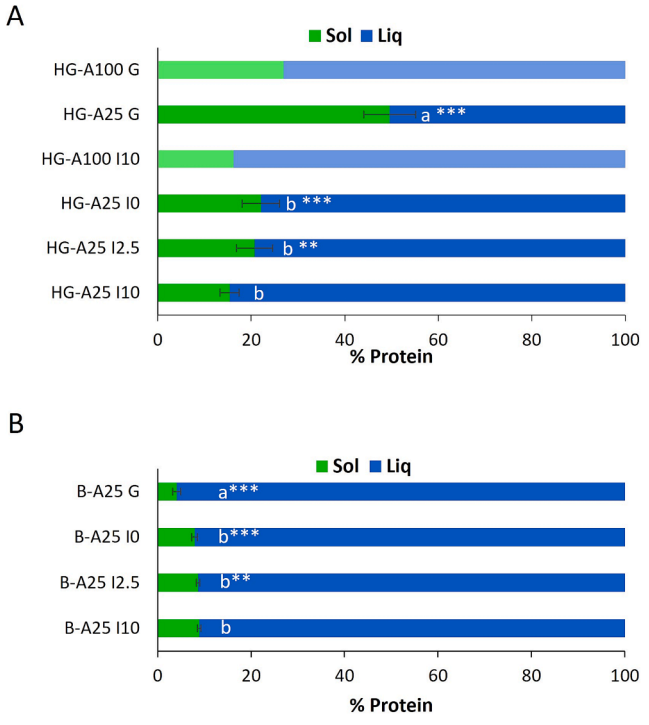

Fig. 1. Protein content distribution in the solid (Sol) and liquid (Liq) fractions after gastric (G) and gastrointestinal (I) digestions with different bile salts concentrations (0 mM, 2.5 mM and 10 mM) in the (A) hybrid agar-casein (HG-A25) and agar hydrogels (HG-A100), and (B) agar-casein blends (B-A25). Different letters (a, b, c) indicate statistically meaningful differences ($p \leq 0.05$) between the protein percentages in the digests from the hybrid systems shown in each graph. On the other hand, asterisks indicate statistically meaningful differences (** $p \leq 0.01$, *** $p \leq 0.001$) between the protein percentage of the digests from hydrogels vs. blends (for samples at the same digestive stage and same bile salts concentration).

Noteworthy disparities were noted between the hydrogels and the blends at identical digestive stages and bile salts concentrations. Except for the samples digested with the highest concentration of bile salts (10 mM), the amount of protein remaining in the solids from the hybrid hydrogels was ca. 2-fold the values estimated in the blends. This supports a protective effect of the hydrogels on the proteolysis of casein, but also evidences that this effect is not so obvious when increasing the amount of bile salts in the digestion medium. Hence, these results may indicate that casein proteolysis was potentiated with higher bile salts concentrations. Such effect has been previously reported for β-lactoglobulin and whole milk (Dulko et al., 2021; Gass et al., 2007). However, whereas bile salts can alter the secondary structure of globular proteins, a different mechanism must take place in the case of casein, as this protein lacks a defined secondary structure. Previous work has demonstrated that the peptidic fragments released upon gastrointestinal digestion of casein are able to interact with bile salts, forming a mixture of lamellae/micelles and vesicular structures (Fontes-Candia et al., 2023); however, the mechanism by which bile salts are able to promote casein hydrolysis remains unknown. A plausible hypothesis is that this interaction between casein and bile salts may expose inaccessible regions of the protein, thereby facilitating the enzymatic activity. In fact, the hydrophobic amino acid residues on the surface of β-casein micelles have been reported to interact with the hydrophobic regions of bile salts through hydrophobic bonds. Additionally, the hydrophilic regions of β-casein micelles establish hydrogen bonds with the hydroxyl and carboxylic groups of bile salts, resulting in a strong complexes (Kuchlyan et al., 2016). These newly organized structures of β-casein micelles combined with bile salts could form mixed micelles. In any case, the protective effect of the hybrid hydrogels might be useful to develop systems with controlled release of peptides upon the intestinal digestion phase.

3.2. *Evaluation of the degree of proteolysis*

The molecular weight (MW) distribution analysis of proteins and peptides was assessed by SDS-PAGE (Fig. 2). Included in the experimental setup are two blank samples corresponding to the gastric (Ctrl G) and intestinal (Ctrl I2.5) digestion media, including the enzymes and bile salts (at a concentration of 2.5 mM). These blank samples facilitate the identification of bands corresponding to digestive enzymes (which are marked with asterisks in Fig. 2), including salivary and pancreatic amylase (glycosylated isoform ~ 62 kDa, and non-glycosylated form ~ 56 kDa) (Ferey-Roux et al., 1998; Fisher et al., 2006), pepsin (34.5 kDa) (Stanforth et al., 2022), trypsin (23.3 kDa) and pancreatic lipase (50

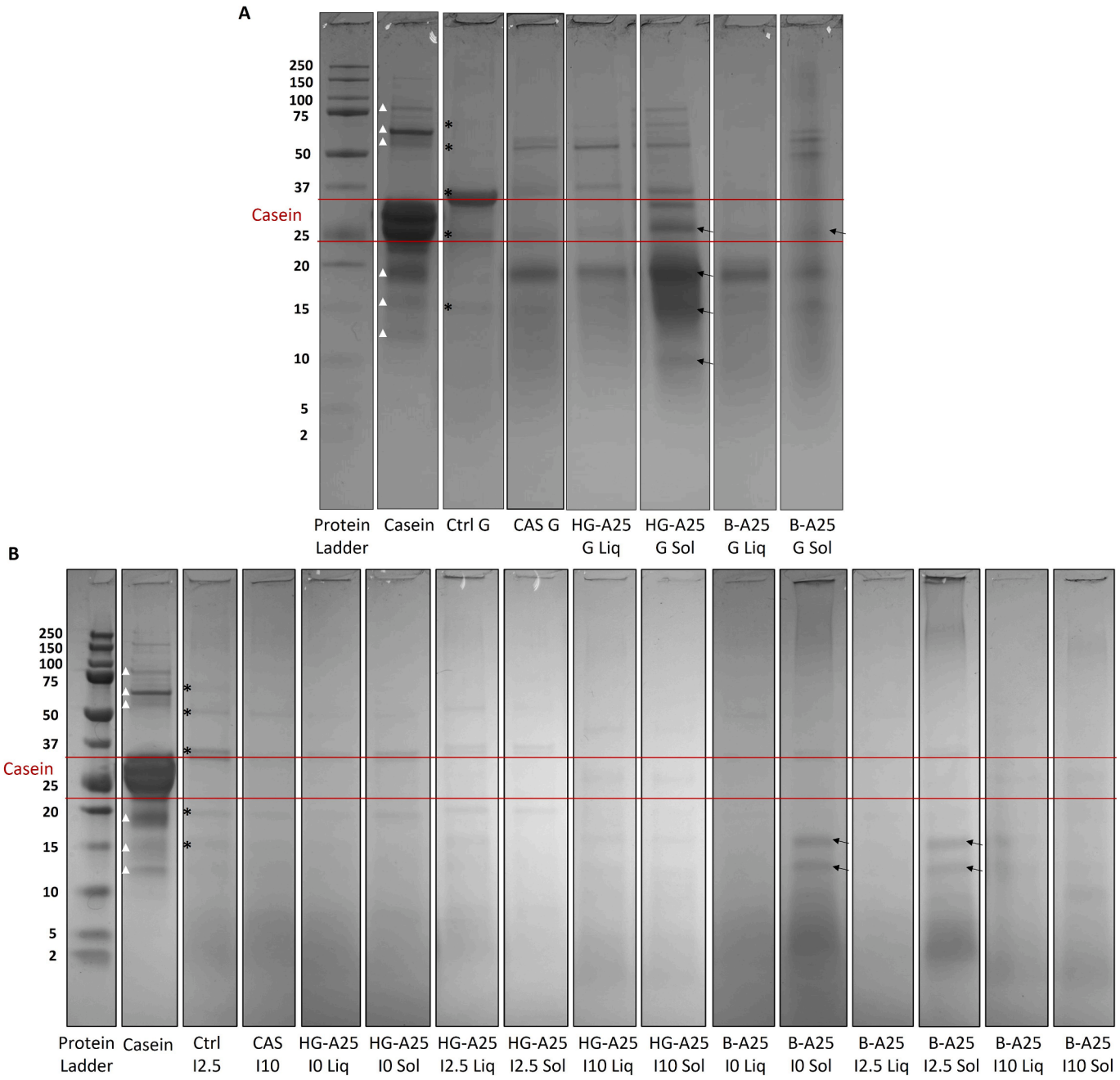

Fig. 2. SDS-PAGE protein profiles of casein, agar-casein hydrogels (HG-A25) and agar-casein blends (B-A25) after (A) gastric and (B) gastrointestinal digestions with different bile salts concentrations (0 mM, 2.5 mM and 10 mM). White triangles mark bands corresponding to some whey proteins present in the micellar casein sample (Lf, BSA, IgG, β-LG and α-LA). Black asterisks mark digestion enzymes (amylase, pepsin and pancreatic enzymes). Arrows point towards protein fragments and peptides.

kDa) (Sanchón et al., 2018).

The SDS-PAGE protein profile of the initial undigested casein (Fig. 2A) showed several bands within the 24–35 kDa molecular weight range, primarily corresponding to α_s-, β- and κ-casein (Wang et al., 2018). Furthermore, bands corresponding to whey proteins, such as lactoferrin (Lf), bovine serum albumin (BSA), immunoglobulin G (IgG), β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) were observed as expected, given that the product used was a commercial micellar casein (Costa et al., 2014). From these bands, the one corresponding to the serum protein β-LG (~18 kDa) was observed in all the gastric samples, which is not surprising since this protein is known to be resistant to gastric pepsin (Egger et al., 2016; Kopf-Bolanz et al., 2012). With regards to the casein fraction, the digesta obtained after gastric digestion (CAS G) appeared to be highly hydrolysed, as bands corresponding to the native caseins were not visible. In turn, some weak smearing appeared in the region under 13 kDa, corresponding to peptides generated as a consequence of proteolysis. The high degree of casein hydrolysis was expected, as it has been reported that the open and flexible structure of this protein makes it highly susceptible to pepsin hydrolysis (Jin et al., 2016; Li et al., 2020). In contrast, in the case of the hybrid casein-agar systems, it can be observed that the protein was distributed within the solid and liquid fractions, as evidenced by the appearance of the β-LG band in all the gastric digests. The solid fraction from the hydrogels exhibited undigested casein, as its distinctive bands were evidently noted, in line with what was previously described by Fontes-Candia et al., 2022. Moreover, bands within the range of 17–7

kDa were observed, suggesting that larger peptides were generated due to a lower degree of proteolysis. In the case of the blends, it seems that a small fraction of casein might have remained undigested in the solid fraction, while the liquid fraction showed a very similar profile to that of the casein digest.

At the conclusion of the intestinal phase (Fig. 2B), the characteristic bands of intact casein were not present in any sample, while bands corresponding to digestion products with molecular weights under 13 kDa were discernible, indicating the substantial hydrolysis of casein into peptides. In general, all the liquid digesta from the hybrid casein-agar systems displayed a similar profile to that of casein alone, indicating that most of the casein present in the liquid fractions was hydrolysed into small peptides and/or free amino acids, regardless of the presence of agar. It should be noted that in the case of the hydrogels, sample handling was very complex, since solubilisation of the proteins was very challenging due to the presence of a gelled agar matrix, as previously reported (Fontes-Candia et al., 2022). Thus, the lanes corresponding to the solid fractions appeared almost empty, although other characterization techniques, such as CLSM, showed that in fact, some partially digested protein was present in these samples. Interestingly, the region corresponding to MW $\leq$ 13 kDa was more intensely stained in the solid fractions from the casein-agar blends digested under lower concentrations of bile salts (0 mM and 2.5 mM), providing further experimental support for the hypothesis that bile salts play a significant role in protein digestion, as at 10 mM concentration most of these peptides were further digested or even converted into free amino acids.

The net charge density of intact casein and agar, as well as the liquid fractions obtained after gastrointestinal digestion, were characterised to determine their surface charge densities through the measurement of ζ potential (Fig. 3). The initial casein exhibited a net negative charge of –23.3 mV, similar to values reported in the literature (Cano-Sarmiento et al., 2018; Wade et al., 1996), as the pH was maintained above its isoelectric point and thus charged amino acid side chains were situated on the protein surface. On the other hand, agar possessed a net negative charge of −28.8 mV, which is ascribed to the presence of sulphate groups in the polysaccharide chains. This value is in accordance with the literature values reported for agar solutions (Boral & B. Bohidar, 2010).

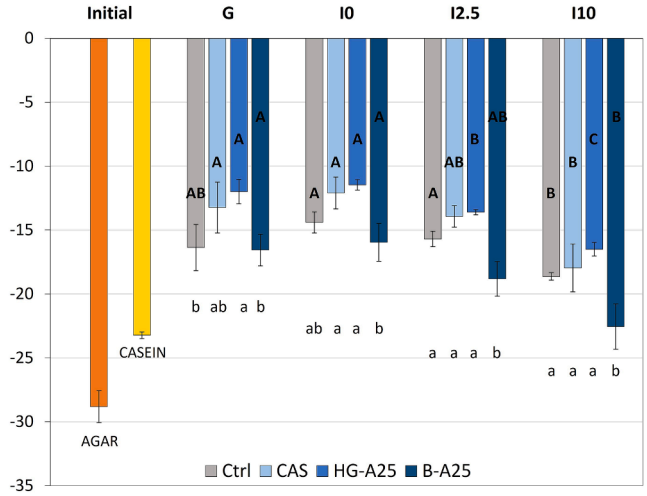

Fig. 3. ζ potential of the initial agar (orange) and casein (yellow) solutions and the digestion products of casein (CAS), agar-casein hydrogels (HG-A25) and agar-casein blends (B-A25) in the liquid fraction after gastric (G) and gastrointestinal (I) digestions with different bile salts concentrations (0 mM, 2.5 mM and 10 mM). Ctrl corresponds to gastric and gastrointestinal digestion fluids within enzymes and bile salts. Different capital letters (A, B, C) indicate statistically meaningful differences ($p \leq 0.05$) between the ζ potential of the digests from the same sample within different digestive stages and bile salts concentrations. Different letters (a, b, c) indicate statistically meaningful differences ($p \leq 0.05$) between the ζ potential of the digests from different samples at the same digestive stage and same bile salts concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

After the gastric phase, the surface charge values of the liquid fraction digests were also measured and compared with the values of the simulated gastric fluid (Ctrl). While the digests from casein and the hybrid hydrogels had less negative values than the control fluid, the digests from casein blends presented more negative values, similar to the control. This could be ascribed to the presence of a proportion of agar solubilized in the liquid fraction providing negative charges. This suggests that the polysaccharide-protein interactions were not so strong in the blends as compared to the hydrogels. Interestingly, despite the presence of negatively charged agar in the digests from the hydrogels, their ζ potential was less negative than that from the casein digests, which could be related to the higher proteolysis of casein when in the absence of the polysaccharide, hence increasing the number of charged amino acids and peptides (Cebrián-Lloret et al., 2024; Mahmoud et al., 1992).

After the intestinal phase, the simulated gastrointestinal fluid (Ctrl) showed more negative values when increasing the bile salts concentration, which was expected due to their negative charge contribution, as already reported in the literature (Wickham et al., 1998). Upon analysing the samples within each range of bile salt concentrations, similarly to the gastric phase, while casein and hydrogel liquid digests had a lower surface charge (less negative ζ potential) than the control liquids, the digests from casein-agar blends had more negative values, probably due to the presence of solubilized agar. Once again, it was observed that the casein digests presented slightly more negative values than those from the hydrogels. Regarding the different concentrations of bile salts, an increase in the negative charges of the digests was observed. This could be attributed to two factors: (i) the presence of increasing amounts of salts in the digesta (Zhang et al., 2017) and (ii) a greater degree of proteolysis induced by bile salts (Cebrián-Lloret et al., 2024), likely due to the exposure of previously inaccessible regions of the protein, thereby facilitating enzymatic activity, as previously hypothesized. From Fig. 3, it can be noted that the difference between the ζ potential values from the digests and their respective control fluids diminished as the bile salts concentration was increased, indicating that casein hydrolysis was contributing to a greater extent to the negative ζ potential values at higher bile salt concentrations. The decrease in the ζ potential when increasing the bile salts concentration from 0 mM to 10 mM was ca. 48 % in the case of casein, while it was 44 % in the hydrogel liquid digests, suggesting that the proteolytic effect of bile salts was mitigated to some extent in the case of the hydrogels.

3.3. *Structural characterization of the digests*

3.3.1. *Microstructure of the solid fractions*

The process of degradation and structural assembly of digestion products generated during gastrointestinal digestion was evaluated through confocal laser scanning microscopy and representative images of the structural evolution of the protein fraction in the casein digests and in the solid fractions from hydrogels and blends are shown in Fig. 4. Initially, the commercial micellar casein exhibited structures corresponding to casein aggregates (micelles could not be visualized due to their small size, which typically ranges within 100–150 nm) (Dalgleish, 2011; Jarunglumlert et al., 2015), the size of which decreased throughout the gastrointestinal digestion. On the other hand, in the hybrid hydrogels, larger aggregates of casein were identified, reflecting a distinct structural conformation of the casein when incorporated into the agar gelling network.

After the gastric phase, very few large particles were observed in the casein digests, which could in fact correspond to aggregates from the non-hydrolysed or partially-hydrolysed proteins, such as β-LG (Nicolai et al., 2011), as indicated by the SDS-PAGE results. This fact confirms that casein is highly susceptible to the hydrolytic action of pepsin (Wang

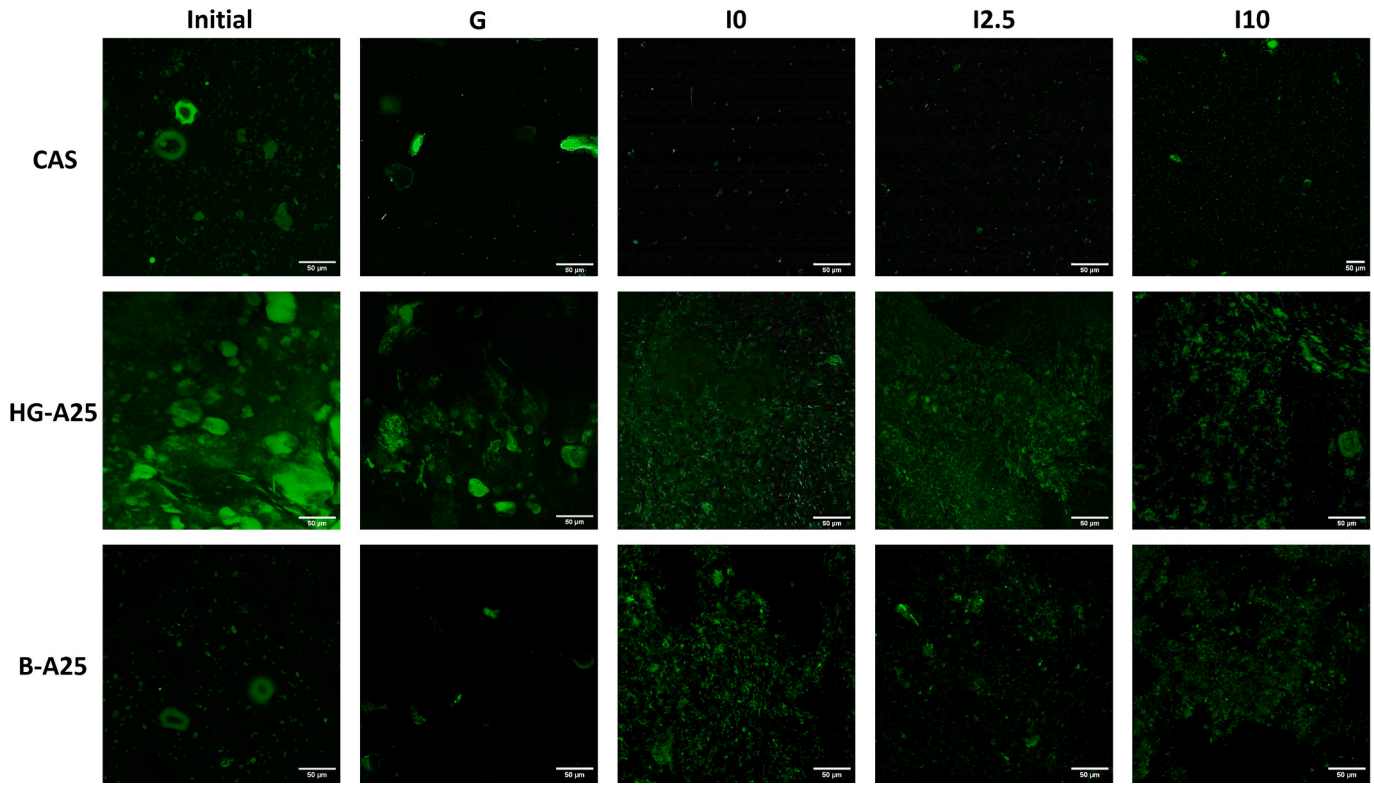

Fig. 4. Confocal laser scanning microscopy (CLSM) images of the initial casein dilution (CAS), agar-casein hydrogel (HG-A25) and agar-casein blend (B-A25) and the solid fraction from digestion products, after gastric (G) and gastrointestinal (I) digestions with different bile salts concentrations (0 mM, 2.5 mM and 10 mM). Proteins were stained with Fast Green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2018). Regarding the hybrid structures, very few large structures were observed in the solid phase from the blends, likely corresponding to β-LG aggregates and partially digested casein aggregates, as deduced from the SDS-PAGE results. The solid fractions from the hydrogels showed larger particles similar to the ones observed in the sample before digestion, hence suggesting the presence of poorly digested casein, which forms larger aggregates. This result is in agreement with SDS-PAGE, where the characteristic bands of intact casein were observed in the solid fraction, demonstrating the protective effect of the hydrogels against the hydrolytic action of pepsin, as previously reported (Fontes-Candia et al., 2022).

After the subsequent intestinal phase, only very small structures could be observed in the casein digests, since following the action of digestive enzymes, the generated hydrolysed products are not capable of forming large aggregates. However, the increase in bile salts concentration led to the appearance of some bigger particles in the casein digests, likely due to the assembly of peptides and bile salts into ordered structures, as recently reported (Fontes-Candia et al., 2023), which could in turn associate into larger particles. In the case of the hybrid casein-agar systems, the solid fractions showed some particles and aggregated structures. These structures were probably originated from the assembly of peptides into aggregates, being more abundant in the hydrogel samples, due to their protective effect. In the hydrogels, it was evident that a decrease in the overall amount of protein remaining in the solid fraction was produced. The smaller protein particles were removed from the solid and only larger aggregates remained when the concentration of bile salts was increased. This effect was not noted in the blends, where the overall amount of protein fraction remaining in the solid was quite similar regardless of the bile salts concentration, which is in line with the mass balance results. This indicates that bile salts seem to induce a greater diffusion of protein towards the liquid in the case of the hydrogels.

3.3.2. *Nanostructures and molecular interactions in the liquid fractions*

To investigate further on the nature/type of nanostructures formed by the assembly of the digestion products, transmission electron microscopy (TEM) was employed to characterise the intestinal liquid fractions, enabling the direct visualization of the particles in the samples. During the gastric phase, the formation of relatively large branched structures was evident in the gastric fluid (Figure S2) due to the presence of high concentrations of salts. These branched structures also appeared in all the gastric digests, as evidenced in Fig. 5.

After the intestinal phase, the structures found in the intestinal fluids were completely different to those seen in the gastric phase. Specifically, much smaller particles were observed, which started to form larger aggregates when bile salts were present. These aggregates showed an average size of around 28 nm in diameter for 2.5 mM, and even larger aggregates (~1000 nm) were noted when increasing the bile salts concentration to 10 mM. Bile salts are known to present a self-assembly behaviour (Maldonado-Valderrama et al., 2011), being capable of forming ordered lamellar/micellar structures in the nanometer scale. Apparently, these nanostructures tend to associate to form larger structures, especially when increasing the concentration.

With the release of digestion products towards the liquid medium, different types of structures were formed, with average sizes ranging from 70 nm up to ~500 nm, due to the interplay amongst peptides, polysaccharides, and constituents dissolved in the physiological fluids (Fontes-Candia et al., 2023). In general, it was noted for all the samples that the overall amount of particles in the liquid phase decreased when increasing the bile salts concentration, which can be related to the higher degree of hydrolysis induced by bile salts, but also to the self-association of bile salts into larger structures, as noted in the control intestinal fluids. In the case of the micellar casein, particles with average sizes from 68 to 227 nm were detected. When adding bile salts at 2.5 mM, these particles tended to agglomerate into larger structures, although some of the smaller structures were also observed. Increasing

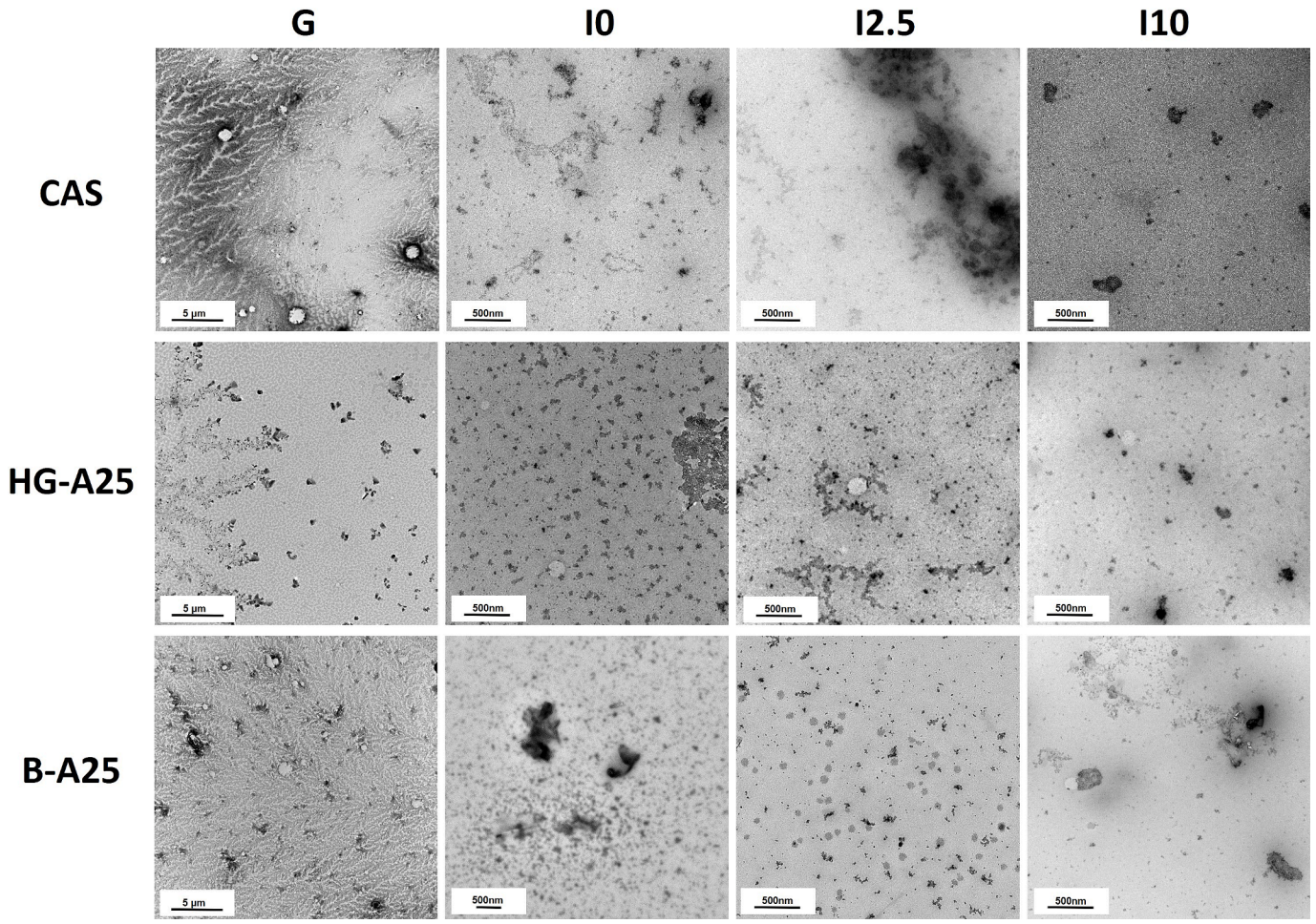

Fig. 5. TEM images of the gastrointestinal digestion products in the liquid fraction from digestion products of casein (CAS), agar-casein hydrogels (HG-A25) and agar-casein blends (B-A25), after gastric (G) and gastrointestinal (I) digestions with different bile salts concentrations (0 mM, 2.5 mM and 10 mM).

further the bile salts concentration up to 10 mM led to the formation of two distinct populations of particles, small particles of ca. 16 nm and larger globular particles of ca. 198 nm. The fact that larger structures were formed at a bile salts concentration of 2.5 mM might be related to the association of peptides with bile salts (Bellesi & Pilosof, 2021). It seems that at this particular concentration, the amount of peptides present in the digesta can associate with bile salts to form ordered structures. On the other hand, increasing further the bile salts concentration leads to a higher degree of protein hydrolysis, reducing the amount of peptides and increasing the proportion of free amino acids. In that case, bile salts tend to self-associate instead. In the case of the hybrid hydrogels, it was noted that a lesser degree of particle agglomeration took place at a bile salts concentration of 2.5 mM, as compared to the micellar casein. This is probably due to the fact that a lower proportion of soluble peptides and free amino acids were released towards the liquid medium, as part of the protein was retained within the solid fraction, as evidenced by mass balance and CLSM. On the other hand, the blends showed very large particles in the absence of bile salts, which could correspond to a proportion of agar present in the liquid fraction. Larger particles than those detected in the hydrogels were in general observed for bile salts concentrations of 2.5 mM and 10 mM. This may be indicative of the polysaccharide being able to establish interactions with bile salts.

The nanostructure of the digestion products in the liquid digesta was also investigated through SAXS experiments and the obtained scattering patterns are shown in Fig. 6. As observed, all the digests presented a small scattering peak located at approximately 0.13 $Å^{-1}$, which corresponds to a real distance of ca. 5 nm. This peak has been previously detected in the gastrointestinal digests from different types of samples and was attributed to the presence of ordered bile salt structures (e.g. lamellae, micelles) in the digestion medium (Fontes-Candia et al., 2023; Fontes-Candia et al. 2022). The fact that this peak was detected also in the liquids from the digestions performed without adding bile salts to the digestion medium can be explained by the fact that the pancreatin added during the intestinal phase contained a certain amount of residual bile salts, as deduced from Figure S3. This peak was detected in the pancreatin solution and in the bile salts solution used to prepare the simulated intestinal fluids. In the gastrointestinal digestion mediums, the peak characteristic from micellar/lamellar structures was observed for all the bile salts concentrations, although it was less evident for the one with the highest concentration (10 mM). This could be explained by the presence of other salts and additional components affecting the bile salts critical micellar concentration (CMC), i.e. the concentration above which micelles are destabilized and can aggregate into larger structures (Miyagishi et al., 2001; Qazi et al., 2020). Indeed, the TEM images (Fig. 5) corroborated a greater degree of aggregation of the bile salts when increasing their concentration up to 10 mM.

As already mentioned, the liquid digests from all the samples presented the bile salts scattering peak, however, the appearance of this peak was affected differently by the bile salts concentration depending on the system. In the case of casein, the position and intensity of the peak was almost identical to that observed in the respective control digestion mediums for the three bile salt concentrations, indicating that the small peptides and amino acids released upon digestion did not have a

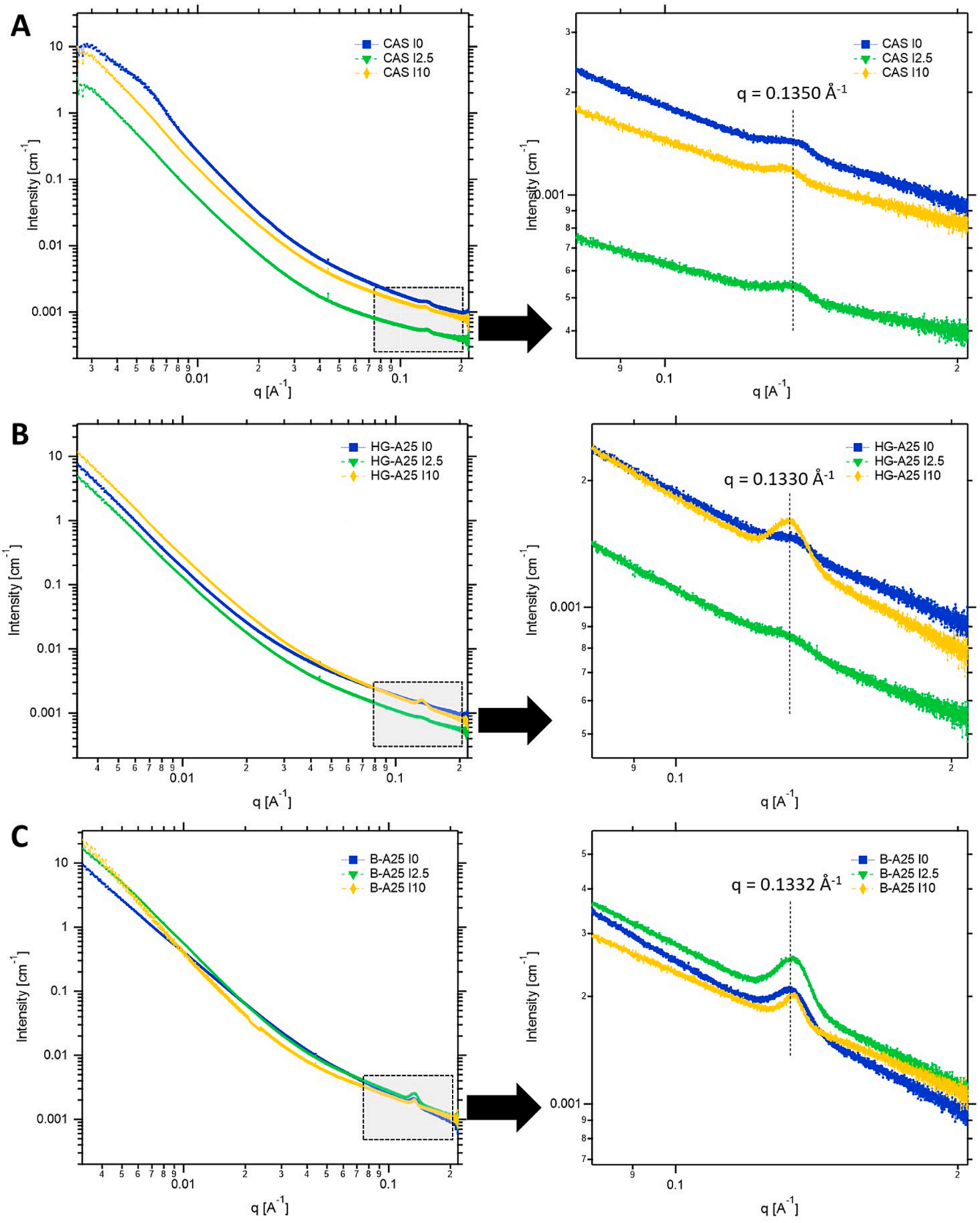

Fig. 6. SAXS patterns from the liquid fraction obtained after the gastrointestinal digestion for the casein (A), the agar-casein hydrogels (HG-A25) (B) and the agar-casein blends (B-A25) (C) with different bile salts concentrations (0 mM, 2.5 mM and 10 mM). The q range of interest is displayed in the graphs on the right column.

significant effect in altering the structural conformation of the bile salts micelles. However, it should be noted that a small scattering shoulder was detected in the liquid from the casein digested without the addition of bile salts. This indicates the presence of larger structures (around 100 nm) in the digesta, which were no longer visible when adding bile salts. It should be noted that the q range from the performed SAXS experiments only covers nanostructures up to ca. 200 nm and thus, structures larger than that cannot be determined in the scattering data. For instance, the aggregates of bile salts at 10 mM observed in the TEM images are out of the range covered by SAXS.

In the case of the hydrogels, the liquid digests obtained at 0 and 2.5 mM of bile salts were very similar to those from the casein, suggesting the presence of analogous nanostructures in the liquid fractions from both systems. However, at the highest bile salts concentration, the scattering peak became more intense and defined, indicating a greater concentration of ordered bile salt structures in that case. A plausible explanation for this is that at low bile salt concentrations a significant proportion of partially digested casein remained trapped within the solid fraction, as evidenced by the CLSM images, and the fraction of casein which was relased towards the liquid medium was digested to a similar extent as that from the control casein sample. In contrast, a further increase in the bile salts concentration promoted a greater release of the casein towards the liquid, most likely in the form of peptide-polysaccharide complexes which could interact with the bile salts. Surprisingly, in the case of the agar-casein blends the scattering peak was clearly more intense, especially for the 0 and 2.5 mM bile salts concentrations. This could be due to the fact that some agar was solubilized into the liquid fraction, which may have promoted the formation of ordered structures through interaction of the polysaccharide with the bile salts. In fact, some polysaccharides are known to interact with bile salts, affecting the digestion of lipids and limiting the absorption of cholesterol in the small intestine (Fontes-Candia et al. 2022; Gunness & Gidley, 2010). Fig. 7 shows schematically the proposed nanostructural assembly undergone by the digestion products from the casein and the two different hybrid systems in the presence of increasing concentration of bile salts.

To investigate the molecular interactions which might have been established between the protein and polysaccharide in the intestinal digests, the FT-IR spectra from the liquid fractions, as well as the native micellar casein and the agar, were collected. As observed in Figure S4, the spectra from the intestinal digests from the casein and the hybrid hydrogels were very similar, whereas the digests from the agar-casein blends showed some notable differences, especially within the range of 700–1700 cm^{-1}. For clarity, Fig. 8 shows the spectra from the digests and the micellar casein and agar within that specific wavenumber region. As expected, due to the high degree of hydrolysis, the protein characteristic bands were strongly modified in the intestinal digests from the micellar casein. In particular, the amide I band, located at ~1640 cm^{-1}, was replaced by a broader band with its maximum absorbance at ~1580 cm^{-1}. Moreover, the relative intensities of that band and the amide II band, located at ~1515 cm^{-1}, were reduced as the bile salts concentration in the intestinal phase was increased. These changes can be attributed to the disruption of the protein secondary structure, as previously reported (Fontes-Candia et al., 2023). Therefore, in agreement with the nitrogen distribution (Fig. 1) and ζ potential (Fig. 3) results, it is clear that in the case of the micellar casein, higher concentrations of bile salts induced a greater extent of proteolysis, leading to the formation of smaller peptides and free amino acids. A

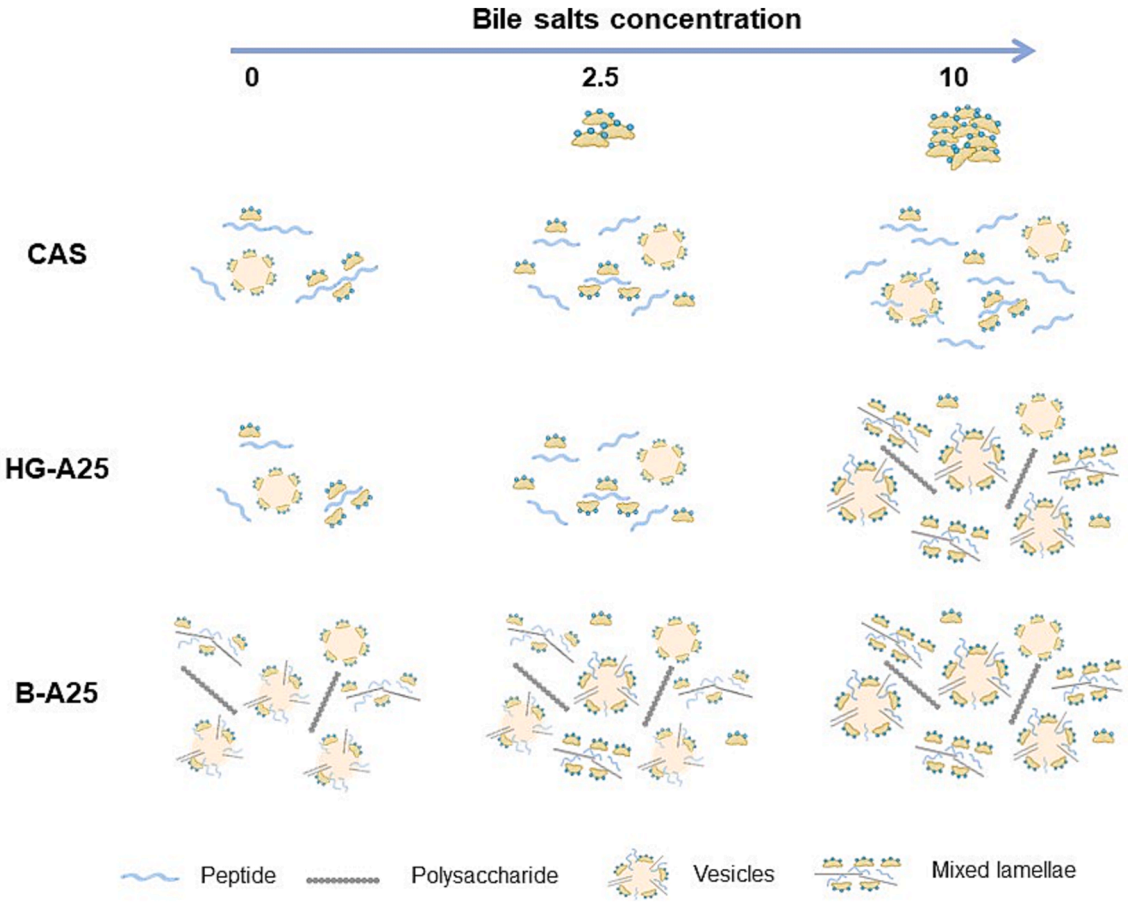

Fig. 7. Schematic representation of the proposed nanostructural assembly of the digestion products from the micellar casein (CAS), hybrid agar-casein hydrogels (HG-A25) and casein-agar blends (B-A25) in the presence of different bile salts concentrations.

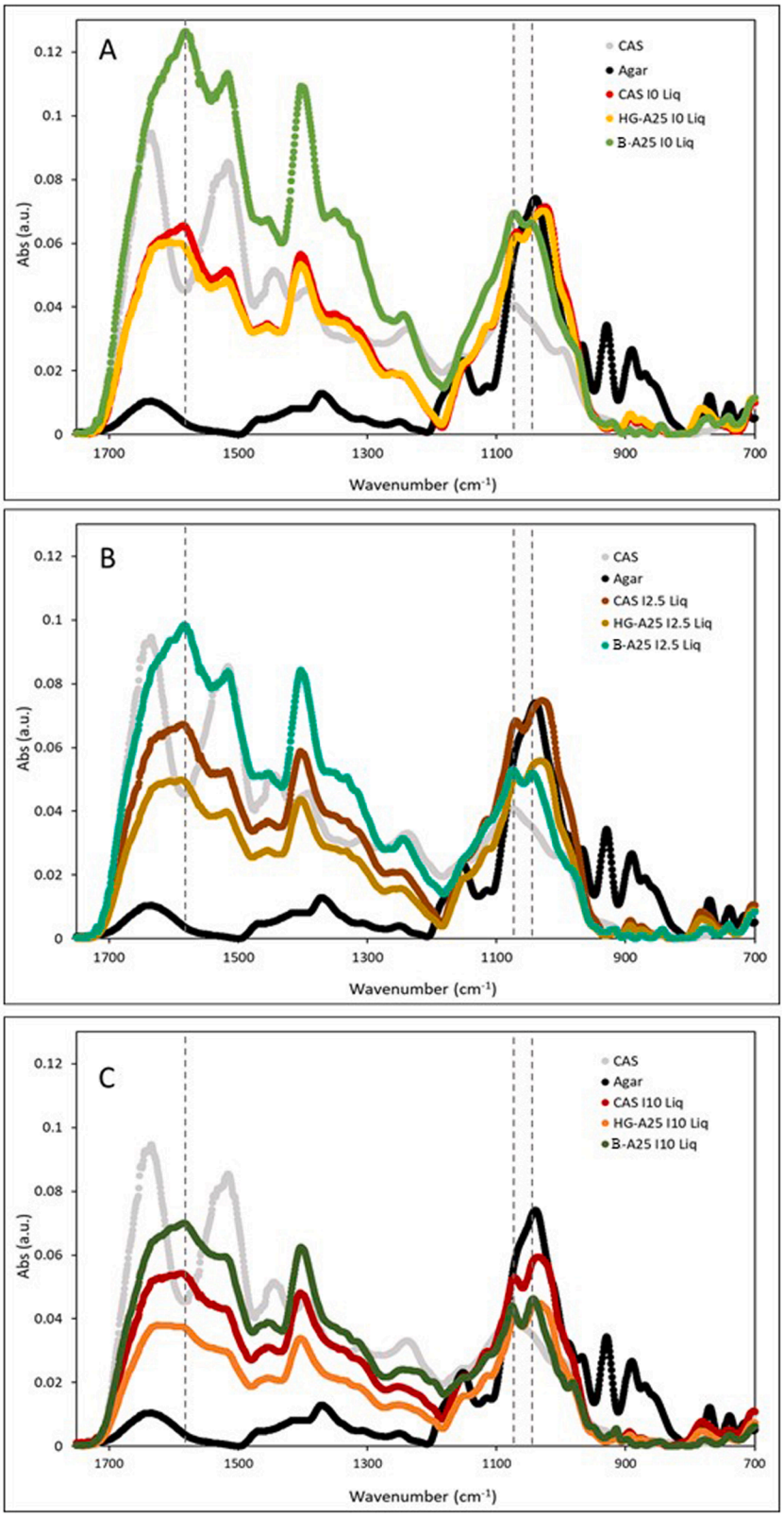

Fig. 8. FT-IR spectra from the micellar casein and agar and the liquids fractions obtained after the gastrointestinal digestions of casein (CAS), hybrid agar-casein hydrogels (HG-A25) and casein-agar blends (B-A25) with bile salts concentrations of (A) 0 mM, (B) 2.5 mM and (C) 10 mM.

similar effect was noted in the case of the hybrid hydrogels, although in that case the change in the relative intensities of the bands was only evident when increasing the bile salts concentration from 2.5 to 10 mM. According to SAXS and CLSM, it seems that a greater release of the casein from the hydrogel structure was promoted at the highest bile salts concentration, which were most likely released in the form of peptide-polysaccharide complexes capable of interacting with the bile salts to form ordered structures. The formation of such complexes may be also

responsible for the decrease in the relative intensity of the bands associated to the amide I and II bands. Interestingly, in the case of the blends, the relative intensity of these bands was much higher, which can be related to the presence of greater proportions of peptides in their liquid fractions, originated by the formation of complexes between the bile salts and the solubilized agar. In fact, the presence of agar in the liquid fractions from the blends digesta was evidenced by the appearance of a band at 1040 cm^{-1}, due to the coupling of the C-O or the C–C stretching modes with the C-O–H bending modes (Guerrero et al., 2014), which was only visible in the case of the blends.

4. Conclusions

Bile salts have been demonstrated to be a key factor for the intestinal digestion step, as they are able to interact with proteins and polysaccharides and intervene in the nanostructural assembly of the digestion products generated. Our results have confirmed that in the case of micellar casein, bile salts potentiate proteolysis. In the presence of agar, this effect of increased proteolysis is somehow limited, due to the competitive interactions between bile salts and the polysaccharide. Hybrid gel-like structures limit casein hydrolysis, especially during the gastric phase, due to their capacity to retain casein trapped within their gel network. Bile salts promote the diffusion of casein towards the digestion medium, hence promoting a greater degree of proteolysis. On the other hand, casein-agar blends limit proteolysis to a certain extent due to competitive interactions between the polysaccharide and the bile salts.

Another important consideration is that bile salts can self-associate to form ordered lamellar/micellar structures, and are also able to interact with the released peptides and/or polysaccharides. The concentration of the bile salts in the medium will determine type of nanostructures formed, which can agglomerate into larger structures depending on the amount of peptides/polysaccharides interacting with the salts. In particular, agar seems to be able to promote the formation of ordered lamellar/micellar structures. Furthermore, the presence of peptides and/or solubilized agar can reduce the aggregation of bile salts which takes place at higher concentrations of 10 mM.

All these results evidence the importance of bile salts on the digestion mechanism and the nanostructural assembly process. Future studies will aim to correlate the formed nanostructures with transepithelial transport, potentially revealing the mechanisms for the transport of nutrients through the intestinal epithelium, hence controlling the nutritional quality and metabolic responses induced by different types of foods. Thus, these findings will contribute to the development of strategies for the formulation of food ingredients with targeted metabolic responses.

CRediT authorship contribution statement

Laura Díaz-Piñero: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Cynthia Fontes-Candia:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Estefanía Rodríguez-Dobreva:** Investigation, Formal analysis, Data curation. **Isidra Recio:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Data curation, Conceptualization. **Marta Martínez-Sanz:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgements

This work was funded by European Union (ERC, PRODIGEST, ERC-2022-COG 101086483). Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or the European Research Council Executive Agency. Neither the European Union nor the granting authority can be held responsible for them. Laura Díaz-Piñero is recipient of a pre-doctoral FPU grant from the Spanish Ministry of Universities (FPU21/04504). Cynthia Fontes-Candia was supported by a Margarita Salas Postdoctoral Fellowship from Universitat Politectica de Valencia, funded by Ministerio de Universidades and the Eropean Union-Next Generation EU. Fig. 7 was created with BioRender.com. Synchrotron experiments were performed at NCD beamline at ALBA Synchrotron with the collaboration of ALBA staff (proposal 2022025569).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.115179.

References

Alavi, F., Emam-Djomeh, Z., Yarmand, M. S., Salami, M., Momen, S., & Moosavi-Movahedi, A. A. (2018). Cold gelation of curcumin loaded whey protein aggregates mixed with k-carrageenan: Impact of gel microstructure on the gastrointestinal fate of curcumin. *Food Hydrocoll., 85*, 267–280. https://doi.org/10.1016/J.FOODHYD.2018.07.012

Association of Official Analytical Chemists (AOAC). (1969). *Official Method for Protein (Crude) in animal feed. Dumas method. (1969). Test Method: AOAC 968.06-1969.*

Bellesi, F. A., & Pilosof, A. M. R. (2021). Potential implications of food proteins-bile salts interactions. *Food Hydrocoll., 118*, Article 106766. https://doi.org/10.1016/J.FOODHYD.2021.106766

Boral, S., & B. Bohidar, H. (2010). Effect of Ionic Strength on Surface-Selective Patch Binding-Induced Phase Separation and Coacervation in Similarly Charged Gelatin−Agar Molecular Systems. *The Journal of Physical Chemistry B*, *114*(37), 12027–12035. doi: 10.1021/jp105431t.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., … Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols, 14*(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Carter, B. G., Cheng, N., Kapoor, R., Meletharayil, G. H., & Drake, M. A. (2021). Invited review: Microfiltration-derived casein and whey proteins from milk. *J. Dairy Sci., 104*(3), 2465–2479. https://doi.org/10.3168/JDS.2020-18811

Cebrián-Lloret, V., Martínez-Abad, A., Recio, I., López-Rubio, A., & Martínez-Sanz, M. (2024). In vitro digestibility of proteins from red seaweeds: Impact of cell wall structure and processing methods. *Food Res. Int., 178*, Article 113990. https://doi.org/10.1016/J.FOODRES.2024.113990

Chiang, J. Y. L. (2013). Bile Acid Metabolism and Signaling. *Compr Physiol., 3*(3), 1191–1212. https://doi.org/10.1002/cphy.c120023

Clarysse, S., Tack, J., Lammert, F., Duchateau, G., Reppas, C., & Augustijns, P. (2009). Postprandial evolution in composition and characteristics of human duodenal fluids in different nutritional states. *J. Pharm. Sci., 98*(3), 1177–1192. https://doi.org/10.1002/JPS.21502

Cano-Sarmiento, C., Téllez-Medina, D. I., Viveros-Contreras, R., Cornejo-Mazón, M., Figueroa-Hernández, C. Y., García-Armenta, E., Alamilla-Beltrán, L., García, H. S., & Gutiérrez-López, G. F. (2018). Zeta Potential of Food Matrices. In *Food Engineering Reviews* (Vol. 10, Issue 3, pp. 113–138). Springer New York LLC. doi: 10.1007/s12393-018-9176-z.

Costa, F. F., Vasconcelos Paiva Brito, M. A., Moreira Furtado, M. A., Martins, M. F., Leal De Oliveira, M. A., Mendonça De Castro Barra, P., Amigo Garrido, L., & De Oliveira Dos Santos, A. S. (2014). Microfluidic chip electrophoresis investigation of major milk proteins: Study of buffer effects and quantitative approaching. *Analytical Methods*, *6*(6), 1666–1673. doi: 10.1039/c3ay41706a.

Cremers, C. M., Knoefler, D., Vitvitsky, V., Banerjee, R., & Jakob, U. (2014). Bile salts act as effective protein-unfolding agents and instigators of disulfide stress in vivo. In *Proceedings of the National Academy of Sciences of the United States of America* (p. 111 (16).). https://doi.org/10.1073/pnas.1401941111

Cummings, J. H., & Englyst, H. N. (1995). Gastrointestinal effects of food carbohydrate. *Am. J. Clin. Nutr., 61*(4), 938S–S945. https://doi.org/10.1093/AJCN/61.4.938S

Dalgleish, D. G. (2011). On the structural models of bovine casein micelles—review and possible improvements. *Soft Matter, 7*(6), 2265–2272. https://doi.org/10.1039/C0SM00806K

Dulko, D., Staroń, R., Krupa, L., Rigby, N. M., Mackie, A. R., Gutkowski, K., Wasik, A., & Macierzanka, A. (2021). The bile salt content of human bile impacts on simulated

intestinal proteolysis of β-lactoglobulin. *Food Res. Int., 145*. https://doi.org/10.1016/j.foodres.2021.110413

Egger, L., Ménard, O., Delgado-Andrade, C., Alvito, P., Assunção, R., Balance, S., Barberá, R., Brodkorb, A., Cattenoz, T., Clemente, A., Comi, I., Dupont, D., Garcia-Llatas, G., Lagarda, M. J., Le Feunteun, S., JanssenDuijghuijsen, L., Karakaya, S., Lesmes, U., Mackie, A. R., & Portmann, R. (2016). The harmonized INFOGEST in vitro digestion method: From knowledge to action. *Food Res. Int., 88*, 217–225. https://doi.org/10.1016/J.FOODRES.2015.12.006

Ferey-Roux, G., Perrier, J., Forest, E., Marchis-Mouren, G., Puigserver, A., & Santimone, M. (1998). The human pancreatic α-amylase isoforms: Isolation, structural studies and kinetics of inhibition by acarbose. *Biochim. Biophys. Acta Protein Struct. Mol. Enzymol., 1388*(1), 10–20. https://doi.org/10.1016/S0167-4838(98)00147-2

Fisher, S. Z., Govindasamy, L., Tu, C., Agbandje-McKenna, M., Silverman, D. N., Rajaniemi, H. J., & McKenna, R. (2006). Structure of human salivary α-amylase crystallized in a C-centered monoclinic space group. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun., 62*(2), 88–93. https://doi.org/10.1107/S1744309105042491

Fontes-Candia, C., Díaz-Piñero, L., Carlos Martínez, J., Gómez-Mascaraque, L. G., López-Rubio, A., & Martínez-Sanz, M. (2023). Nanostructural changes in Polysaccharide-Casein Gel-Like structures upon in vitro gastrointestinal digestion. *Food Res. Int., 169*, Article 112862. https://doi.org/10.1016/J.FOODRES.2023.112862

Fontes-Candia, C., Jiménez-Barrios, P., Miralles, B., Recio, I., López-Rubio, A., & Martínez-Sanz, M. (2022). Development of polysaccharide-casein gel-like structures resistant to in vitro gastric digestion. *Food Hydrocoll., 127*. https://doi.org/10.1016/j.foodhyd.2022.107505

Fontes-Candia, C., Martínez, J. C., López-Rubio, A., Salvia-Trujillo, L., Martín-Belloso, O., & Martínez-Sanz, M. (2022). Emulsion gels and oil-filled aerogels as curcumin carriers: Nanostructural characterization of gastrointestinal digestion products. *Food Chemistry, 387*. https://doi.org/10.1016/j.foodchem.2022.132877

Gass, J., Vora, H., Hofmann, A. F., Gray, G. M., & Khosla, C. (2007). Enhancement of Dietary Protein Digestion by Conjugated Bile Acids. *Gastroenterology, 133*(1), 16–23. https://doi.org/10.1053/j.gastro.2007.04.008

Gribble, F. M., & Reimann, F. (2019). Function and mechanisms of enteroendocrine cells and gut hormones in metabolism. In *Nature Reviews Endocrinology* (Vol. 15, Issue 4, pp. 226–237). Nature Publishing Group. doi: 10.1038/s41574-019-0168-8.

Guerrero, P., Etxabide, A., Leceta, I., Peñalba, M., & De La Caba, K. (2014). Extraction of agar from Gelidium sesquipedale (Rodhopyta) and surface characterization of agar based films. *Carbohydr. Polym., 99*, 491–498. https://doi.org/10.1016/J.CARBPOL.2013.08.049

Gunness, P., & Gidley, M. J. (2010). Mechanisms underlying the cholesterol-lowering properties of soluble dietary fibre polysaccharides. *Food Funct., 1*(2), 149–155. https://doi.org/10.1039/C0FO00080A

Ilavsky, J., & Jemian, P. R. (2009). Irena : Tool suite for modeling and analysis of small-angle scattering. *J. Appl. Cryst., 42*(2), 347–353. https://doi.org/10.1107/S0021889809002222

Jarunglumlert, T., Nakagawa, K., & Adachi, S. (2015). Influence of aggregate structure of casein on the encapsulation efficiency of β-carotene entrapped via hydrophobic interaction. *Food Struct., 5*, 42–50. https://doi.org/10.1016/J.FOOSTR.2015.05.001

Jin, Y., Yu, Y., Qi, Y., Wang, F., Yan, J., & Zou, H. (2016). Peptide profiling and the bioactivity character of yogurt in the simulated gastrointestinal digestion. *J. Proteomics, 141*, 24–46. https://doi.org/10.1016/J.JPROT.2016.04.010

Kieffer, J., & Wright, J. P. (2013). PyFAI: A Python library for high performance azimuthal integration on GPU. *Powder Diffraction, 28*(S2), S339–S350. https://doi.org/10.1017/S0885715613000924

Kopf-Bolanz, K. A., Schwander, F., Gijs, M., Vergéres, G., Portmann, R., & Egger, L. (2012). Validation of an In Vitro Digestive System for Studying Macronutrient Decomposition in Humans3. *J. Nutr., 142*(2), 245–250. https://doi.org/10.3945/JN.111.148635

Kuchlyan, J., Roy, A., Dutta, R., Sen, S., & Sarkar, N. (2016). Effect of the submicellar concentration of bile salts on structural alterations of β-casein micelles. *RSC Adv., 6*(76), 71989–71998. https://doi.org/10.1039/C6RA14804B

Li, S., Hu, Q., Chen, C., Liu, J., He, G., Li, L., Wu, J., & Ren, D. (2020). Formation of bioactive peptides during simulated gastrointestinal digestion is affected by αs1-casein polymorphism in buffalo milk. *Food Chem., 313*, Article 126159. https://doi.org/10.1016/J.FOODCHEM.2020.126159

Li, X., Su, Y., Liu, S., Tan, L., Mo, X., & Ramakrishna, S. (2010). Encapsulation of proteins in poly(l-lactide-co-caprolactone) fibers by emulsion electrospinning. *Colloids Surf. B Biointerfaces, 75*(2), 418–424. https://doi.org/10.1016/J.COLSURFB.2009.09.014

López-Fandiño, R., Martos, G., Contreras, P., & Molina, E. (2010). Egg white ovalbumin digestion mimicking physiological conditions. *J. Agric. Food Chem., 58*(9), 5640–5648. https://doi.org/10.1021/jf904538w

Lovegrove, A., Edwards, C. H., De Noni, I., Patel, H., El, S. N., Grassby, T., Zielke, C., Ulmius, M., Nilsson, L., Butterworth, P. J., Ellis, P. R., & Shewry, P. R. (2017). Role of polysaccharides in food, digestion, and health. *Crit. Rev. Food Sci. Nutr., 57*(2), 237–253. https://doi.org/10.1080/10408398.2014.939263

MacIerzanka, A., Sancho, A. I., Mills, E. N. C., Rigby, N. M., & MacKie, A. R. (2009). Emulsification alters simulated gastrointestinal proteolysis of β-casein and β-lactoglobulin. *Soft Matter, 5*(3), 538–550. https://doi.org/10.1039/b811233a

Mahmoud, M. I., Malone, W. T., & Cordle, C. T. (1992). *Enzymatic Hydrolysis of Casein: Effect of Degree of Hydrolysis on Antigenicity and Physical Properties*.

Maldonado-Valderrama, J., Wilde, P., MacIerzanka, A., & MacKie, A. (2011). The role of bile salts in digestion. *Adv. Colloid Interface Sci., 165*(1), 36–46. https://doi.org/10.1016/j.cis.2010.12.002

McClements, D. J. (2017). Recent progress in hydrogel delivery systems for improving nutraceutical bioavailability. *Food Hydrocoll., 68*, 238–245. https://doi.org/10.1016/J.FOODHYD.2016.05.037

Ménard, O., Chauvet, L., Henry, G., Dupont, D., Gaudichon, C., Calvez, J., & Deglaire, A. (2023). The use of 15N-labelled protein to account for the endogenous nitrogen contribution to in vitro protein digestibility measurement. *Food Res. Int., 173*, Article 113242. https://doi.org/10.1016/J.FOODRES.2023.113242

Miralles, B., Sanchón, J., Sánchez-Rivera, L., Martínez-Maqueda, D., Le Gouar, Y., Dupont, D., Amigo, L., & Recio, I. (2021). Digestion of micellar casein in duodenum cannulated pigs. Correlation between in vitro simulated gastric digestion and in vivo data. *Food Chem., 343*, Article 128424. https://doi.org/10.1016/j.foodchem.2020.128424

Miyagishi, S., Okada, K., & Asakawa, T. (2001). Salt Effect on Critical Micelle Concentrations of Nonionic Surfactants, N-Acyl-N-methylglucamides (MEGA-n). *J. Colloid Interface Sci., 238*(1), 91–95. https://doi.org/10.1006/JCIS.2001.7503

Moghimipour, E., Ameri, A., & Handali, S. (2015). Absorption-Enhancing Effects of Bile Salts. *Molecules, 20*(8), 14451–14473. https://doi.org/10.3390/molecules200814451

Nicolai, T., Britten, M., & Schmitt, C. (2011). β-Lactoglobulin and WPI aggregates: Formation, structure and applications. *Food Hydrocoll., 25*(8), 1945–1962. https://doi.org/10.1016/J.FOODHYD.2011.02.006

Qazi, M. J., Schlegel, S. J., Backus, E. H. G., Bonn, M., Bonn, D., & Shahidzadeh, N. (2020). Dynamic Surface Tension of Surfactants in the Presence of High Salt Concentrations. *Langmuir, 36*(27), 7956–7964. https://doi.org/10.1021/acs.langmuir.0c01211

Riethorst, D., Mols, R., Duchateau, G., Tack, J., Brouwers, J., & Augustijns, P. (2016). Characterization of Human Duodenal Fluids in Fasted and Fed State Conditions. *J. Pharm. Sci., 105*(2), 673–681. https://doi.org/10.1002/JPS.24603

Sanchón, J., Fernández-Tomé, S., Miralles, B., Hernández-Ledesma, B., Tomé, D., Gaudichon, C., & Recio, I. (2018). Protein degradation and peptide release from milk proteins in human jejunum. Comparison with in vitro gastrointestinal simulation. *Food Chem., 239*, 486–494. https://doi.org/10.1016/J.FOODCHEM.2017.06.134

Santos-Hernández, M., Amigo, L., & Recio, I. (2020). Induction of CCK and GLP-1 release in enteroendocrine cells by egg white peptides generated during gastrointestinal digestion. *Food Chem., 329*, Article 127188. https://doi.org/10.1016/J.FOODCHEM.2020.127188

Selvasekaran, P., & Chidambaram, R. (2021). Food-grade aerogels obtained from polysaccharides, proteins, and seed mucilages: Role as a carrier matrix of functional food ingredients. *Trends in Food Science & Technology, 112*, 455–470. https://doi.org/10.1016/J.TIFS.2021.04.021

Stanforth, K. J., Wilcox, M. D., Chater, P. I., Brownlee, I. A., Zakhour, M. I., Banecki, K. M. R. M., & Pearson, J. P. (2022). Pepsin properties, structure, and its accurate measurement: A narrative review. *Annals of Esophagus, 5*. https://doi.org/10.21037/aoe-20-95

Sudha, P. N., Aisverya, S., Nithya, R., & Vijayalakshmi, K. (2014). Industrial Applications of Marine Carbohydrates. *Adv. Food Nutr. Res., 73*, 145–181. https://doi.org/10.1016/B978-0-12-800268-1.00008-1

Wade, T., Beattie, J. K., Rowlands, W. N., & Augustin, M. A. (1996). Electroacoustic determination of size and zeta potential of casein micelles in skim milk. *Journal of Dairy Research, 63*(3), 387–404. https://doi.org/10.1017/s0022029900031915

Wang, X., Ye, A., Lin, Q., Han, J., & Singh, H. (2018). Gastric digestion of milk protein ingredients: Study using an in vitro dynamic model. *J. Dairy Sci., 101*(8), 6842–6852. https://doi.org/10.3168/JDS.2017-14284

Wickham, M., Garrood, M., Leney, J., Wilson, P. D. G., & Fillery-Travis, A. (1998). Modification of a phospholipid stabilized emulsion interface by bile salt: Effect on pancreatic lipase activity. *J. Lipid Res., 39*(3), 623–632. https://doi.org/10.1016/s0022-2275(20)33300-9

Zhang, Z., Zhang, R., & McClements, D. J. (2017). Control of protein digestion under simulated gastrointestinal conditions using biopolymer microgels. *Food Res. Int., 100*, 86–94. https://doi.org/10.1016/J.FOODRES.2017.08.037