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Running title: Proteins and pure carotenoid bioaccessibility

ABSTRACT

Proteins could alter carotenoid bioaccessibility through altering their fate during digestion, due to emulsifying properties of resulting peptides, or influencing access of digestion enzymes to lipid droplets. In this investigation, we studied whether whey protein isolate (WPI), soy protein isolate (SPI), sodium caseinate (SC) and gelatin (GEL), added at various concentrations (expressed as percentage of recommended dietary allowance (RDA): 0, 10, 25 and 50%) would influence the bioaccessibility of lycopene, β-carotene or lutein, added as pure carotenoids solubilized in oil, during simulated gastro-intestinal (GI) digestion. Protein and lipid digestion as well as selected physico-chemical parameters including surface tension, ζ-potential and micelle size were evaluated. Adding proteins influenced positively the bioaccessibility of β-carotene, by up to 189% ($p<0.001$), but it resulted in generally decreased bioaccessibility of lutein, by up to 50% ($p<0.001$), while for lycopene, the presence of proteins did not influence its bioaccessibility, except for a slight increase with WPI, by up to 135% ($p<0.001$). However, the effect depended significantly on the type of protein ($p<0.001$) and its concentration ($p<0.001$). While β-carotene bioaccessibility was greatly enhanced in the presence of SC, compared to WPI and GEL, the presence of SPI strongly decreased carotenoid bioaccessibility. Neglecting individual carotenoids, higher protein concentration correlated positively with carotenoid bioaccessibility ($R=0.57$, $p<0.01$), smaller micelle size ($R=-0.83$, $p<0.01$), decreased repulsive forces (ζ-potential, $R=-0.72$, $p<0.01$), and higher surface tension ($R=0.44$, $p<0.01$). In conclusion, proteins differentially affected carotenoid bioaccessibility during digestion depending on carotenoid and protein species, with both positive and negative interactions occurring.

Key-words: Interfacial properties, physicochemical characteristics; mixed micelles; oil-in-water emulsion; in vitro digestion; emulsifiers; protein intake.

INTRODUCTION

Carotenoids are naturally occurring tetraterpenoid pigments synthesized by plants, algae, and several bacteria and fungi. Despite the fact that humans cannot produce them, and that these secondary plant compounds are not essential for humans, they have always been part of the food chain, as carotenoid-rich food sources include a large variety of fruits and vegetables, as well as certain animal-derived food items such as salmon and eggs [1]. Furthermore, several carotenoids are included in fortified foods or are available as supplements.

Despite their non-essentiality, these phytochemicals contribute to important biochemical and physiological functions in the human body [2]. Some carotenoids are provitamin A compounds, such as β-carotene, α-carotene and β-cryptoxanthin, and in many countries with low animal derived food intake and for vegetarians, carotenoids are the main source of dietary vitamin A. Furthermore, many of these pigments have been associated in epidemiological studies with reduced incidence of chronic diseases, such as lowered risk of coronary heart disease [3], certain types of cancer [4], type 2 diabetes [5], and eye-health related diseases [6, 7]. Though the exact mechanisms are often unclear, this is attributed to, at least in part, their influence on the cellular level, acting on transcription factors and nuclear factors [8], which may also contribute to their anti-inflammatory and antioxidant activities [9-11].

Given these potential health benefits for humans, the bioavailability of carotenoids, i.e. the fraction that is absorbed and is available for physiological function or storage, has been high on the agenda of carotenoid-oriented research. In fact, following their dietary intake, carotenoids are liberated from food matrices, solubilized in lipid droplets[12] and are finally incorporated into mixed micelles. The micellization of carotenoids is crucial for their uptake by enterocytes, which is a prerequisite for their intestinal absorption and further biodistribution [13].

However, in part due to their poor water-solubility, the bioavailability of carotenoids is low and variable [14, 15], and many dietary as well as host-related factors are known to interfere with their

absorption [13, 16]. Regarding dietary factors, it has been acknowledged that lipids can enhance carotenoid absorption efficiency, by improving their micellization and fostering chylomicron sequestration [17, 18]. In contrast, dietary fibers negatively affect their bioavailability, likely by hampering the transition of lipid droplets into mixed micelles, also affecting the activity of digestion enzymes [19, 20].

One factor that has not yet received much attention is the influence of co-digested proteins on carotenoid solubility and/or absorption. Indeed, several proteins have exhibited emulsifying properties during digestion, being highly surface-active, stabilizing emulsions by forming a viscoelastic phase, and adsorb to the oil droplets, forming a physical barrier[12] and preventing coalescence [21]. Thus, depending on digestive conditions, proteins, via their interaction with lipid droplets during the digestion process, may influence carotenoid bioaccessibility by modulating their transfer from lipid droplets into mixed micelles [12]. Such interactions may be positive due to the stabilization of emulsions or negative, due to hampered enzymatic access to lipid droplets [22].

For instance, it has been reported that sodium caseinates (SC) stabilized oil-in-water (o/w) emulsions and facilitated β-carotene uptake [23, 24]. In another study employing milk-protein stabilized fat droplets, these droplets remained small throughout the digestion process, suggesting that they were stable against coalescence, facilitating the formation of smaller lipid droplets [25], likely enhancing carotenoid bioaccessibility as demonstrated earlier [26]. In our previous study, the addition of whey protein isolate (WPI) during simulated GI digestion influenced the bioaccessibility of β-carotene positively as well as negatively, depending on the digestion conditions [22]. While positive effects appeared to dominate under more complete GI conditions, additional WPI reduced β-carotene bioaccessibility at lower enzymatic activity of pancreatin, lower concentration of bile and reduced peristalsis. However, most studies have focused only on the very apolar β-carotene and especially on WPI.

Thus, in the current study, we aimed to investigate the effect of several frequently consumed proteins of various hydrophobicity on the bioaccessibility of commonly ingested carotenoids. By means of a European consensus model for static *in vitro* digestion [27, 28], we focused on the influence of WPI, soy protein isolate, sodium caseinate and gelatin on the bioaccessibility of individual carotenoids, i.e. β-carotene, lutein and lycopene. In order to provide additional insights about the interaction of proteins and carotenoids during digestion, we also investigated lipid and protein digestion efficiency, as well as physico-chemical properties of the digesta, including surface tension, viscosity, and micelle size. Insights obtained from such experiments can be valuable for fortifying/enriching food items with these health-associated pigments and to determine the optimal food matrix to support carotenoid bioavailability.

EXPERIMENTAL

Chemicals and carotenoid standards

Standards of *all-trans*-β-carotene (≥97% purity) and *all-trans*-lycopene (≥85%) were purchased from Sigma-Aldrich (Overijse, Belgium), while lutein (≥95%) was obtained from Extrasynthese (Genay, France). Pepsin from porcine gastric mucosa (≥250 U/mg, Art. No. P7000), pancreatin from porcine pancreas (activity equivalent to 4x USP specifications, Art. No. P1750) and porcine bile extract (Art. No. B8631) were obtained from Sigma-Aldrich (Overijse, Belgium).

Unless otherwise specified, all chemicals were of analytical grade or superior. Potassium chloride (≥99%), potassium dihydrogen phosphate (≥99%), sodium hydrogen carbonate (≥99%), sodium chloride (≥99.5%), magnesium chloride hexahydrate, ammonium carbonate, sodium hydroxide solution (1 M), calcium chloride dihydrate (≥99%), Nile red, fluorescein isothiocyanate isomer I (FITC), and phenolphthalein were acquired from Sigma-Aldrich. Hexane (≥95%), acetone (≥99%), and hydrochloric acid (1 M) were obtained from VWR (Leuven, Belgium).

Pure proteins and dietary lipid sources

Whey protein isolate (WPI) was acquired from Pure Nutrition USA® (95% purity, California, USA), and soy protein isolate (SPI) was obtained from Self Omninutrition® (≥90% purity, Stockholm, Sweden), whereas casein sodium salt (SC) from bovine milk (Art. No. C8654) and gelatin (GEL) from bovine skin (Art. No. G9391) were both purchased from Sigma-Aldrich.

Peanut oil, typically free of native carotenoids [29] and own blank examinations, was purchased from a local supermarket (Delhaize, Strassen, Luxembourg).

Solubilization of carotenoid standards & preparation of protein and enzyme solutions

All solutions were prepared as previously described [22]. Briefly, carotenoid standard solutions were produced at a final concentration of 0.2 mg/mL. For this, β-carotene, lycopene and lutein were first dissolved in hexane. Peanut oil was added to foster the dissolution of pure carotenoids. Then, the solutions were sonicated at 50/60 Hz for 5 min. (Ultrasonic Cleaner, VWR Symphony®, Massachusetts, USA) and warmed up at 30 °C until complete dissolution. The hexane was finally removed by evaporation (TurboVap LV from Biotage®, Uppsala, Sweden) and the carotenoid standard solutions were made by the further addition of peanut oil to reach the targeted concentration of 0.2 mg/mL.

Regarding the protein solutions, the amounts of proteins tested were 0, 3, 7.5, 15 g/L, corresponding to 0, 10, 25 and 50% of the recommended dietary allowance (RDA) within 1 test meal, being 0.83 g per kg of body weight per day for adults (≈ 60g/d) [30], by dissolving/emulsifying each protein type in pure water.

The simulated gastric fluid (SGF), the simulated intestinal fluid (SIF), as well as the enzyme solutions were prepared as recommended earlier [27]. Pepsin solution was prepared in SGF at a concentration of 2000 U/mL of the final gastric mixture, pancreatin and bile extract were prepared in the same SIF solution, at a concentration of 200 U/mL and 6.8 mg/mL, respectively. Based on

150 our previous study [22], pancreatin amount was doubled (200 U/mL based on trypsin activity) in order
151 to enhance protein digestion.
152
153 **Simulation of gastro-intestinal digestion and extraction of carotenoids**
154 *In vitro* simulated GI digestion was carried out according to the harmonized INFOGEST
155 protocol, with slight modifications for pancreatin concentrations [22]. The model was used to test the
156 influence of proteins at four different concentrations on the bioaccessibility of pure carotenoids.
157 The oral phase was omitted as the matrix employed did not include significant amounts of
158 carbohydrates and was liquid.
159 **Gastric phase:** Each sample comprised a total of 6.5 mL of gastric phase solution containing the
160 desired amounts of proteins, i.e. 0, 78, 195 or 390 mg (equivalent to 0, 10, 25 or 50% of the RDA),
161 also including 150 µL of oil containing carotenoid standards (approx. 30 µg of carotenoid standard
162 per digestion), similar to former *in vitro* experiments [31]. The oil containing carotenoids was mixed
163 with the gastric phase by brief sonication for 5 min at 50 Hz to obtain oil-in-water emulsions. Prior
164 to the addition of SGF (1.25 x concentrate), 2000 U/mL of pepsin was added to the sample. Then,
165 0.075 mM of calcium dichloride was added in the final mixture, and pH was adjusted to 3 by
166 hydrochloric acid (1 M), bringing the volume of each sample to 13 mL with pure water in order to
167 reach a final ratio of matrix (protein + carotenoid solutions) to simulated gastric fluid of 50:50
168 (v/v). Samples were then incubated in a shaking water bath (GFL 1083 from VEL®, Leuven,
169 Belgium) for 2 h at 37 °C, with a shaking speed of 100 rounds per minute (rpm).
170 **Intestinal phase:** At the end of the gastric incubation, SIF, pancreatin (200 U/mL) and bile
171 extract (6.8 mg/mL) were added to the chyme. Then, calcium dichloride was added at a
172 concentration of 0.3 mM in the final mixture. The ratio of gastric chyme to SIF of 50:50 (v/v) was
173 obtained by filling up the sample to 26 mL with pure water, the pH was adjusted to 7 by the

addition of sodium hydroxide solution (1 M), then the samples were incubated for 2 h at 37 °C, maintaining a shaking speed of 100 rpm.

Analyses of final products of digestion

Extraction and analysis of carotenoids of the bioaccessible fraction: Carotenoid extraction and analyses were carried out as described previously [22]. Briefly, at the end of the intestinal incubation, the intestinal digestion was stopped by immediately transferring the digests on ice. 12 mL of the digesta were centrifuged for 1 h at 3200×g (4 °C), and then 5 mL were collected from the middle aqueous phase and filtered through 0.2 μm nylon membrane syringe filters (Corning Incorporated® Life Sciences, Tewksbury, Massachusetts, USA). The extraction started by adding 6 mL of hexane:acetone (2:1, v:v) to 2 mL of the filtered aqueous phase. After brief centrifugation, the supernatant hexane phase was collected, and the extraction process was repeated two times with pure hexane. All extracts were combined in the same tube, dried under a stream of nitrogen, and stored under argon at -80 ^{o}C until the spectrophotometric analyses.

The absorbance spectrum was measured between 300 and 600 nm (GENESYS^{TM} 10S UV-Vis Spectrophotometer, Thermo Fisher Scientific, Massachusetts, USA). Carotenoid concentration was calculated by applying the Beer-Lambert law. The percentage of carotenoid micellization was used as a measure of bioaccessibility, and was expressed as the percentage of amount of carotenoids present in the micellar phase of the filtered digesta after *in vitro* GI digestion, compared to the initial amount added to the sample.

SDS-PAGE electrophoresis: Sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) was carried out as previously described [22] to study the extent of protein digestion. Samples were denatured at 95^{o}C for 5 min. and were then loaded to a 15% acrylamide and 0.4% bisacrylamide gel for the samples obtained after complete GI digestion, or to a 10% acrylamide/0.3% bisacrylamide gel for the digesta obtained following only gastric digestion. The

running time was 30 min. at 80 V followed by 90 min. at 100 V. Protein marker was Invitrogen novex see blue plus 2 (Invitrogen, Carlsbad, CA). After each run, fixation/staining was done with methanol/acetic acid/Coomassie brilliant blue R (50%, 10%, 0.1%) for 30 minutes, and destained in methanol/glacial acetic acid/water (30%, 10%, 60%, 3 h). The gels were scanned by a Kodak Gel Logic 2200 imaging system (Kodak, Rochester, NY).

Free fatty acid determination: Lipid hydrolysis was evaluated by measuring the amount of free fatty acids (FFAs) released in the digesta after GI digestion. This was determined by Cayman's Free Fatty Acid Fluorometric Assay (Cayman Chemical, Art. No. 700310, Ann Arbor, MI) according to the manufacturer's protocol.

Macroviscosity and surface tension analysis

Flow curves were determined as previously described [22], using an Anton-Paar rheometer (MCR 302, WESP, Graz, Austria), equipped with a double gap cell at 5°C. A regression flow curve was calculated for the shear rate range between 0 s^{-1} and 100 s^{-1} for the shear stress respectively between 1 s^{-1} and 100 s^{-1} for the viscosity. The regression curves were plotted as viscosity and shear stress as a function of the shear rate.

Surface tension of digesta samples, pre-conditioned at 25 ± 0.1 °C, were determined by the weight-drop method as previously described [32]. The air-water interfacial properties of the digesta were calculated as follows:

$$\sigma_{digesta} = \frac{m_{digesta}}{m_{H2O}} \text{ x } \sigma H_2O$$

where σ_{H2O}= 71.99 dyn cm^{-1} is the surface tension of pure water.

Confocal laser scanning microscopy

Confocal imaging of emulsion structures after GI digestion was carried out at room temperature with a confocal laser scanning microscope (Zeiss LSM 880, Airyscam SR, Jena, Germany), using a

63x objective. The aliquots were dyed with Nile red dissolved in ethanol, and FITC dissolved in acetone. Both were used for fat and protein staining, respectively, at a concentration of 1 µg/mL. An Argon 488 nm laser excited the fluorescent dyes. The emitted light was collected at 500-540 nm for proteins and 590-650 nm for the fat phase. The resulting images were processed and de-noised by using a modular image-processing and analysis software for digital microscopy (Zen 2.3 blue edition, Carl Zeiss Microscopy GmbH, Jena, Germany).

Micelle size and ζ-potential analysis

Aliquots of the filtered aqueous micellar fraction were used for the analysis of the micelle size and ζ-potential, and the measurements were done at room temperature with at least four replicates. The intensity-weighted mean hydrodynamic radius and ζ-potential were determined by dynamic light scattering and laser Doppler micro-electrophoresis, respectively, by using a Zetasizer Nano Zs instrument (Malvern Instruments, Malvern, UK).

Statistical analyses and data treatment

In order to minimize day-to-day variations between experiments, bioaccessibility of pure carotenoids was normalized to a daily control which was assessed for each digestion. Unless otherwise stated, all values are expressed as the mean ± standard deviation. Replicates were obtained from at least 2 individual sets of analyses obtained at different days ($N \geq 2$), and at least 4 replicates ($n > 4$) were obtained for each digestive condition during 1 set of analyses.

Statistical analysis was performed using SPSS 22 software (SPSS Inc., Chicago, IL). Normal distribution of data was checked by Q-Q -plots and equality of variance by box-plots and Levene's test. For statistical evaluation, carotenoid bioaccessibility values were log-transformed to achieve a normal distribution. Linear mixed models were developed with the effect of type and concentration of proteins (quantitative variable) and carotenoid species as fixed independent factors and log-

carotenoid bioaccessibility as the observed dependent factor. When significant interactions were obtained, additional linear mixed models were run to keep certain of these parameters constant and to better allow for all further group-wise comparisons. P-values < 0.05 were considered statistically significant different (2-sided). Where needed, ANOVA Fisher F-tests were followed by post hoc tests (Tukey's test, for comparing >3 groups) or LSD tests (for comparison of ≤ 3 groups). For correlation analyses, Pearson correlation coefficients were calculated between major outcomes.

RESULTS

General influence of proteins on the bioaccessibility of carotenoids

Bioaccessibility differed significantly between carotenoid species. In fact, under control conditions (no protein added), the bioaccessibility of lutein was significantly higher (81.3% ±1.2) compared to that of β-carotene (26.4% ±0.7) ($p>0.001$), while the lowest bioaccessibility ($p<0.001$) was obtained for lycopene (20.2% ±0.9).

The addition of various proteins to the simulated GI digestion impacted the bioaccessibility differently. All carotenoids and all protein concentrations applied considered, i.e. evaluated together statistically (without applying post-hoc tests for group-wise comparisons), the addition of SC resulted overall in slightly higher bioaccessibility (43.7%) compared to WPI and GEL (41.9% and 41.7%, respectively) ($p<0.001$), while the lowest bioaccessibility was clearly engendered by SPI (35.1%) ($p<0.001$).

Influence of individual proteins on the bioaccessibility of carotenoids

Whey protein isolate: Following simulated GI digestion, the overall bioaccessibility of lycopene and β-carotene was positively influenced by the presence of WPI (all concentrations considered), showing an average increase to 24.1% ($p<0.001$) and 30.3% ($p<0.001$), respectively, compared to the respective controls (see average control values above), while the bioaccessibility of lutein dropped to 71.5% ($p<0.001$). More specifically, the addition of WPI at 10 and 25% RDA decreased

the bioaccessibility of lutein to 63.3% ±0.5 and 61.2% ±1.1, respectively ($p<0.001$, Fig. 1A), while the same concentrations enhanced the bioaccessibility of lycopene up to 27.4% ±1.1 and 25.0% ±1.2 ($p<0.001$ and $p<0.05$, respectively). β-Carotene bioaccessibility was only enhanced by 50% RDA of WPI, to 39.0% ± 1.7 ($p<0.001$), while the other concentrations had no significant effect compared to the control.

Soy protein isolate: The addition of SPI (all concentrations considered) to the simulated GI digestion enhanced the bioaccessibility of β-carotene (to 31.3%, $p<0.001$), while it decreased the bioaccessibility of lycopene (to 15.8%, $p<0.001$) and lutein (to 58.2%, $p<0.001$) (Fig. 1B). In detail, at 10 and 25% RDA of SPI, the bioaccessibility of β-carotene increased to 36.9% ±2.5 and 34.6% ±1.6, respectively ($p<0.05$). Conversely, the addition of SPI drastically decreased the bioaccessibility of lutein in a dose-dependent manner, to 41.0% ±1.2 ($p<0.001$). Lycopene was less drastically influenced, with a reduced bioaccessibility to 14.3% ±0.8 at 50% RDA ($p<0.001$).

Sodium caseinate: No significant effect engendered by the presence of SC (all concentrations considered) regarding the bioaccessibility of lycopene (Fig. 1C), while an average increase up to 39.8% of β-carotene bioaccessibility was observed ($p<0.001$). As with other proteins, the addition of SC decreased the bioaccessibility of lutein to an average of 70.2% ($p<0.001$). More specifically, at high protein concentration (50% RDA), SC reduced lutein bioaccessibility to 62.9% ±1.2 ($p<0.001$). For β-carotene, a drastic increase in bioaccessibility to 49.8% ±0.9 was observed ($p<0.001$).

Gelatin: The co-digestion of pure carotenoids with GEL resulted in very similar effects regarding the bioaccessibility as observed with SC. Briefly, when considering all protein concentrations, the presence of GEL in the digested matrix (all concentrations applied) enhanced the bioaccessibility of β-carotene to an average of 32% ($p<0.001$). However, the bioaccessibility of lutein was brought down to an average of 72.3% ($p<0.001$). Again, there was no significant change regarding the bioaccessibility of lycopene compared to the control (Fig. 1D). The negative influence of GEL

299 regarding the bioaccessibility of lutein appeared to be dose-dependent, reaching a minimum
300 bioaccessibility of 63.4% ±1.2, while the bioaccessibility of β-carotene was improved in turn in a
301 dose-dependent manner, with an increase to 36.6% ±2.4 of bioaccessibility ($p<0.001$).
302
303 **Protein and lipid analysis**
304 The images of the gels revealed that all bands disappeared after complete GI digestion (Fig. 2),
305 except for SPI, where some low-size polypeptide fragments remained. By way of comparison, the
306 bands observed after the gastric phase were of variable size, but always corresponded to lower
307 molecular weights compared to their native proteins. Nevertheless, more marked bands were
308 observed in case of WPI (Fig.2 A), corresponding mostly to β-lactoglobulin according to the native
309 undigested WPI. Comparatively, SPI was found less digestible (Fig. 2B), while SC (Fig. 2C) and
310 GEL (Fig. 2D) were apparently more readily digested. We observed that there were no protein
311 bands present under control condition (0% RDA), only those corresponding to the enzymes.
312 Regarding lipid digestion (Fig. 3.), it is obvious that proteins influenced differentially the release
313 of FFAs. At 10% RDA of WPI and SC, the release of FFAs was found higher compared to the other
314 concentrations (Fig. 3A, $p<0.05$; Fig. 3C, $p<0.001$, respectively), i.e. the presence of these proteins
315 at higher concentrations negatively influenced lipid digestion, resulting in a significant negative
316 correlation ($R=-0.857$, -0.867, respectively, $p<0.001$). On the other hand, SPI had a positive
317 influence, in a dose-dependent manner ($R=0.952$, $p<0.001$, Fig. 3B), enhancing FFAs release more
318 than twice compared to the control ($p<0.001$). Conversely, the addition of GEL at different
319 concentrations did not show any significant influence on FFAs release (Fig. 3D).
320
321 **Physicochemical characteristics of the digesta**
322 Surface tension is an important indicator of the emulsifying capacity of proteins during digestion,
323 as they have been proposed to reduce the surface tension also in the GI tract [21]. Overall, the addition

of proteins to the simulated GI digestion influenced differentially the surface tension of the digesta, depending on the type of protein, even though absolute changes in surface tension remained rather small. The presence of SPI in the digesta (all concentrations considered) led to the highest surface tension (39.5 dyn/cm), followed by SC and GEL (38.7 dyn/cm), and finally WPI (38 dyn/cm). A significant difference was found between the three protein groups (SPI > SC=GEL > WPI, $p<0.001$) (OSM Fig. 1). Regarding the effect of protein concentration (individual or all proteins considered), we observed a significant increase on the surface tension at 50% RDA for all proteins (except for WPI), compared to the control ($p<0.001$).

Viscosity is a measure of the resistance of a fluid to deformation under shear stress [33]. A high viscosity can reduce the emulsification of dietary lipids [34]. As proteins could change the viscosity of the digesta, the transport of carotenoids within lipid droplets and their incorporation into mixed micelles could be affected. In the present study, the viscosity of digested and non-digested SC and WPI solutions did not change with the different concentrations (Fig. 4A). The same effect was observed in digested GEL solutions, which remained in the viscosity range of the protein-free solution. In contrary, the viscosity of undigested GEL solutions increased exponentially with its concentration. Such a viscosity response was expected due to the presence of a gel-like structure in the solution (Fig. 4A). The shear stress of samples digested without additional proteins (0% RDA) showed a very small yield point of approximatively 1 mPa (Fig. 4B). However, the digested samples containing WPI and SC showed a higher yield point of about 10 mPa, while digested samples with GEL had a higher yield point reaching 10 Pa, in line with the polymeric property of GEL at low temperature (Fig. 4B). Due to sedimentation and the fact that a double gap cell was used for this experiment, we preferred not to analyze the samples digested with SPI, as this would involve changing parameters that were used during digestion of the proteins (such as requiring additional heating, ultrasonication), in order to obtain a homogeneous solution.

Lipid digestibility may vary depending of the emulsion characteristics, such as interfacial composition and oil droplet size, influencing micelle formation and the associated carotenoid bioaccessibility [35]. Here, the presence of proteins in the simulated GI digestion resulted in different emulsion structures, though all proteins remarkably reduced the size of the emulsion (Fig. 5). Overall, at 10% RDA, SPI, SC and GEL showed a higher degree of aggregation compared to WPI, while the addition of these proteins at a concentration of 25% RDA resulted in a clear decrease of microstructure sizes, with a smaller emulsion in case of WPI. However, increasing the concentration to 50% RDA resulted in very homogeneous emulsions, for all protein types, compared to the other concentrations (Fig. 5). With all proteins, we observed a co-localization of the labelled compounds (proteins and lipids) present in the matrix, possibly indicating an interaction between these during the simulated GI digestion.

The produced mixed micelles following the simulated GI digestion were significantly affected by the presence of proteins ($p<0.001$). WPI and SPI had a stronger influence on the size of mixed micelles (all concentrations considered), compared to GEL and SC ($p<0.001$). In fact, the addition of WPI and SPI (OSM Fig. 2A and Fig. 2B, respectively) to the simulated digestion at a concentration of 25% RDA reduced significantly the size of mixed micelles, compared to the control ($p<0.001$), while no effect was observed following the addition of SC and GEL at this concentration. However, increasing the concentration to 50% RDA, all proteins, including GEL and SC, decreased significantly the size of the micelles, compared to the control ($p<0.001$).

ζ-potential, which may direclty affect the stability of emulsions, was also measured [36]. The ζ-potential determines the effective electric charge on the particles' surface, by measuring the degree of electrostatic repulsion between adjacent particles [37]. Interestingly, the average of the absolute ζ-potential (all proteins considered) was reduced in a dose-dependent manner (mean $R = 0.581$, $p<0.001$); the results were similar for the different proteins. At 10% RDA of proteins, a significant difference was seen only for WPI and SPI ($P<0.001$), while at concentrations of 25 and 50% RDA,

a significant difference was found in the presence of each individual protein, compared to the control ($p>0.001$) (Fig. 6).

DISCUSSION

In the present study, we investigated the influence of different proteins (WPI, SPI, SC and GEL) at various concentrations equivalent to 0, 10, 25 and 50% of the recommended dietary allowance (RDA), on the bioaccessibility of pure carotenoids (lycopene, β-carotene and lutein). The presence of proteins during the simulated GI digestion influenced the bioaccessibility of carotenoids, either positively or negatively. These effects depended on the type and concentration of protein, but also on the type of carotenoid.

Proteins are surface-active molecules present within the aqueous phase surrounding the lipid droplets during digestion [38]. Once adsorbed, they form a viscoelastic layer that stabilizes emulsions [39]. They have been proclaimed to aid in emulsifying liposoluble dietary constituents [23] and to enable the incorporation of non-polar components into emulsions during GI digestion [38]. However, it has been reported that high protein concentrations at the lipid surface could affect the stability of emulsions, negatively influencing lipid degradation, impacting the incorporation of lipophilic constituents into lipid droplets during GI digestion [12, 21, 40]. Indeed, in our previous study, we found that β-carotene bioaccessibility was influenced positively by WPI due to the stabilization of emulsions or negatively, due to hampered enzymatic access to lipid droplets [22].

In the present investigation, the addition of proteins enhanced the bioaccessibility of β-carotene by up to 50% compared to the protein-free control, while hampering lutein bioaccessibility. In case of lycopene, adding proteins to the simulated digestion did not influence its bioaccessibility, except in the presence of WPI, with a slight increase compared to the control.

Physicochemical properties of carotenoids, such as polarity, could arbitrate the extent of micellization [41]. Borel et *al.* have shown that carotenoids behave differently in biological emulsions,

depending on their polarity [42]. Indeed, the more polar zeaxanthin, which has a chemical structure very close to lutein, was preferentially solubilized at the lipid droplet surface, while the apolar β-carotene was solubilized almost exclusively in the core of the lipid droplets. Therefore, the presence of proteins at the interface may result in a stronger negative interaction of proteins and the more polar xanthophylls, though this may happen also before micelle incorporation [12].

Regarding individual proteins, WPI at lower concentrations (10 and 25% RDA) enhanced lycopene bioaccessibility, possibly due to its emulsion forming ability [43], while at 50% RDA it only improved the bioaccessibility of β-carotene. It was reported that β-lactoglobulin was largely resistant to digestion by pepsin during the gastric phase [44], and remained adsorbed at lipid droplet surfaces [40], being in line with our results. Another study demonstrated that β-carotene can be bound by β-lactoglobulin with high affinity in the internal cavity of the β-barrel [45], suggesting that this major WPI fraction could play a transporter role for some carotenoids. Such interactions appear specific to a certain structure, i.e. β-ionone cycle and isoprenoid chain [45]; consequently, carotenoids with only a hydrocarbon chain such as lycopene may show low binding affinity, perhaps explaining the absence of positive effects of higher WPI concentration on its bioaccessibility. In addition, WPI significantly decreased the release of FFAs. We can only speculate that in these emulsions, large peptides adsorbed to lipid droplet surfaces could form a biological barrier to digestive enzymes, rendering WPI-stabilized emulsions more resistant to lipolysis [40], finally interfering with mixed micelle formation and lycopene micellization.

In case of SC, the bioaccessibility of β-carotene increased in a dose-dependent manner, clearly a different behavior compared to WPI. SDS-PAGE showed that SC was almost completely digested following gastric phase. It is acknowledged that caseins are rapidly hydrolyzed into small peptides, covering the surface of produced emulsions [46, 47]. Furthermore, FFAs release was reduced in the presence of SC, possibly due to a film formed at o/w interface, with high viscoelasticity [48]. Thus, we hypothesize that the presence of these surface-active molecules enhanced the bioaccessibility of

β-carotene (and for lycopene, though not significantly), by stabilizing the emulsions and preventing the aggregation of droplets.

Similar mechanisms seemed to occur with GEL, enhancing β-carotene bioaccessibility at 25 and 50% RDA, while only a slight increase was observed for lycopene. GEL was also fully digested at the end for the GI digestion, and its presence did not influence lipid digestion. Recent nanoemulsion applications have demonstrated the ability of GEL to adsorb on the o/w interfaces and produce emulsions that are stable toward coalescence [49], finally promoting the micellization of the encapsulated β-carotene [50].

Low concentrations of SPI enhanced β-carotene bioaccessibility, while reducing it at 50% RDA. The protein was incompletely digested following the gastric phase. SPI in the digesta led to the formation of insoluble fractions which precipitated and accumulated at the bottom of the digested phases, likely due to the low solubility of SPI [51, 52]. It was earlier shown that SPI influenced the physicochemical behaviour of o/w emulsions during *in vitro* digestion; affecting emulsion stability and extensively increasing lipid digestion, mainly dictated by the type of the liberated peptides [44]. This increased lipid digestion is in accordance with our results regarding FFAs release. On one hand, if being partially digested, i.e. at low concentrations (10 and 25% RDA), the resulting short peptides were able to emulsify lipid droplets and prevented their aggregation, enhancing lipolysis as well as β-carotene bioaccessibility. This is similar to hydrolyzed SPI which stabilized emulsions thanks to a thick elastic film formed on lipid droplet surfaces [48]. On the other hand, the accumulation of remaining long peptide chains from SPI digestion may aggregate in the aqueous phase, especially at high concentrations, due to hydrophobic interactions between partially denatured proteins. These may be adsorbed on individual lipid droplets [53], and carotenoids could be trapped in these aggregates, negatively affecting their transfer into mixed micelles.

Despite the ambivalent influence that proteins had on carotenoid bioaccessibility, the viscosity of the digested protein solutions did not depend on the protein concentration and remained in the

viscosity range of the control solution. In addition, the analysis of the complete shear rate range revealed that the presence of proteins did not affect shear thinning, except a slight apparent increase in the presence of GEL, which is typical for polymer solutions. Therefore, the drop of viscosity in combination with the lack of shear thinning could be taken as indication for full protein hydrolysis (except for SPI) due to digestive enzymes.

Surprisingly, adding proteins to the simulated GI digestion increased surface tension, most strongly by SPI. However, the absolute increase compared to water was quite small. A previous study reported similar findings, assuming that the increase in surface tension is reflecting the effects of pepsinolysis on the adsorbed proteins at the interface [54], and the incorporation of intestinal proteases exerted an additional hydrolysis of adsorbed proteins. Consequently, protein digestion reduced the interfacial layer and caused an increment of the surface tension [55]. Furthermore, other studies reported that these changes could be associated with the very complex characteristics of the interface, such as the adsorption of bile salts to the interface, the interaction between bile salts and protein fragments, or the adsorption and activity of pancreatic lipase [56-58].

The investigated physico-chemical properties such as micelle size, ζ-potential as well as confocal microscopy visualization, yielded additional insights regarding the stability of emulsions. At 25% RDA, the presence of WPI and SPI during the simulated GI digestion outperformed other proteins (SC and GEL) in reducing mixed micelle size. In addition, the absolute ζ-potential of WPI and SPI was lower, compared to SC and GEL, indicating weaker repulsive forces and probably less stable micelles. However and interestingly, microscopic visualization of the emulsions following GI digestion showed that all investigated proteins reduced lipid droplet size dose-dependently. These results suggest that various processes govern the emulsifying properties of the investigated proteins, with perhaps ζ-potential not playing a major role.

Previous studies have reported that in emulsions stabilized by hydrolyzed soy or whey proteins, the repulsive forces between the droplets were greatly lessened, and even no longer strong enough

to resist the attractive inter-droplet interactions, i.e. van der Waals and hydrophobic interactions [59, 60]. This could lead to the formation of WPI- or SPI-stabilized emulsions, forming a network of bridged lipid droplets, as a result of enhanced hydrophobic interactions between the adsorbed proteins on individual droplets, without changing their individual integrities [61, 62]. Our results indicated that besides the low solubility of SPI, and pepsinolysis-resistance of β-lactoglobulin in case of WPI, these proteins could have higher surface hydrophobicity, a crucial parameter determining their emulsification performance [62]. Thus, the biological barrier at the interface could hinder the transfer and incorporation of carotenoids into mixed micelles, reducing their bioaccessibility. On the other hand, SC and GEL, which were better hydrolyzed even in the early stage of digestion, could be involved in the formation of emulsions with more dispersed oil droplets, as the repulsive forces were slightly stronger, enhancing the bioaccessibility of carotenoids.

Taken together, the low solubility of SPI resulted in a reducted protein digestion due to the formation of aggregates in the aqueous phase. Accordingly, a higher surface tension and a decreased ζ-potential were observed, adversely affecting carotenoid bioaccessibility. This could also be due to entrapment of carotenoids in the aggregates. GEL and SC showed a better solubility. Their digestion was effective even during the early stage of digestion, associated with greater repulsive forces between the particles and a better emulsification, as shown by the higher ζ-potential and the lower surface tension, respectively, leading to emulsions with more dispersed lipid droplets. Consequently, GEL and SC promoted carotenoid bioaccessibility compared to SPI, despite a slightly reduced lipid digestion. The same negative effect on lipid digestion was found for WPI, perhaps due to the pepsinolysis-resistance of β-lactoglobulin, preventing enzymatic access to the surface of the lipid droplets at the early stage of intestinal digestion, though this effect disappeared during intestinal digestion, resulting in complete digestion of WPI and fairly high carotenoid bioaccessibility.

CONCLUSIONS

Proteins added to the simulated GI digestions significantly influenced carotenoid bioaccessibility, either positively or negatively. The effects depended on the type and concentration of the protein, but also on the type of carotenoid. Proteins enhanced β-carotene bioaccessibility by up to 189%, while a decrease of up to 50% was observed for lutein. The influence was somewhat limited regarding lycopene bioaccessibility (increase by up to 135%). On the one hand, differences in polarity of each carotenoid are reflected in a different rate of micellization [41, 42] and on the other hand, the contribution of proteins, via their emulsifying properties, depended on their type and concentration [21, 63]. This study also investigated the association between the digestion of macronutrients (proteolysis and lipolysis) and their impact on digesta characteristics such as surface tension and macroviscosity, which in turn could influence emulsion stability, repulsive forces, mixed micelle size, all of which could also impinge on carotenoid bioaccessibility. The results highlight the effects of proteins on carotenoid micellization, constituting an important stage in their bioavailability. However, studying further potential interactions of proteins or/with carotenoid-rich food-matrices would be of great interest. It also remains to be investigated which interactions occur following the interaction of carotenoids from a more mixed diet containing various types of proteins. In addition, investigating the subsequent stages of bioaccessibility, i.e. cellular uptake, will certainly provide additional insights, and of course, final confirmation of these results *in vivo* should be targeted.

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The authors declare no conflict of interest.

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689 **TABLES**

690

691 **Table 1.** Overview of the applied conditions and parameters investigated following the simulated
692 gastro-intestinal (GI) digestion.
693
694

Conditions of the simulated *in vitro* GI digestion				Aspects investigated following the *in vitro* GI digestion				
					Characterization of the digesta			
					Macromolecules		Physicochemical characteristics	
Digestion steps	Protein type digested	Protein concentration (% RDA*)	Carotenoid type studied	Micellar phase				
				Carotenoids	Proteins	Lipids	Solution	Particles
Gastric & intestinal phases	Whey protein isolate	0 10 25 50	Lycopene	Carotenoid bioaccessibility	Protein degradation	Free fatty acid release	Macro-viscosity Surface tension	ζ-Potential Micelle size Confocal microscopy visualization
	Soy protein isolate	0 10 25 50	β-Carotene					
	Sodium caseinate Gelatin	0 10 25 50	Lutein					

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699 *RDA: Recommended dietary allowance.
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FIGURE HEADINGS

Fig. 1. Influence of whey protein isolate (panel A), soy protein isolate (panel B), sodium caseinate (panel C) and gelatin (panel D) at various concentrations on the bioaccessibility of pure carotenoids following simulated gastro-intestinal digestions. β-Carotene, lycopene and lutein were examined in a digestion system either in the absence or presence of WPI, SPI, SC or GEL at different concentrations (0, 10, 25 and 50% of protein recommended dietary allowance (RDA)). Bioaccessibility is expressed as the percentage of pure carotenoids recovered from the aqueous micellar fraction at the end of the *in vitro* GI digestion, compared to the amount of the individual carotenoid added at the beginning of digestion. Values represent means ± SD of $n \geq 4$. Labeled means without a common superscript (alphabetic letters or roman numbers) differ significantly, $P < 0.001$.

Fig. 2. SDS-PAGE results showing the protein bands of pure whey protein isolate (panel A), soy protein isolate (panel B), sodium caseinate (panel C) and gelatin (panel D), compared to gastro-intestinal digestions and gastric digestions only in either presence or absence of proteins. Marker bands are shown on each side. G: gastric digestion of the matrix in the absence (0%) or presence of proteins at a concentration of 50% RDA. GI: gastrointestinal digestion of the matrix in the absence (0%) or presence of proteins at a concentration of 50% RDA. Pure protein represents native undigested protein. Some known polypeptides have been identified to confirm the integrity and type of the proteins. Panel A) 1: β-Lactoglobulin; 2: α-Lactalbumin. Panel B) 1: β-Conglycinin polypeptides (α, α', β subunits); 2: Acid glycinin subunit polypetides; 3: Basic glycinin subunit polypeptides. Panel C) 1: α_{S2}-Casein; 2: α_{S1}-Casein; 3: β-Casein; 4: κ-Casein.

Fig. 3. Influence of proteins on the release of free fatty acids (FFAs) during simulated gastro-intestinal digestion. Whey protein isolate (panel A), soy protein isolate (panel B), sodium caseinate

731 (panel C) and gelatin (panel D) were digested at various concentrations (0, 10, 25 and 50% of the
732 recommended dietary allowance (RDA)), and the release of FFAs was evaluated at the end of GI
733 digestion by means of Cayman's Free Fatty Acid Fluorometric Assay. Values represent means ± SD
734 of n=3. Labeled means without a common superscript (alphabetic letters) differ significantly,
735 P<0.05.
736
737 **Fig. 4.** Effect of pure proteins at various concentrations on the macroviscosity (panel A) and shear
738 stress (panel B) of the digesta following gastro-intestinal digestion. Panel A represents the averaged
739 viscosity curves (n≥3) as a function of the shear (1 – 100 s^{-1}) rate for non-digested and digested
740 whey protein isolate (WPI), sodium caseinate (SC) or gelatin (GEL), at different concentrations
741 representing 0 and 50% of the recommended dietary allowance (RDA). Panel B represents the
742 averaged Bingham regressions (n≥3) of the shear stress (0 – 100 Pa) as a function of the shear (1 –
743 100 s^{-1}) rate for non-digested and digested WPISC or GEL at different concentrations, representing
744 0 and 50% of the RDA.
745
746 **Fig. 5.** Confocal microscopy images taken after gastro-intestinal digestion with whey protein isolate
747 (WPI), soy protein isolate (SPI), sodium caseinate (SC), and gelatin (GEL). Confocal imaging of
748 emulsion structures was carried out at room temperature with a confocal laser scanning microscope
749 (Zeiss LSM 880, Airyscam SR, Jena, Germany), using a 63x objective. The fluorescent dyes were
750 excited by an Argon laser (488 nm) and the emitted light was collected at 522 nm for protein and
751 635 nm for the fat phase. Lipids were labelled with Nile red (red colour) and proteins were labelled
752 with fluorescent isothiocyanate (FITC) (green colour). Of note, the combination of red and green is
753 yellow in additive colour configurations. The white-grey bar indicates 10 μm.
754

Fig. 6. Effect of whey protein isolate (panel A), soy protein isolate (panel B), sodium caseinate (panel C) and gelatin (panel D) at various concentrations on the mixed micelle ζ-potential following gastro-intestinal *in vitro* digestions. β-Carotene, lycopene and lutein were examined in a digestion system in the presence of WPI, SPI, SC or GEL at different concentrations (0, 10, 25 and 50% of the recommended dietary allowance (RDA)). Filtered aliquots of the aqueous micellar fraction were used to determine mixed micelle size by using photon correlation spectroscopy (Zetasizer Nano Zs, Malvern Instruments) at room temperature. Values represent means ± SD of n=4. Labeled means without a common superscript (alphabetic letters) differ significantly, P<0.001.

FIGURES

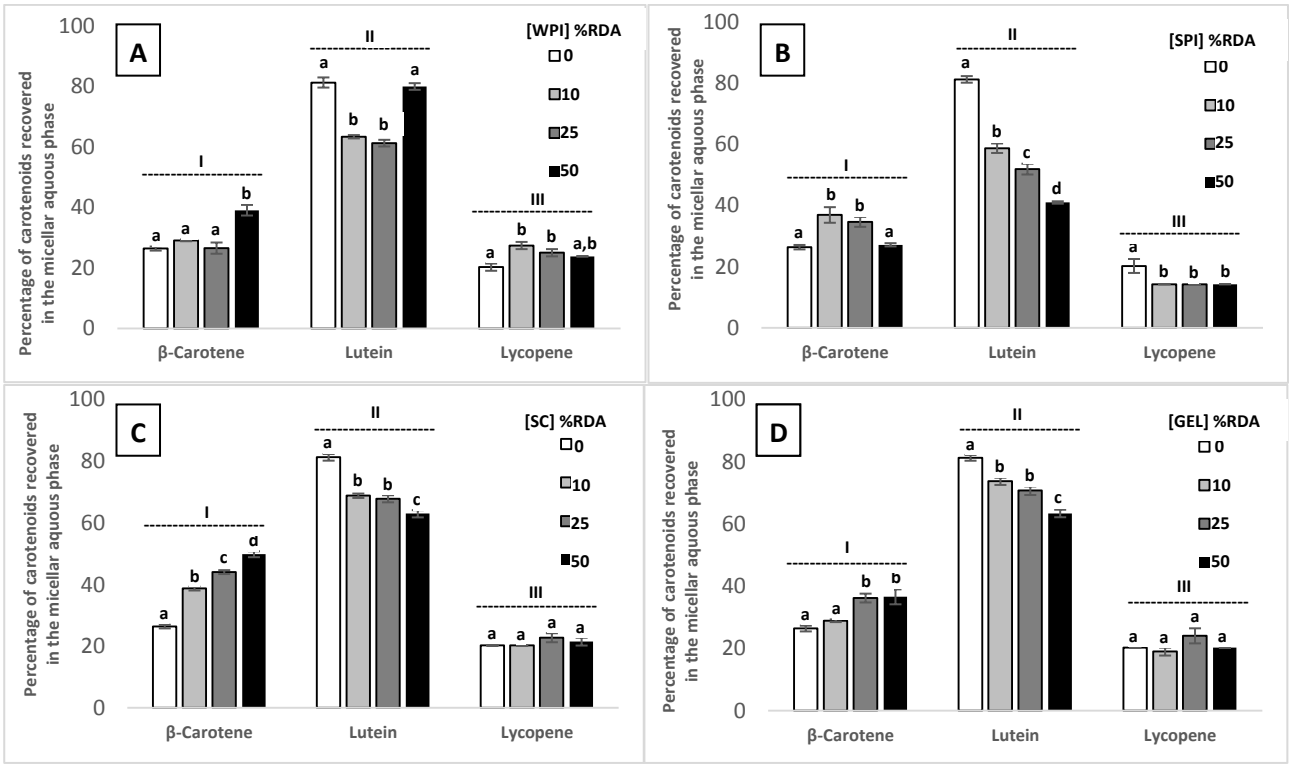


Fig. 1

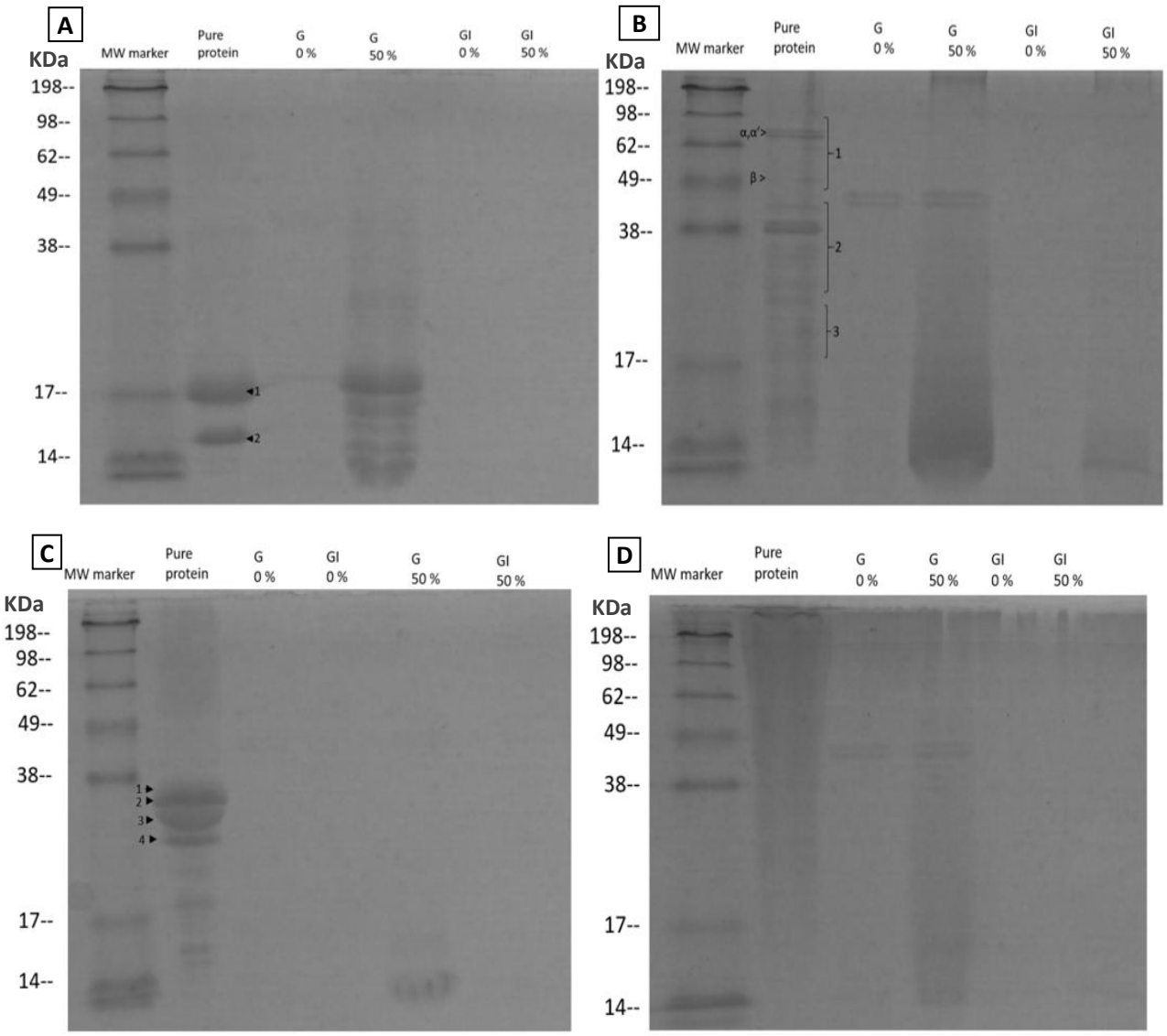

A
KDa
MW marker
Pure protein
G 0 %
G 50 %
GI 0 %
GI 50 %
198--
98--
62--
49--
38--
17--
14--
1
2
B
KDa
MW marker
Pure protein
G 0 %
G 50 %
GI 0 %
GI 50 %
198--
98--
62--
49--
38--
17--
14--
α,α'>
β>
1
2
3
C
KDa
MW marker
Pure protein
G 0 %
GI 0 %
G 50 %
GI 50 %
198--
98--
62--
49--
38--
17--
14--
1
2
3
4
D
KDa
MW marker
Pure protein
G 0 %
G 50 %
GI 0 %
GI 50 %
198--
98--
62--
49--
38--
17--
14--

Fig. 2

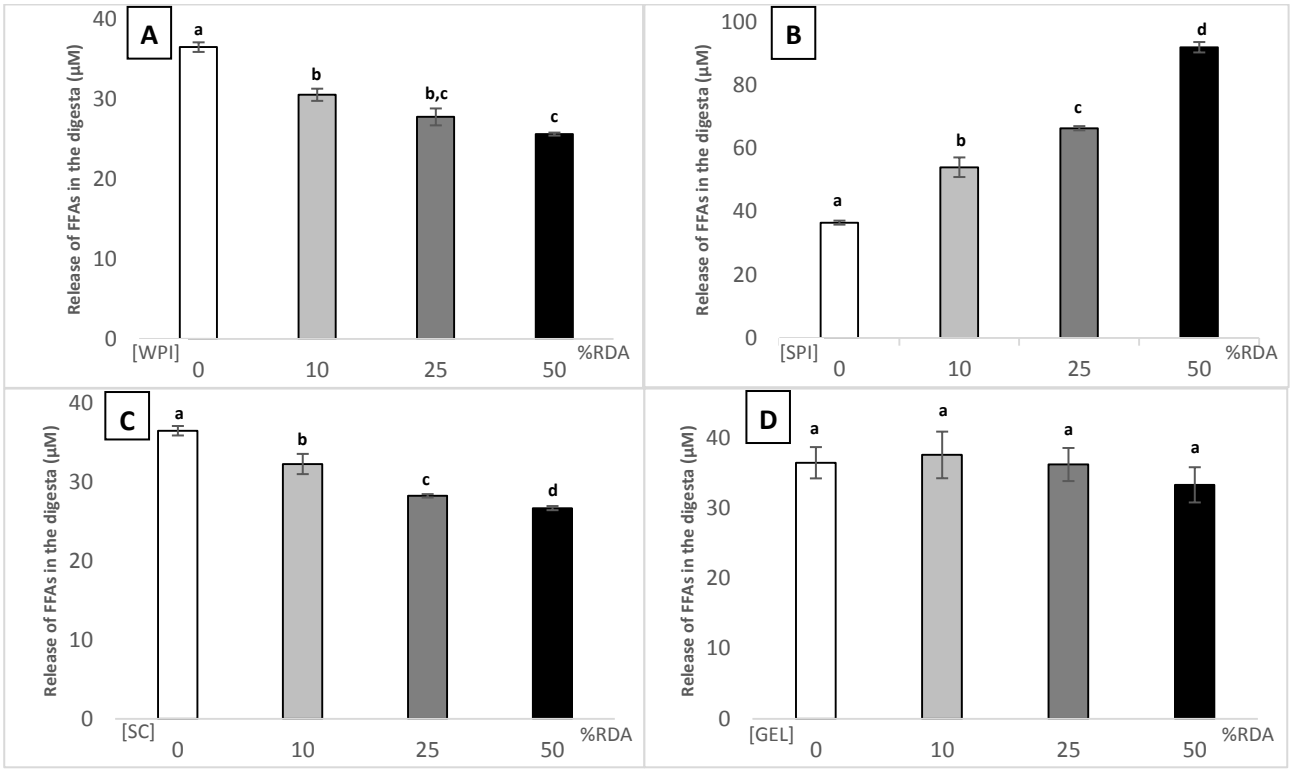

A
Release of FFAs in the digesta (µM)
40
30
20
10
0
a
b
b,c
c
[WPI]
0
10
25
50
%RDA
B
Release of FFAs in the digesta (µM)
100
80
60
40
20
0
a
b
c
d
[SPI]
0
10
25
50
%RDA
C
Release of FFAs in the digesta (µM)
40
30
20
10
0
a
b
c
d
[SC]
0
10
25
50
%RDA
D
Release of FFAs in the digesta (µM)
40
30
20
10
0
a
a
a
a
[GEL]
0
10
25
50
%RDA

Fig. 3

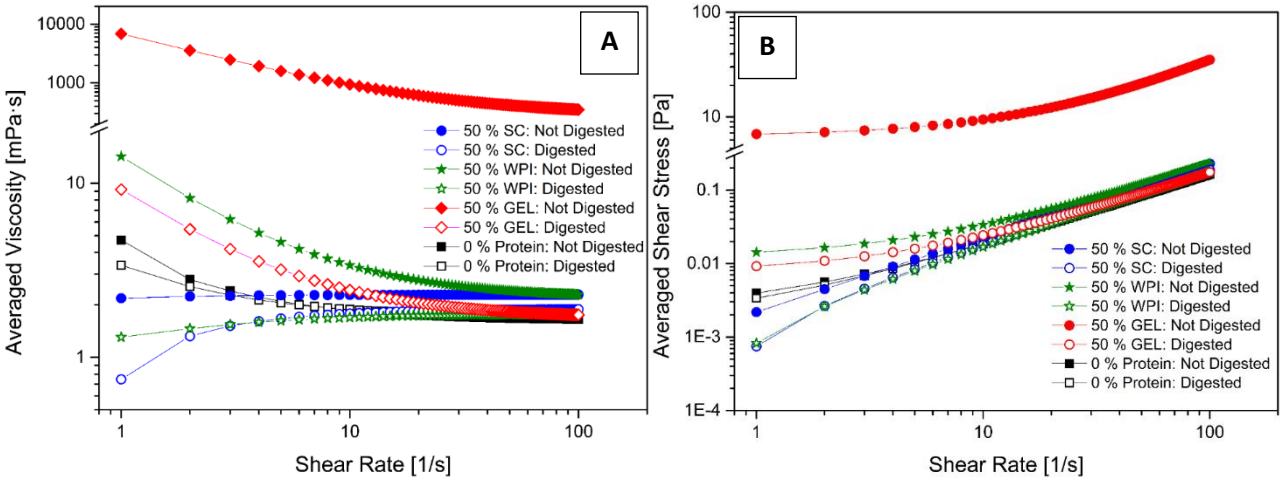

A
Averaged Viscosity [mPa·s]
10000
1000
10
1
1
10
100
Shear Rate [1/s]
50 % SC: Not Digested
50 % SC: Digested
50 % WPI: Not Digested
50 % WPI: Digested
50 % GEL: Not Digested
50 % GEL: Digested
0 % Protein: Not Digested
0 % Protein: Digested
B
Averaged Shear Stress [Pa]
100
10
0.1
0.01
1E-3
1E-4
1
10
100
Shear Rate [1/s]
50 % SC: Not Digested
50 % SC: Digested
50 % WPI: Not Digested
50 % WPI: Digested
50 % GEL: Not Digested
50 % GEL: Digested
0 % Protein: Not Digested
0 % Protein: Digested

Fig. 4

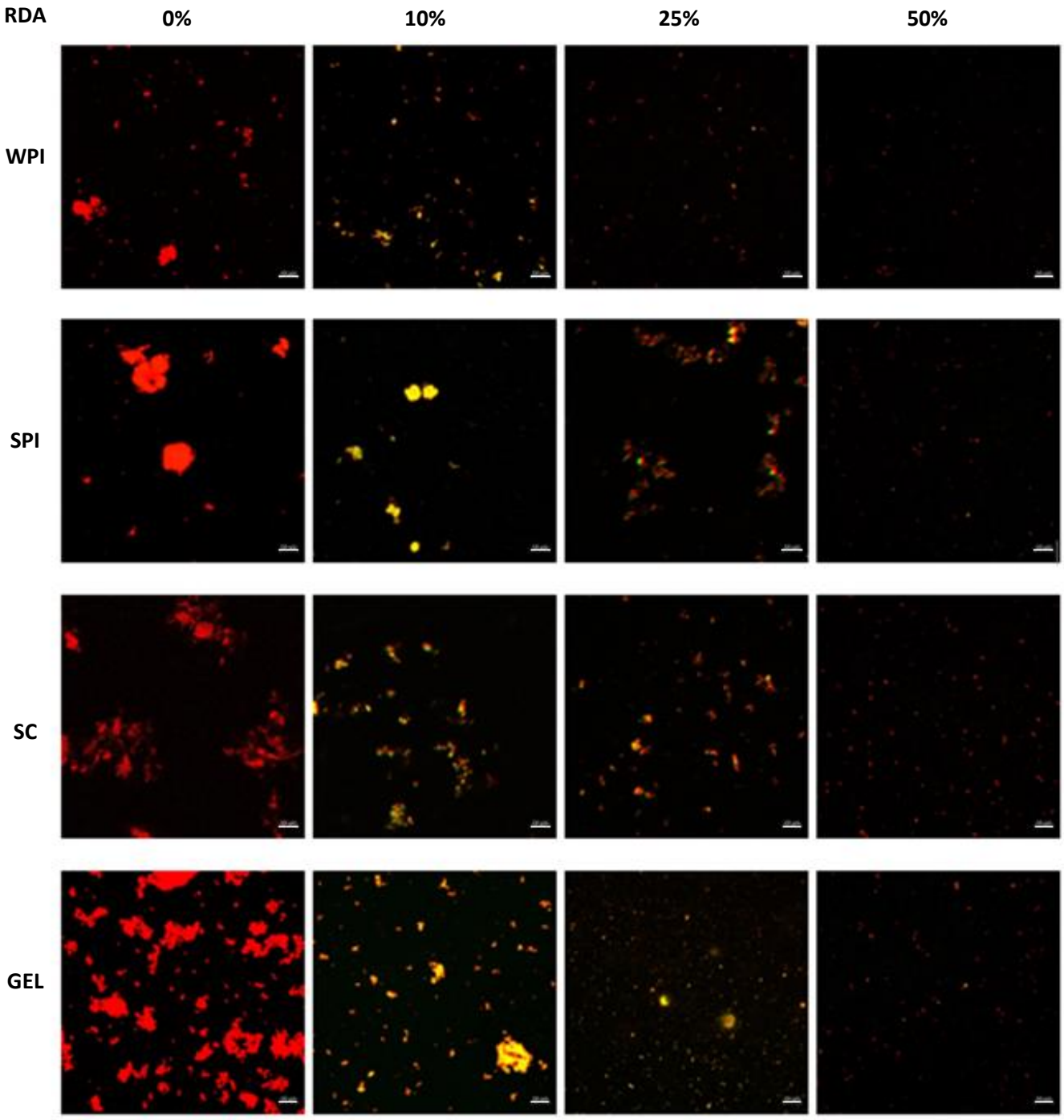


Fig. 5

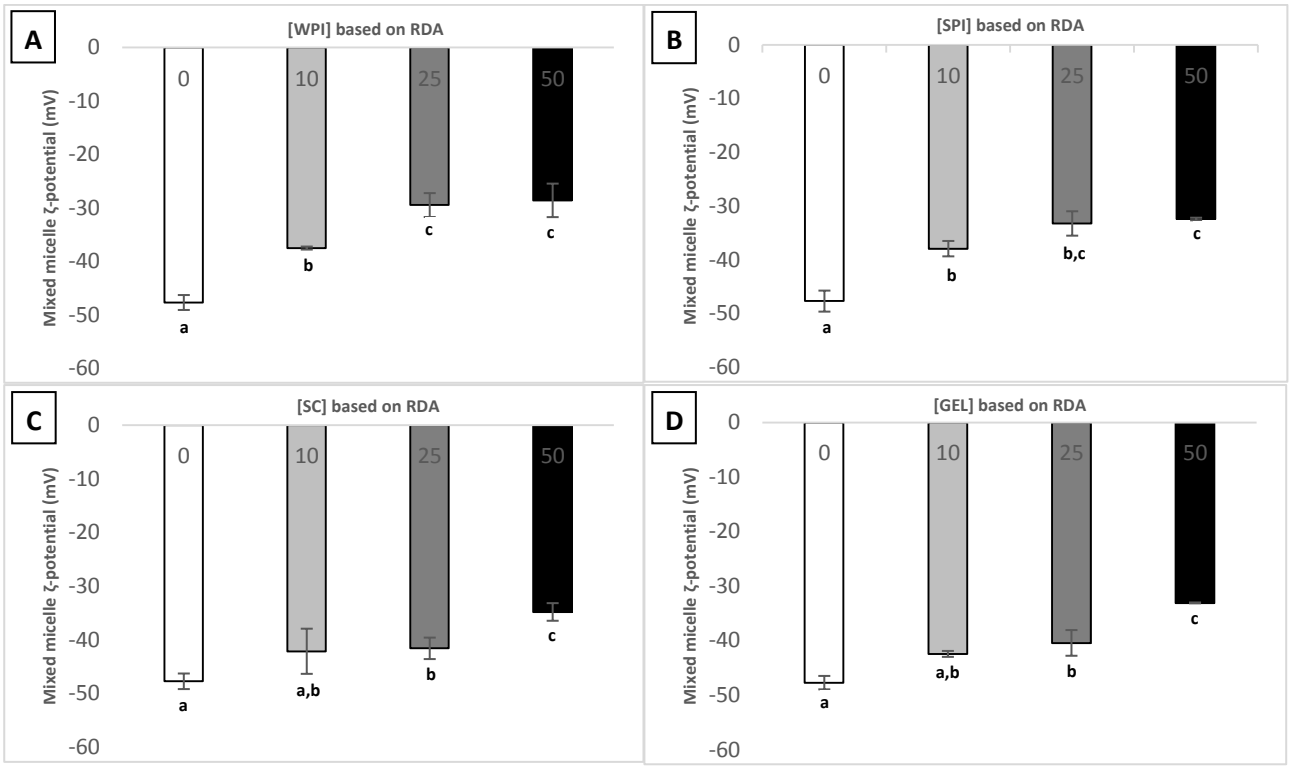
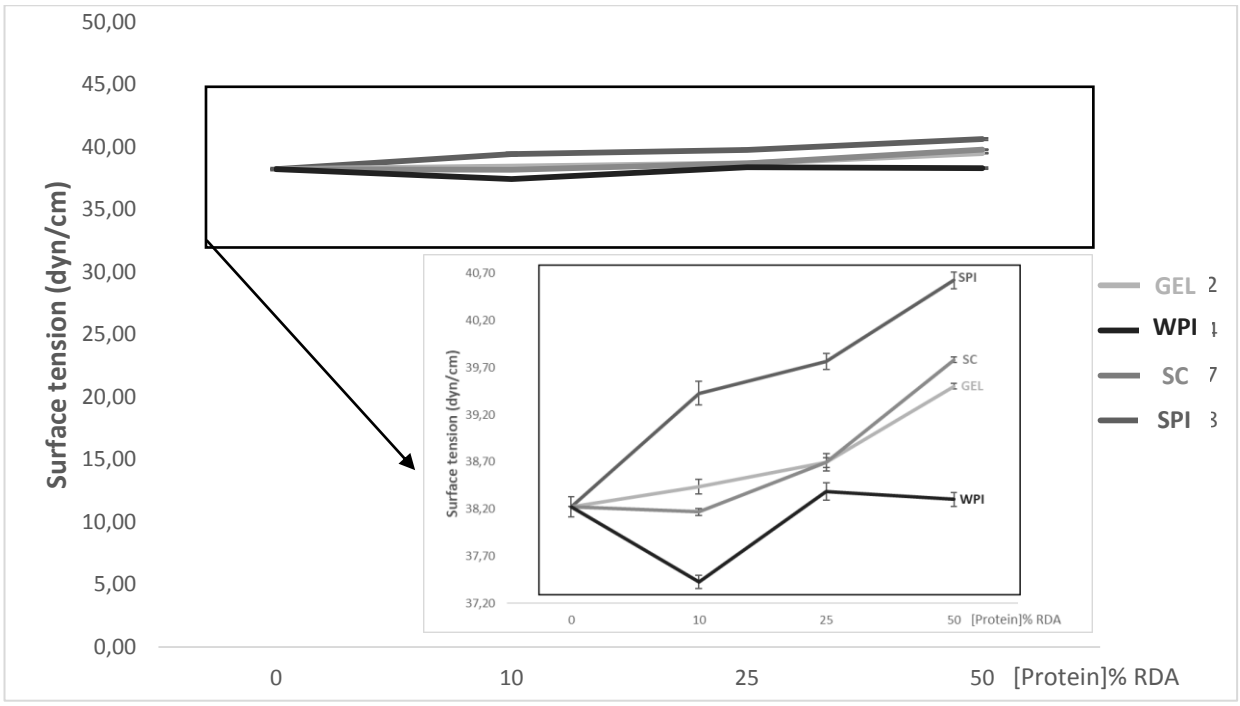
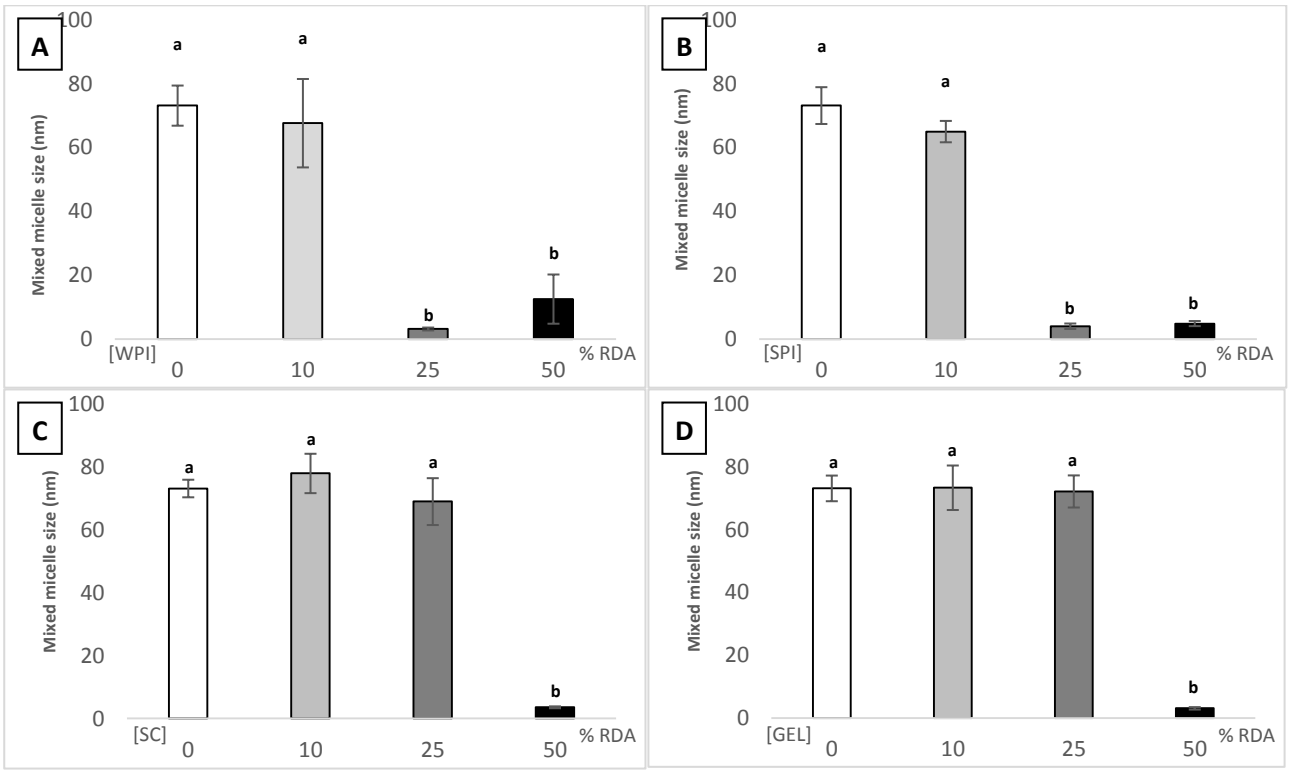

A
[WPI] based on RDA
Mixed micelle ζ-potential (mV)
0
-10
-20
-30
-40
-50
-60
0
10
25
50
a
b
c
c
B
[SPI] based on RDA
a
b
b,c
c
C
[SC] based on RDA
a
a,b
b
c
D
[GEL] based on RDA
a
a,b
b
c

Fig. 6

Online supporting material (OSM)



OSM - Fig. 1. Effect of pure proteins at various concentrations on the surface tension of the digesta (value of pure water: 71.99 dyn/cm), following gastro-intestinal digestion. Whey protein isolate (WPI), soy protein isolate (SPI), sodium caseinate (SC) or gelatin (GEL) were subjected to simulated GI digestion at different concentrations (0, 10, 25 and 50% of the recommended dietary allowance (RDA)). The surface tension of digesta, pre-conditioned at 25 ± 0.1 °C, was determined by the weight-drop method. Values represent means ± SD of n = 16 replicates.



OSM – Fig. 2. Effect of whey protein isolate (WPI, panel A), soy protein isolate (SPI, panel B), sodium caseinate (SC, panel C) and gelatin (Gel, panel D) at various concentrations on the size of the produced mixed micelles following gastro-intestinal *in vitro* digestions. WPI, SPI, SC or GEL were subjected to the simulated digestion at different concentrations (0, 10, 25 and 50% of the recommended dietary allowance (RDA)). Filtered aliquots of the aqueous micellar fraction were used to determine mixed micelle size by photon correlation spectroscopy (Zetasizer Nano Zs, Malvern Instruments) at room temperature. Values represent means ± SD of $n \geq 12$. Labeled means without a common superscript (alphabetic letters) differ significantly, $P<0.05$.