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1 **Influence of Soy and Whey Protein, Gelatin and Sodium Caseinate on Carotenoid**  
2 **Bioaccessibility**

3

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23

24 **Running title:** Proteins and pure carotenoid bioaccessibility

25

26     **ABSTRACT**

27     Proteins could alter carotenoid bioaccessibility through altering their fate during digestion, due to  
28     emulsifying properties of resulting peptides, or influencing access of digestion enzymes to lipid  
29     droplets. In this investigation, we studied whether whey protein isolate (WPI), soy protein isolate  
30     (SPI), sodium caseinate (SC) and gelatin (GEL), added at various concentrations (expressed as  
31     percentage of recommended dietary allowance (RDA): 0, 10, 25 and 50%) would influence the  
32     bioaccessibility of lycopene,  $\beta$ -carotene or lutein, added as pure carotenoids solubilized in oil,  
33     during simulated gastro-intestinal (GI) digestion. Protein and lipid digestion as well as selected  
34     physico-chemical parameters including surface tension,  $\zeta$ -potential and micelle size were evaluated.  
35     Adding proteins influenced positively the bioaccessibility of  $\beta$ -carotene, by up to 189% ( $p<0.001$ ),  
36     but it resulted in generally decreased bioaccessibility of lutein, by up to 50% ( $p<0.001$ ), while for  
37     lycopene, the presence of proteins did not influence its bioaccessibility, except for a slight increase  
38     with WPI, by up to 135% ( $p<0.001$ ). However, the effect depended significantly on the type of  
39     protein ( $p<0.001$ ) and its concentration ( $p<0.001$ ). While  $\beta$ -carotene bioaccessibility was greatly  
40     enhanced in the presence of SC, compared to WPI and GEL, the presence of SPI strongly decreased  
41     carotenoid bioaccessibility. Neglecting individual carotenoids, higher protein concentration  
42     correlated positively with carotenoid bioaccessibility ( $R=0.57$ ,  $p<0.01$ ), smaller micelle size ( $R=-$   
43      $0.83$ ,  $p<0.01$ ), decreased repulsive forces ( $\zeta$ -potential,  $R=-0.72$ ,  $p<0.01$ ), and higher surface tension  
44     ( $R=0.44$ ,  $p<0.01$ ). In conclusion, proteins differentially affected carotenoid bioaccessibility during  
45     digestion depending on carotenoid and protein species, with both positive and negative interactions  
46     occurring.

47

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

50

51       **INTRODUCTION**

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

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

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

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

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

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

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

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

110

## 111 **EXPERIMENTAL**

### 112 **Chemicals and carotenoid standards**

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

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

124

125 **Pure proteins and dietary lipid sources**

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

130 Peanut oil, typically free of native carotenoids<sup>29</sup> and own blank examinations, was purchased  
131 from a local supermarket (Delhaize, Strassen, Luxembourg).

132

133 **Solubilization of carotenoid standards & preparation of protein and enzyme solutions**

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

142 Regarding the protein solutions, the amounts of proteins tested were 0, 3, 7.5, 15 g/L,  
143 corresponding to 0, 10, 25 and 50% of the recommended dietary allowance (RDA) within 1 test  
144 meal, being 0.83 g per kg of body weight per day for adults ( $\approx 60\text{g/d}$ )<sup>30</sup>, by dissolving/emulsifying  
145 each protein type in pure water.

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

150 our previous study<sup>22</sup>, 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<sup>22</sup>. 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<sup>31</sup>. 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<sup>®</sup>, 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

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

176

## 177 **Analyses of final products of digestion**

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

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

194 **SDS-PAGE electrophoresis:** Sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-  
195 PAGE) was carried out as previously described <sup>22</sup> to study the extent of protein digestion. Samples  
196 were denatured at 95°C for 5 min. and were then loaded to a 15% acrylamide and 0.4%  
197 bisacrylamide gel for the samples obtained after complete GI digestion, or to a 10%  
198 acrylamide/0.3% bisacrylamide gel for the digesta obtained following only gastric digestion. The

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

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

208

#### 209 **Macroviscosity and surface tension analysis**

210 Flow curves were determined as previously described<sup>22</sup>, using an Anton-Paar rheometer (MCR  
211 302, WESP, Graz, Austria), equipped with a double gap cell at 5°C. A regression flow curve was  
212 calculated for the shear rate range between 0 s<sup>-1</sup> and 100 s<sup>-1</sup> for the shear stress respectively between  
213 1 s<sup>-1</sup> and 100 s<sup>-1</sup> for the viscosity. The regression curves were plotted as viscosity and shear stress as  
214 a function of the shear rate.

215 Surface tension of digesta samples, pre-conditioned at 25 ± 0.1 °C, were determined by the  
216 weight-drop method as previously described<sup>32</sup>. The air-water interfacial properties of the digesta  
217 were calculated as follows:

$$218 \quad \sigma_{digesta} = \frac{m_{digesta}}{m_{H_2O}} \times \sigma_{H_2O}$$

219 where  $\sigma_{H_2O} = 71.99 \text{ dyn cm}^{-1}$  is the surface tension of pure water.

220

#### 221 **Confocal laser scanning microscopy**

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

224 63x objective. The aliquots were dyed with Nile red dissolved in ethanol, and FITC dissolved in  
225 acetone. Both were used for fat and protein staining, respectively, at a concentration of 1 µg/mL.  
226 An Argon 488 nm laser excited the fluorescent dyes. The emitted light was collected at 500-540 nm  
227 for proteins and 590-650 nm for the fat phase. The resulting images were processed and de-noised  
228 by using a modular image-processing and analysis software for digital microscopy (Zen 2.3 blue  
229 edition, Carl Zeiss Microscopy GmbH, Jena, Germany).

230

### 231 **Micelle size and $\zeta$ -potential analysis**

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

237

### 238 **Statistical analyses and data treatment**

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

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

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

255

## 256 **RESULTS**

### 257 **General influence of proteins on the bioaccessibility of carotenoids**

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

262 The addition of various proteins to the simulated GI digestion impacted the bioaccessibility  
263 differently. All carotenoids and all protein concentrations applied considered, i.e. evaluated together  
264 statistically (without applying post-hoc tests for group-wise comparisons), the addition of SC  
265 resulted overall in slightly higher bioaccessibility (43.7%) compared to WPI and GEL (41.9% and  
266 41.7%, respectively) ( $p < 0.001$ ), while the lowest bioaccessibility was clearly engendered by SPI  
267 (35.1%) ( $p < 0.001$ ).

### 268 **Influence of individual proteins on the bioaccessibility of carotenoids**

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

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

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

286 **Sodium caseinate:** No significant effect engendered by the presence of SC (all concentrations  
287 considered) regarding the bioaccessibility of lycopene (Fig. 1C), while an average increase up to  
288  $39.8\%$  of  $\beta$ -carotene bioaccessibility was observed ( $p < 0.001$ ). As with other proteins, the addition  
289 of SC decreased the bioaccessibility of lutein to an average of  $70.2\%$  ( $p < 0.001$ ). More specifically,  
290 at high protein concentration (50% RDA), SC reduced lutein bioaccessibility to  $62.9\% \pm 1.2$   
291 ( $p < 0.001$ ). For  $\beta$ -carotene, a drastic increase in bioaccessibility to  $49.8\% \pm 0.9$  was observed  
292 ( $p < 0.001$ ).

293 **Gelatin:** The co-digestion of pure carotenoids with GEL resulted in very similar effects regarding  
294 the bioaccessibility as observed with SC. Briefly, when considering all protein concentrations, the  
295 presence of GEL in the digested matrix (all concentrations applied) enhanced the bioaccessibility of  
296  $\beta$ -carotene to an average of  $32\%$  ( $p < 0.001$ ). However, the bioaccessibility of lutein was brought  
297 down to an average of  $72.3\%$  ( $p < 0.001$ ). Again, there was no significant change regarding the  
298 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  $63.4\% \pm 1.2$ , while the bioaccessibility of  $\beta$ -carotene was improved in turn in a  
301 dose-dependent manner, with an increase to  $36.6\% \pm 2.4$  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  $\beta$ -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<sup>21</sup>. Overall, the addition

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

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

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

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

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

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

375

## 376 **DISCUSSION**

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

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

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

396 Physicochemical properties of carotenoids, such as polarity, could arbitrate the extent of  
397 micellization<sup>41</sup>. Borel *et al.* have shown that carotenoids behave differently in biological emulsions,

398 depending on their polarity <sup>42</sup>. Indeed, the more polar zeaxanthin, which has a chemical structure  
399 very close to lutein, was preferentially solubilized at the lipid droplet surface, while the apolar  $\beta$ -  
400 carotene was solubilized almost exclusively in the core of the lipid droplets. Therefore, the presence  
401 of proteins at the interface may result in a stronger negative interaction of proteins and the more  
402 polar xanthophylls, though this may happen also before micelle incorporation <sup>12</sup>.

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

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

423  $\beta$ -carotene (and for lycopene, though not significantly), by stabilizing the emulsions and preventing  
424 the aggregation of droplets.

425 Similar mechanisms seemed to occur with GEL, enhancing  $\beta$ -carotene bioaccessibility at 25 and  
426 50% RDA, while only a slight increase was observed for lycopene. GEL was also fully digested at  
427 the end for the GI digestion, and its presence did not influence lipid digestion. Recent nanoemulsion  
428 applications have demonstrated the ability of GEL to adsorb on the o/w interfaces and produce  
429 emulsions that are stable toward coalescence <sup>49</sup>, finally promoting the micellization of the  
430 encapsulated  $\beta$ -carotene <sup>50</sup>.

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

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

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

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

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

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

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

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

498

## 499 **CONCLUSIONS**

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

518

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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.

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

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699 \*RDA: Recommended dietary allowance.

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706 **FIGURE HEADINGS**

707 **Fig. 1.** Influence of whey protein isolate (panel A), soy protein isolate (panel B), sodium caseinate  
708 (panel C) and gelatin (panel D) at various concentrations on the bioaccessibility of pure carotenoids  
709 following simulated gastro-intestinal digestions.  $\beta$ -Carotene, lycopene and lutein were examined in  
710 a digestion system either in the absence or presence of WPI, SPI, SC or GEL at different  
711 concentrations (0, 10, 25 and 50% of protein recommended dietary allowance (RDA)).  
712 Bioaccessibility is expressed as the percentage of pure carotenoids recovered from the aqueous  
713 micellar fraction at the end of the *in vitro* GI digestion, compared to the amount of the individual  
714 carotenoid added at the beginning of digestion. Values represent means  $\pm$  SD of  $n \geq 4$ . Labeled  
715 means without a common superscript (alphabetic letters or roman numbers) differ significantly,  $P <$   
716 0.001.

717

718 **Fig. 2.** SDS-PAGE results showing the protein bands of pure whey protein isolate (panel A), soy  
719 protein isolate (panel B), sodium caseinate (panel C) and gelatin (panel D), compared to gastro-  
720 intestinal digestions and gastric digestions only in either presence or absence of proteins. Marker  
721 bands are shown on each side. G: gastric digestion of the matrix in the absence (0%) or presence of  
722 proteins at a concentration of 50% RDA. GI: gastrointestinal digestion of the matrix in the absence  
723 (0%) or presence of proteins at a concentration of 50% RDA. Pure protein represents native  
724 undigested protein. Some known polypeptides have been identified to confirm the integrity and type  
725 of the proteins. Panel A) 1:  $\beta$ -Lactoglobulin; 2:  $\alpha$ -Lactalbumin. Panel B) 1:  $\beta$ -Conglycinin  
726 polypeptides ( $\alpha$ ,  $\alpha'$ ,  $\beta$  subunits); 2: Acid glycinin subunit polypeptides; 3: Basic glycinin subunit  
727 polypeptides. Panel C) 1:  $\alpha_{S2}$ -Casein; 2:  $\alpha_{S1}$ -Casein; 3:  $\beta$ -Casein; 4:  $\kappa$ -Casein.

728

729 **Fig. 3.** Influence of proteins on the release of free fatty acids (FFAs) during simulated gastro-  
730 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  $\pm$  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 ( $n\geq 3$ ) as a function of the shear ( $1 - 100 \text{ s}^{-1}$ ) 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 ( $n\geq 3$ ) of the shear stress ( $0 - 100 \text{ Pa}$ ) as a function of the shear ( $1 -$   
743  $100 \text{ s}^{-1}$ ) 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  $\mu\text{m}$ .

754

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

763 **FIGURES**

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769 **Fig. 1**

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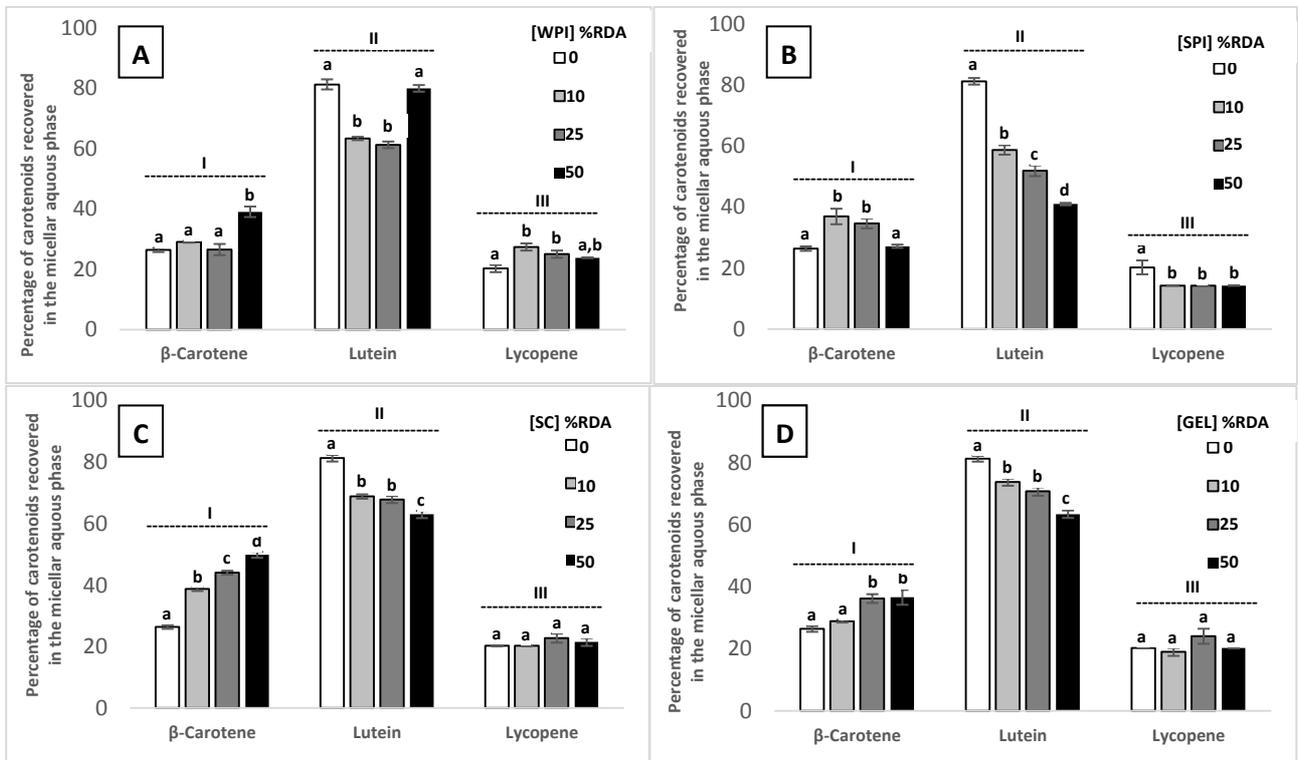
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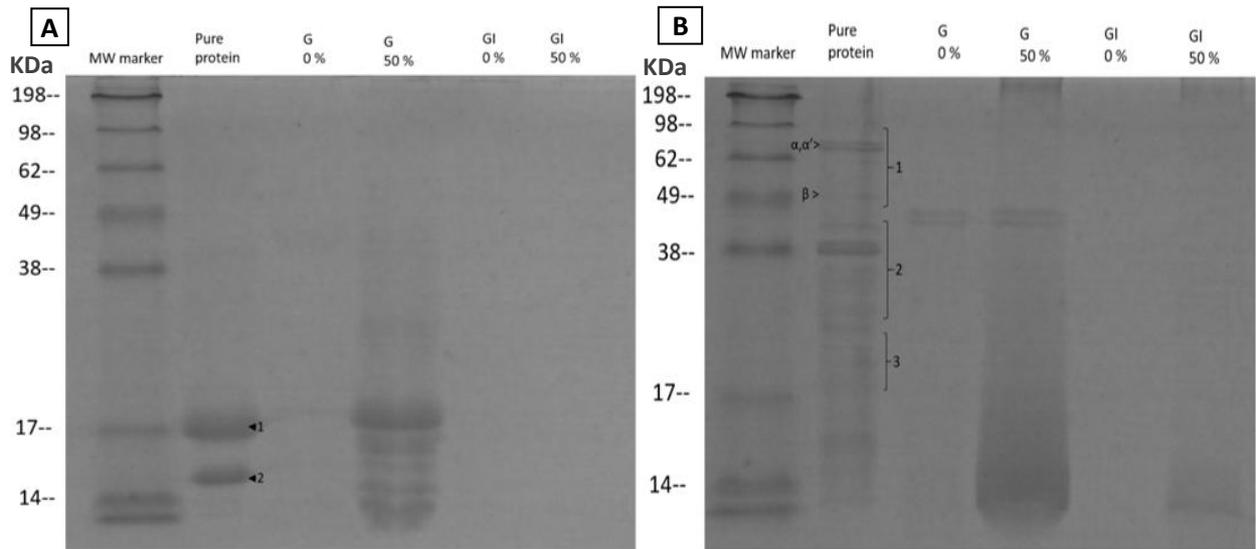
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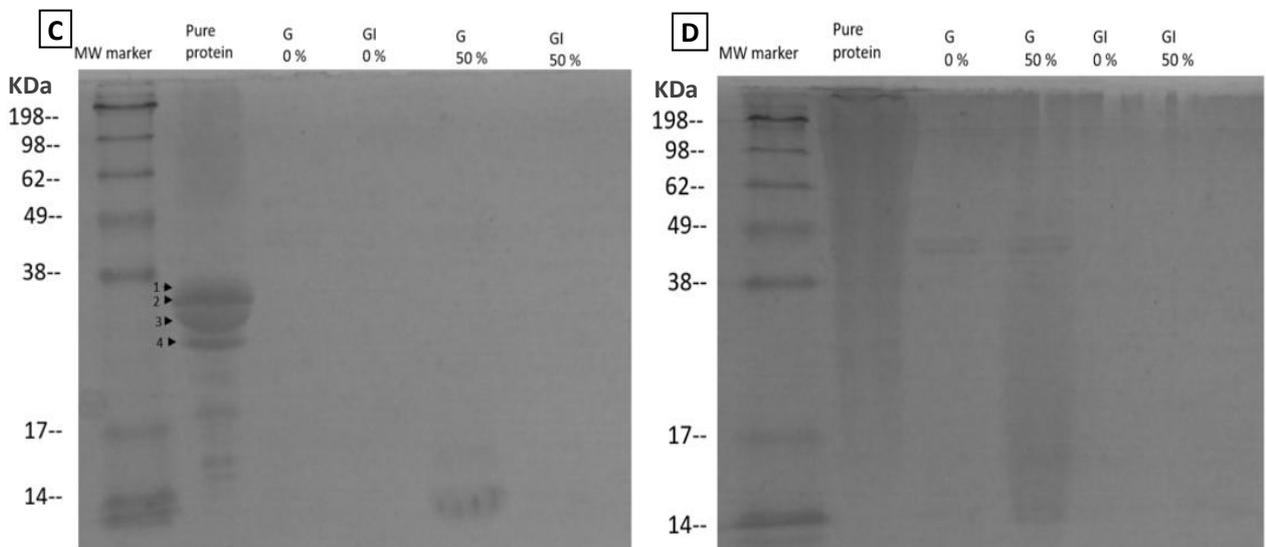
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782 Fig. 2

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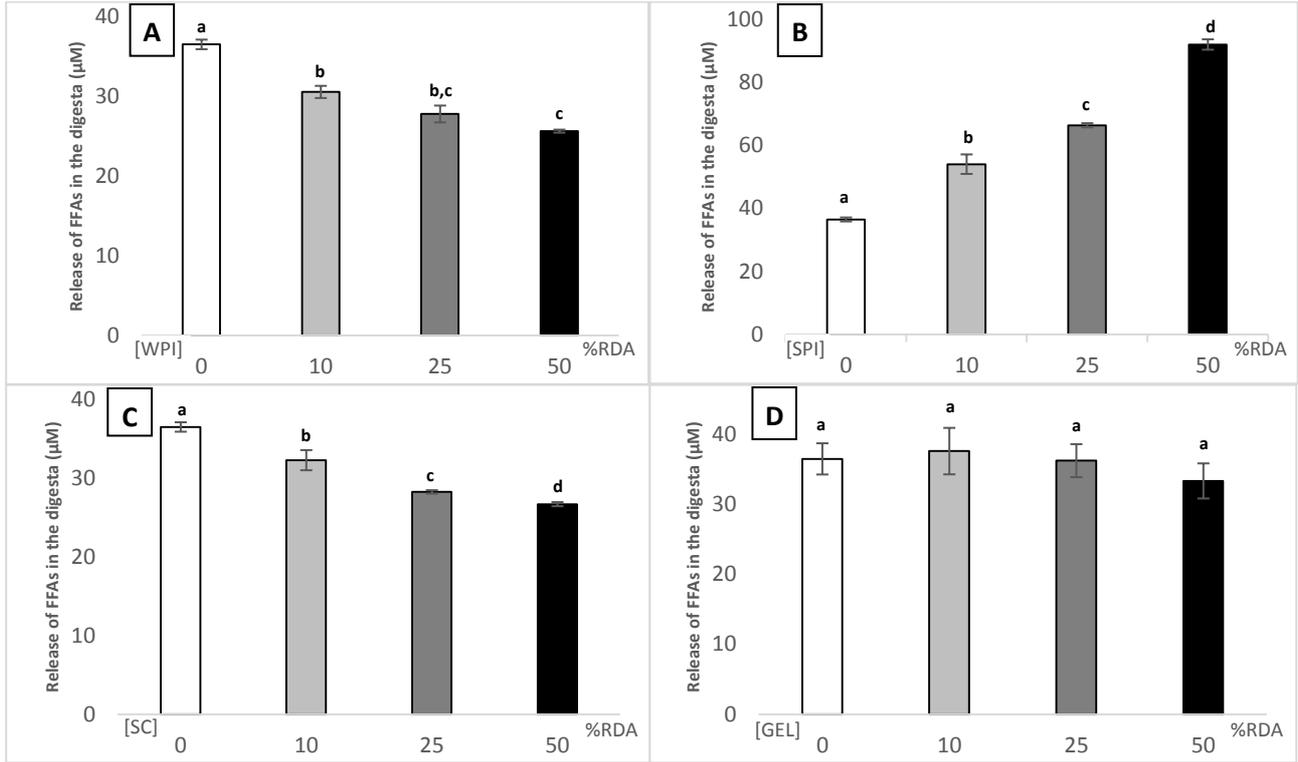
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Fig. 3

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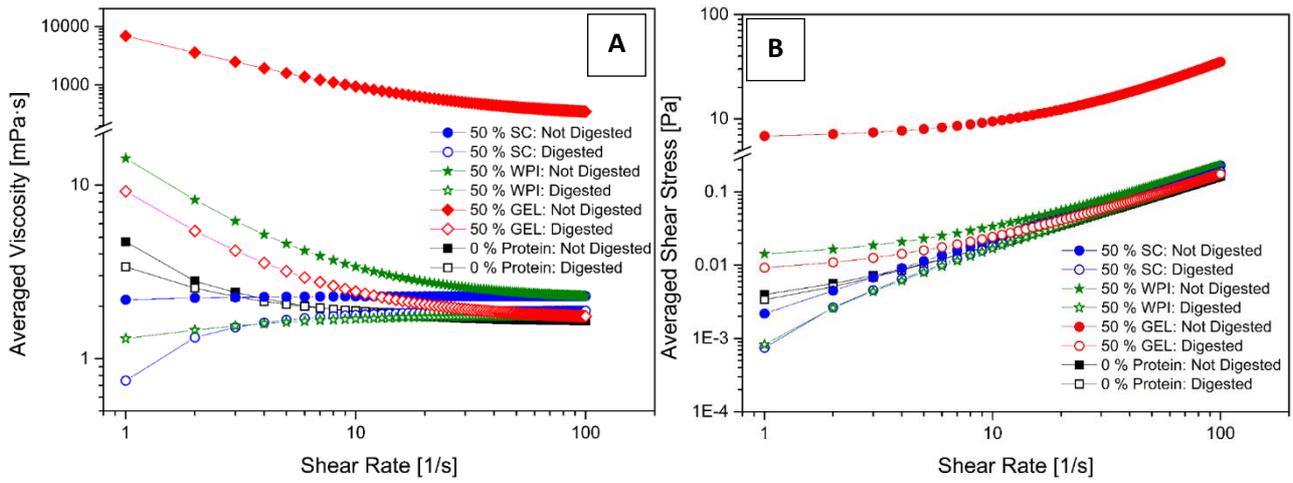
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806 Fig. 4

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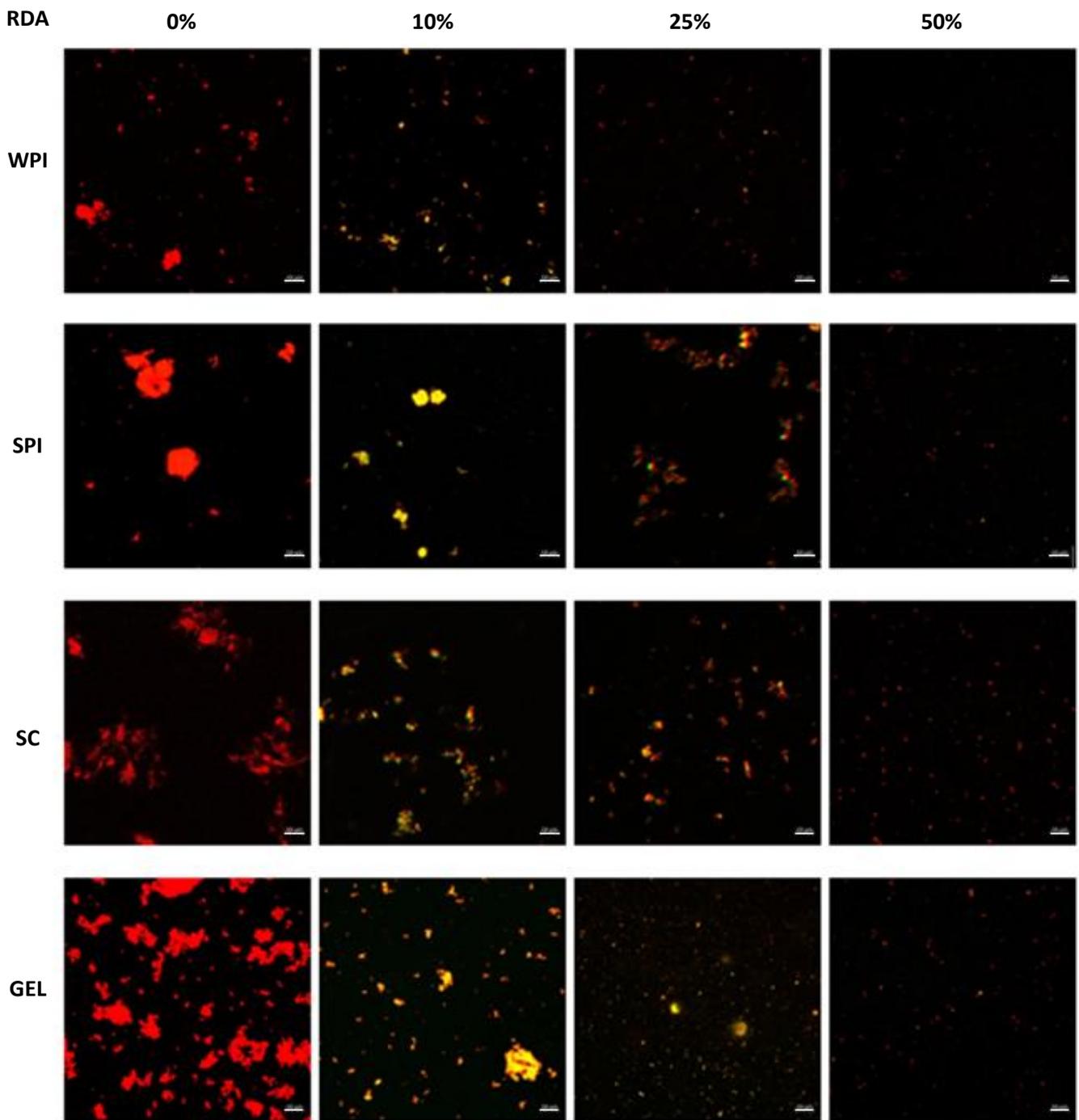
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827 **Fig. 5**

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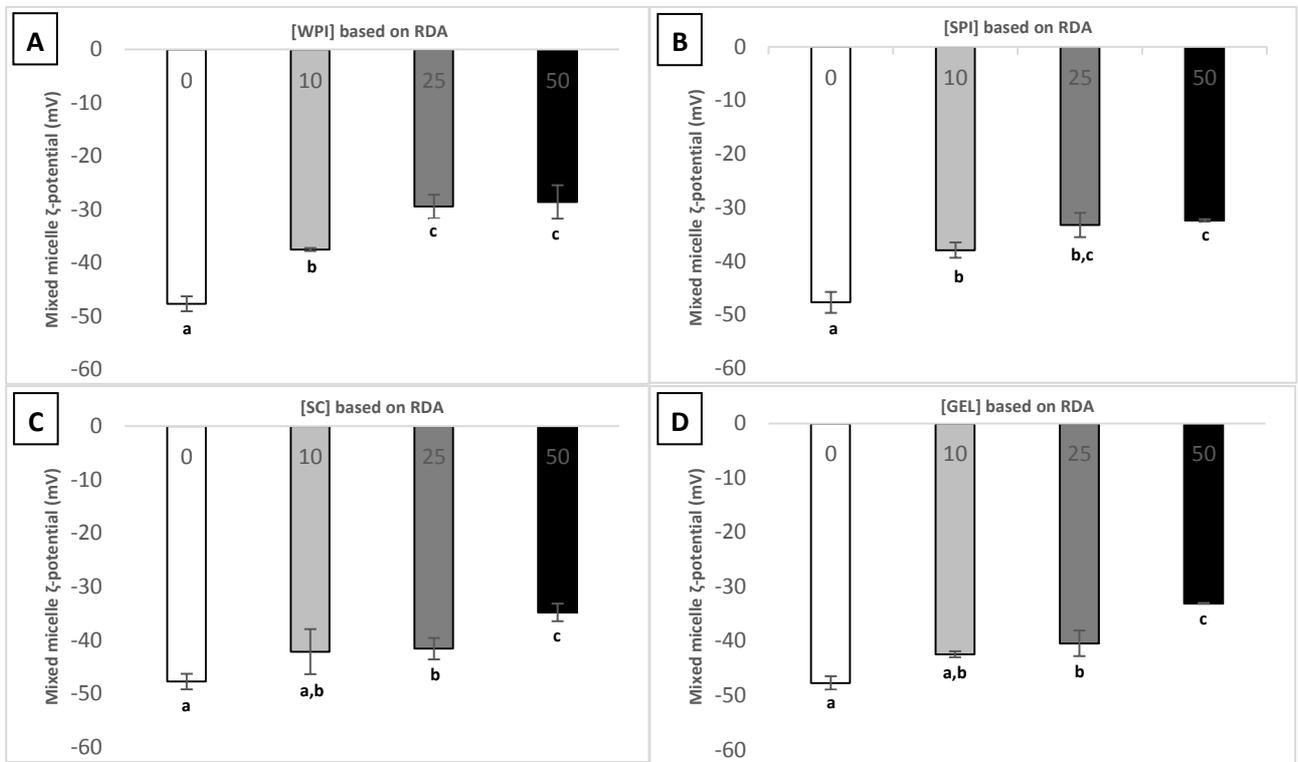
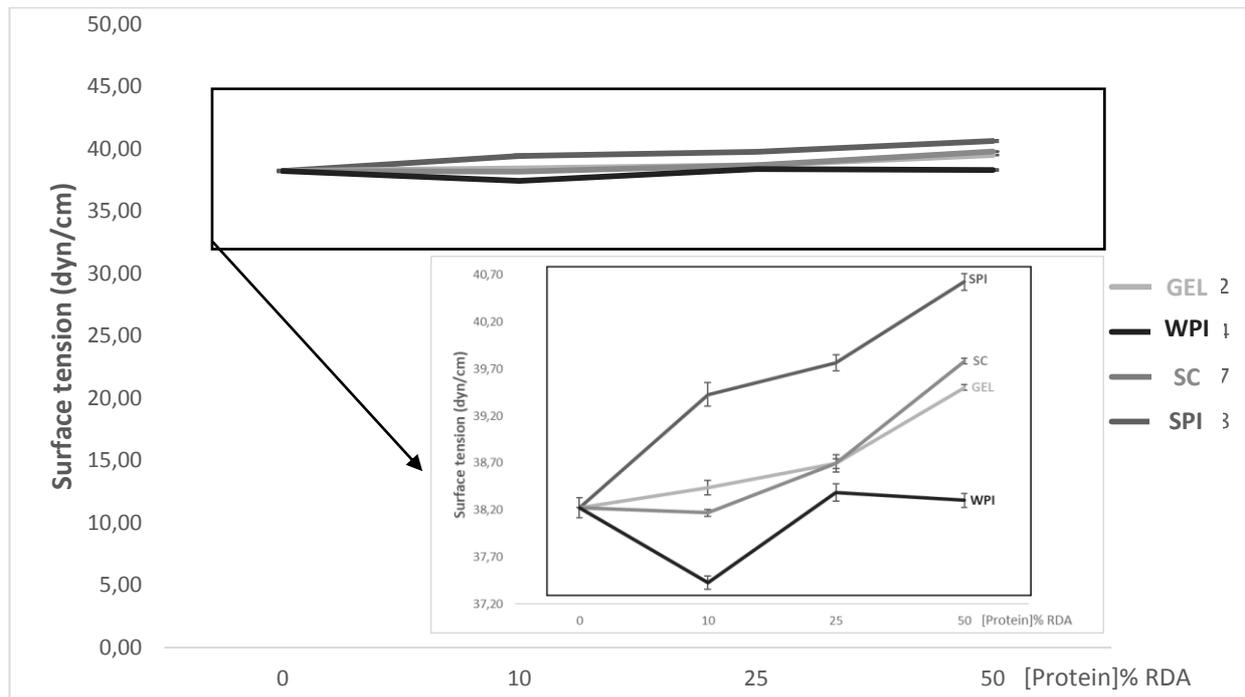


Fig. 6

846 **Online supporting material (OSM)**



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

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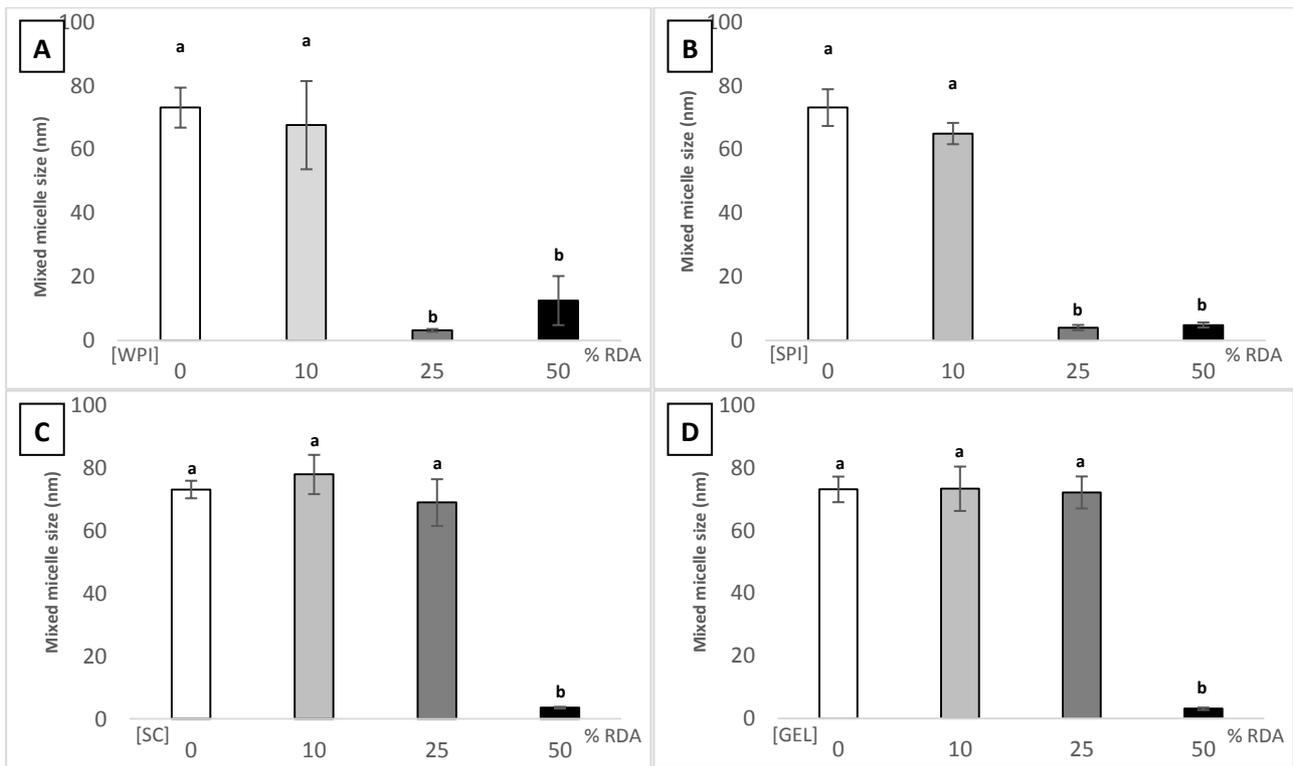
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864 **OSM – Fig. 2.** Effect of whey protein isolate (WPI, panel A), soy protein isolate (SPI, panel B),  
865 sodium caseinate (SC, panel C) and gelatin (Gel, panel D) at various concentrations on the size of  
866 the produced mixed micelles following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

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