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1 **Controlled whey protein aggregates to modulate the texture of fat-free set-type**
2 **yoghurts.**

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23

24 ABSTRACT

25

26 The impact of nanoparticulated whey protein aggregates on the texture of fat-free set-type
27 yoghurts was investigated. Monodisperse (MFA) and polydisperse (PFA) fractal aggregates
28 obtained from heated whey protein isolate (WPI) were added to skimmed milk for yoghurt
29 manufacture at four different concentrations (0.2% to 1.5%, w/w). The impact of the
30 concentration and the polydispersity of the aggregates on fat-free set-type yoghurts were
31 studied by instrumental measurements (rheology, penetrometry, syneresis and microscopy)
32 and sensory analysis. Yoghurt gel strength and firmness increased with the concentration of
33 WPI, MFA and PFA. However, yoghurts enriched with PFA clearly differed from the
34 yoghurts enriched with WPI. Indeed, yoghurts enriched with PFA were characterised by a
35 weak gel, a low firmness and a low-density of the protein network. Sensory analysis
36 confirmed the results obtained by instrumental measurements. The whey protein aggregates
37 studied are thus promising tools to modulate fat-free yoghurt texture while using milk-derived
38 ingredients.

39

40

41 **1. Introduction**

42

43 Yoghurt acceptance and consumption highly depends on texture properties (Huc,
44 Michon, Bedoussac, & Bosc, 2016). Smoothness, creaminess and mouthfeel are considered as
45 the most desirable properties by consumers (Nguyen, Kravchuk, Bhandari, & Prakash, 2017).
46 To meet consumers' expectations of low-fat products and desirable texture, stabilisers (starch,
47 gelatin, pectin) are used by yoghurt manufacturers to reduce the fat content (Cheng et al.,
48 2017). However, over the last several years, special emphasis has been given to naturally
49 made products with as few additives as possible (Thomas & Beeren, 2016). Another solution
50 to improve yoghurt texture is to add milk ingredients considered natural and safe by
51 consumers (skim milk powder, milk protein concentrate, whey protein isolate) (Matumoto-
52 Pintro et al., 2011). However, the amount of milk ingredients that can be added is limited
53 because of a powdery taste, an excessive firmness or a higher whey release that may occur at
54 high concentrations of added ingredients (Aziznia, Khosrowshahi, Madadlou, Rahimi, &
55 Abbasi, 2009; Liu et al., 2016).

56 Whey proteins are mainly composed of β -lactoglobulin and α -lactalbumin and are
57 heat-sensitive ingredients (Donato & Guyomarc'h, 2009; Nicolai, Britten, & Schmitt, 2011).
58 Heating a solution of whey protein isolate (WPI) above 70 °C induces their unfolding and
59 leads to the formation of whey protein aggregates (WPA) by covalent and non-covalent
60 interactions (Lucey, Otter, & Horne, 2017). During yoghurt manufacture, the milk is heated
61 prior to fermentation, leading to the formation of WPA, which are key elements for the gel
62 formation. WPA interact with the casein micelles and enable the formation of a three-
63 dimensional network during fermentation (Xu, Emmanouelidou, Raphaelides, & Antoniou,
64 2008). A means to optimise gel formation, and thus yoghurt texture, could be the control of
65 the properties of WPA. This can be done by preprocessing native WPI in controlled

66 conditions to obtain WPA that can then be incorporated in the milk during yoghurt
67 manufacture.

68 In recent years, several studies focused on the addition of WPA with a size between 1
69 and 10 μm in dairy products (Ipsen, 2017). WPA with a size greater than 1 μm
70 (MicroWPA) have been used in an attempt to substitute fat in low-fat yoghurts (Sandoval-
71 Castilla, Lobato-Calleros, Aguirre-Mandujano, & Vernon-Carter, 2004; Torres et al., 2018;
72 Torres, Janhøj, Mikkelsen, & Ipsen, 2011). In these studies, the addition of MicroWPA had an
73 impact on the structure of the protein network, leading to a less dense and more open
74 structure. This raises the question of the way MicroWPA, and WPA in general, are integrated
75 into the protein network.

76 It can be hypothesised that the impact of the addition of aggregates on the texture
77 depends on the way they are integrated into the protein network (Torres, Mutaf, Larsen, &
78 Ipsen, 2016). In this respect, the addition of WPA with a size less than 1 μm (NanoWPA)
79 could be interesting to improve the texture of yoghurts. Indeed, NanoWPA are more able to
80 interact with other proteins via covalent and non-covalent bindings because of their higher
81 surface area (Liu, Jæger, Nielsen, Ray, & Ipsen, 2017). Gel enhancement and better whey
82 retention have been reported in model systems enriched with NanoWPA (Andoyo,
83 Guyomarc'h, Cauty, & Famelart, 2014). Moreover, a comparison between Nano and
84 MicroWPA showed a higher firmness and viscosity as well as a lower syneresis and a denser
85 microstructure in the systems containing NanoWPA (Liu et al., 2016). NanoWPA seem to be
86 promising to improve texture in dairy products, but few studies have focused on targeted
87 aggregates in this range of size ($<1 \mu\text{m}$) and on their impact on the texture of real food
88 matrices.

89 Producing WPA with good texture functionality highly depends on the experimental
90 conditions used for their production. In this regard, the most important factors are the protein

91 concentration, the heating protocol, the type and concentration of added salts, and the pH
92 (Nicolai et al., 2011). Optimal conditions for the production of WPA have been determined to
93 produce nanoparticulated branched aggregates. These WPA are called fractal aggregates (FA)
94 and are formed from a WPI solution (5%, w/w) heated at 80 °C for 2 h at neutral pH
95 (Loiseleux et al., 2018).

96 FA structure and morphology have been characterised using innovative techniques by
97 Loiseleux et al. (2018), but the textural properties of FA have not been studied yet in complex
98 food matrices. The purpose of this study was to investigate how the presence of fractal WPA
99 affects the textural properties of fat-free set type yoghurts. For that objective, yoghurts were
100 prepared with low heat skimmed milk enriched either with native WPI, as usually performed
101 in the food industry, or with pre-formed FA. The impact on texture was assessed by linking
102 the rheological properties, the texture measurements and the yoghurt microstructure to the
103 sensory characteristics of the yoghurts.

104

105 **2. Materials and methods**

106

107 *2.1. Materials*

108

109 Low-heat spray dried skimmed milk powder (34% proteins, <1.5% fat, 8.5% ash) and
110 WPI (86.51% proteins including 1.98% caseins, 0.4% fat, 1.92% ash) were kindly supplied by
111 local dairy companies (confidential origin). The composition of the powders is given
112 according to the manufacturers information. Food grade sodium chloride (NaCl) and sodium
113 hydroxide were bought at Sigma Aldrich (Saint-Louis, MO, USA). YFL-812 (Chr Hansen,
114 Paris, France) was used as starter culture because of its low ability to produce

115 exopolysaccharides. MilliQ (Merck Millipore, Burlington, VT, USA) water was used for the
116 production of WPI solutions and FA. All ingredients were food-grade.

117

118 2.2. *Experimental design*

119

120 In the following text, the term “yoghurt” will be used to mean “fat-free set-type
121 yoghurt”.

122 The protein content in the yoghurts, the type of protein (WPI or FA) and the
123 polydispersity of FA (MFA and PFA) were the three factors studied. The concentration of
124 protein added in the yoghurts varied from 0.2% (w/w) to 1.5% (w/w) that led to yoghurts with
125 a protein content between 3.6% and 4.9%. Yoghurts were enriched with native WPI,
126 monodisperse fractal aggregates (MFA) or polydisperse fractal aggregates (PFA). The
127 experimental design is shown in Table 1.

128

129 2.3. *Preparation of protein solutions*

130

131 WPI solutions were obtained by dissolving 50 g L⁻¹ of WPI powder in Milli Q water
132 under magnetic stirring. The solutions were stored overnight at 4 °C.

133 The protocol used for the production of FA was based on the protocol reported by
134 Loiseleux et al. (2018). FA were produced from WPI solutions (50 g L⁻¹). To promote protein
135 aggregation, 15 mM and 45 mM of NaCl was added to obtain respectively MFA and PFA. The
136 solution was adjusted to pH 7 with 1 M sodium hydroxide and stored overnight at 4 °C to
137 ensure complete solubilisation. A volume of 200 mL was held in a water bath at 80 °C for 2
138 hours to obtain fractal aggregates. Due to the extensive heating time, a stable dispersion of
139 aggregates is obtained and no native protein remains (Nicolai et al., 2011).

140

141 2.4. *Characterisation of fractal aggregates*

142

143 2.4.1. *Dynamic light scattering*

144 The particle size distribution of the FA was measured by dynamic light scattering
145 (DLS) using a Zetasizer nano ZS (Malvern instruments Ltd, Malvern, England). Analysis was
146 performed on the diluted sample in Milli Q water (1:100) to avoid multiple scattering effects.
147 The diluted solutions were placed in a plastic cell and analysed at 20 °C in a backscattering
148 configuration at 173° for 120 s. Each sample was run in triplicate to measure the
149 hydrodynamic diameter. A refractive index of 1.45 was used for protein particles (Zhang,
150 Arrighi, Campbell, Lonchamp, & Euston, 2016) and the % intensity was used for further
151 analysis.

152

153 2.4.2. *Transmission electron microscopy*

154 Observations using transmission electron microscopy (TEM) were performed based on
155 the method described in Loiseleux et al. (2018). Fractal solutions were diluted to reach a
156 concentration of 0.05 g L⁻¹. A volume of 15 µL of diluted sample was deposited on a carbon
157 grid (200 meshes) and stained with 2% uranyl acetate sodium during 2 min. Samples were
158 rinsed twice with Milli Q water and dried for 20 min at 50 °C. Samples were observed using a
159 transmission electron microscope JEOL JEM-1230 (JEOL, Tokyo, Japan) operating at 80 kV.

160

161 2.5. *Yoghurt manufacture*

162

163 Skimmed milk was reconstituted to 100 g kg⁻¹ milk solids using low-heat spray-dried
164 skimmed milk powder. The reconstituted milk was stored overnight at 4 °C to allow hydration

165 of the powders. After being heated at 90 °C for 5 min, the milk was cooled to the fermentation
166 temperature (43 °C) and inoculated with the yoghurt starter culture. During yoghurt
167 manufacture, the milk was enriched either with WPI or with FA that were added either before
168 or after heat treatment of the milk (Table 1). The mix was conditioned in glass cups (40 mL
169 for sensory analysis or 70 mL for instrumental analysis, except viscoelastic properties) and
170 put in an incubator for fermentation at 43 °C during about 6 h until the pH reached 4.60. pH
171 measurements were carried out at the end of fermentation with a pH 210 Microprocessor pH
172 meter (Hanna Instruments, Woonsocket, RI, USA). Special cylindrical moulds were used for
173 rheology measurements to prepare thin slices of yoghurt without damaging the texture of the
174 gels. It was confirmed that the type of cup used had no influence on the texture properties of
175 yoghurts. The samples were stored at 4 °C for 5 days before analysis.

176

177 2.6. *Rheological measurements*

178

179 Rheological analyses were performed using an AR1000 (TA Instruments, New castle,
180 DE, USA) fitted with a sanded plate (diameter: 14 mm) to avoid slippage and a gap of 1 mm.

181

182 2.6.1. *Viscoelastic properties*

183 To define the linear viscoelastic zone, stress sweeps were performed at a frequency of
184 1 Hz between 0.1 and 1000 Pa. A stress yield (τ_0), defined as the intersection point between
185 the storage modulus G' and the loss modulus G'' , was determined during the stress sweep. A
186 strain of 1.0%, which was within the linear viscoelastic region for all samples, was selected
187 for the frequency sweep experiments (0.1 to 10 Hz) that were performed on new intact
188 yoghurt samples. For a comparative purpose, the value of G' , G'' and $\tan \delta$ at 1 Hz were
189 extracted from the frequency sweep results.

190 2.6.2. *Flow properties*

191 Flow curves were obtained by varying the shear rate logarithmically from 0.1 to 150 s⁻¹.
192 The shear stress data were fitted to the Herschel Buckley model using a nonlinear
193 regression on Excel software:

$$194 \sigma = \sigma_0 + K\dot{\gamma}^n \quad (1)$$

195 where σ is the shear stress (Pa), σ_0 is the yield stress (Pa), K is the consistency index (Pa sⁿ), $\dot{\gamma}$
196 is the shear rate (s⁻ⁿ) and n is the dimensionless flow behaviour index, which indicates the
197 proximity with the Newtonian behaviour of the fluid. Values of K , σ_0 and n were extracted
198 from the equation.

199

200 2.7. *Texture analysis*

201

202 Firmness and mechanical properties of yoghurts were determined using a traction-
203 compression device (INSTRON, Instron S.A., Boston, MA, USA). A cylindrical probe (12
204 mm diameter) was installed on the measurement cell (maximum capacity of 2 N). A 20 mm
205 penetration was applied in the yoghurt at a speed of 1 mm s⁻¹ and returned to the surface of
206 the yoghurt at the same speed. The measurements were done directly in the glass container
207 (45 mm inner diameter) at 10 °C. Data were recorded using Merlin software. Three
208 parameters were extracted from the penetrometry profiles: firmness, thickness and
209 adhesiveness (Fig. 1).

210 Firmness (N) was defined as the first significant discontinuity on the force curve,
211 produced when the probe penetrated into the yoghurt (Salvador & Fiszman, 2004). The area
212 under the curve (N m) was extracted as it is related to the work necessary to break the product
213 and it has been linked to thickness characteristics (Cayot, 2006; Seuvre, Turci, & Voilley,
214 2008). Adhesiveness (N m) was defined as the work necessary to pull the probe away from

215 the sample (Pons & Fiszman, 1996) and corresponded to the area between the negative curve
216 and the x-axis.

217

218 2.8. *Syneresis*

219

220 Syneresis was determined by centrifugation of a sample of yoghurt (10 g) at 222 g for
221 15 min at 4 °C as suggested by Saffon et al. (2013). After centrifugation, the clear supernatant
222 was poured off and weighed. The percentage of syneresis (g of drained whey per g of
223 yoghurt) was determined.

224

225 2.9. *Confocal laser scanning microscopy*

226

227 Rhodamine B isothiocyanate (RITC) was used to stain the proteins. Three hundred μL
228 of a solution of RITC (2%, w/w) was mixed with 50 g of yoghurt just after inoculation of the
229 starter culture. A few drops of the mixture were placed on a slide with a spacer, and a cover
230 slip was placed over the sample. The slide was wrapped in aluminium foil and placed in an
231 incubator at 43 °C for 6 h. The yoghurt samples were studied using a water objective of 40x
232 on a Nikon A1 confocal scanning laser microscope (Nikon, Tokyo, Japan) with a laser
233 emitting at 543 nm. Image analysis was done on ImageJ software and at least 5 representative
234 images (256×256 pixels) of each yoghurt were chosen for image analysis processing to
235 assess the porosity of the network. Porosity corresponds to the ratio between the aqueous
236 phase area and the total area of the image. The aqueous phase was defined as the pixels in the
237 image that do not contribute to the protein network. Images were transformed in grayscale
238 images and a threshold was applied on them to differentiate between the water phase and the

239 protein matrix. The particle analysis calculated the total area of the black holes (aqueous
240 phase) of the image and the % area occupied.

241

242 2.10. *Sensory analysis*

243

244 2.10.1. *Ethics*

245 The sensory tests were conducted in accordance with the Declaration of Helsinki. All
246 applicable institutional and governmental regulations concerning the ethical use of human
247 volunteers were complied with during this research study. Panellists gave written consent
248 after reading detailed information about the study. The sensory tests performed in this study
249 were approved by the ethics evaluation committee of INSERM (IRB00003888,
250 IORG0003254, FWA00005831).

251

252 2.10.2. *Organisation*

253 Panellists were recruited among students and university staff. No training was
254 necessary for the sensory tests. The analysis took place in the sensory laboratory of Oniris,
255 designed in accordance with international standards (ISO NF EN 8589). Paper forms were
256 used.

257

258 2.10.3. *Triangle tests*

259 Triangle tests were performed to determine if differences were perceived among
260 yoghurts enriched either with WPI or with FA. A triangle test consists of assessing which one
261 of the three samples (2 similar and one different) is different from the others. At first,
262 yoghurts enriched with WPI were compared with yoghurts enriched with MFA to determine if
263 texture differences existed. Yoghurts enriched with MFA and PFA were also compared,

264 bringing the overall number of triangle tests to five (Table 4). The tests were performed in 3
265 different sessions with 40 naïve participants each time. Samples were served straight from the
266 refrigerator in coded glass containers in balanced random order to account for order effects.
267 The six triads (AAB, ABA, BAA, BBA, BAB, ABB) were also counterbalanced between the
268 participants. Panellists were instructed to taste the sample to determine the different one and
269 to precise, which characteristic helped them choose (aspect, texture, flavour). They were
270 encouraged to clean their mouth between samples by drinking water (Evian, France).

271

272 *2.10.4. Ranking tests*

273 The goal of the ranking test was to order 6 yoghurts with respect to firmness. Firmness
274 was chosen for this study because it was easily understandable by naïve panellists and it could
275 be related to instrumental measurements of texture in set yoghurts. The 44 panellists recruited
276 for the test received 6 coded-yoghurt samples straight from the refrigerator in a balanced
277 random order. They were instructed to rank the samples from the least firm to the firmest and
278 were encouraged to clean their mouth between samples by drinking water (Evian).

279

280 *2.11. Statistical analysis*

281

282 One-way analysis of variance (ANOVA) was performed on the instrumental data to
283 determine if significant differences exist between samples for texture, rheological parameters
284 and syneresis. If significance was observed, the least significant difference (LSD) test was run
285 to establish differences between means (StatgraphicsCenturion XVII, Statpoint Technologies
286 Inc, Warrenton, VA, USA).

287 Regarding sensory analysis, the statistical analysis for the triangle test was based on
288 the binomial law with a p -value of 5% (Type I error).

289 A Friedman test was performed on the ranking data obtained during the sensory
290 analysis ($\alpha = 5\%$) using the software XLSTAT (Addinsoft, Andernacht, Germany, 2017). If
291 significance was observed, the least significant difference (LSD) test was run to establish
292 differences between means.

293

294 **3. Results and discussion**

295

296 *3.2. Characteristics of fractal aggregates*

297

298 Fig. 2 shows the size distribution (diameter) obtained for FA using DLS. Two types of
299 FA were obtained depending on the NaCl concentration used during production. A thin and
300 monodisperse population of fractal aggregates (MFA) with a Z-average size of 200 nm (± 38
301 nm) was obtained on addition of 15 mM NaCl. The addition of a higher level of NaCl (45 mM)
302 led to a much more polydisperse population of fractal aggregates (PFA) with two distinct sub
303 populations: some aggregates with an average size of 220 nm (± 15 nm) (identical to those
304 obtained with the addition of 15 mM NaCl), and bigger aggregates with an average size of
305 1370 nm (± 257 nm). It is already reported that the aggregation between whey proteins is
306 facilitated by the addition of salt, which leads to a reduction of electrostatic interactions by
307 increasing screening (Nicolai et al., 2011). During heating, the solutions changed
308 progressively from a transparent yellow low viscosity solution to a creamier white solution.
309 The solution with 45 mM NaCl was visually more viscous than the solution at 15 mM NaCl.
310 The difference in viscosity between FA samples obtained with different quantities of NaCl
311 could also indicate the presence of large whey protein aggregates formed during the heating
312 process.

313 TEM images were useful to supplement the DLS results and gave more details about
314 the structure of the different aggregates (Fig. 2a,b). These confirmed the size distributions
315 observed in DLS with a homogeneous population of curved-strand aggregates for small
316 amounts of NaCl (15 mM) and bigger aggregates when more NaCl was added (45 mM), as
317 shown in Fig. 2a and 2b, respectively. The fractal structure of the bigger aggregates was
318 confirmed by TEM. These were characterised by an expanded and branched structure, which
319 is in accordance with the results of Baussay, Bon, Nicolai, Durand and Busnel (2004).
320 Loiseleux et al. (2017) showed that the apparent average density of the FA decreases when
321 the size of FA increases. This is consistent with the branched morphology of large FA
322 observed in our study. This expanded structure corresponds to a second step of aggregation
323 where primary aggregates are linked by disulphide bonds and hydrophobic interactions to
324 form FA (Nicolai et al., 2011).

325

326 3.3. *Effects of FA on the rheological properties of yoghurts*

327

328 Rheological measurements of yoghurt gels were useful to determine the impact of the
329 addition of WPI, MFA and PFA on the mechanical properties of the yoghurt protein network
330 (Table 2). For all the yoghurts, enriched either with WPI, MFA or PFA, the storage modulus
331 G' was higher than the viscous modulus G'' over the whole frequency range studied (Fig.
332 3a,b). Moreover, the G' value increased slightly with frequency. These results imply that the
333 gels displayed a typical behaviour of weak gelled structures.

334 As expected, the viscoelastic parameters increased with the protein concentration for
335 yoghurts enriched with WPI. The heat treatment of milk (5 min at 90 °C) ensured a high level
336 of denaturation of the whey proteins (Kester & Richardson, 1984). As a consequence, strong
337 interactions between denatured whey proteins and casein micelles appeared. Increasing whey

338 protein concentration will lead to more interactions, enabling the formation of a denser and
339 stronger network and thus leading to higher G' values (Chua, Deeth, Oh, & Bansal, 2017;
340 Krzeminski, Großhable, & Hinrichs, 2011; Torres et al., 2011). The increase in G' value was
341 also noted for yoghurts enriched with MFA and PFA as shown in Fig. 3a for MFA. It can be
342 noted that the rise of MFA concentration led to a sharp increase of gel strength (from 0.5% to
343 1.0%, G' was increased by 61% for MFA versus 25% for WPI) (Table 2).

344 Storage modulus G' and yield stress (τ_0) gave indications on gel strength (Table 2). G'
345 and τ_0 values of yoghurts enriched with PFA were the lowest, indicating that the addition of
346 PFA led to weaker gels compared with WPI and MFA. Fig. 3b shows the differences in the
347 viscoelastic properties of the yoghurts enriched with 1% of WPI, MFA or PFA. Yoghurts
348 enriched with PFA had a 32% lower G' compared with MFA-enriched yoghurts. These results
349 confirmed and extended the conclusions of a previous study about the impact of whey protein
350 aggregates in model WPI gels (Purwanti et al., 2011). At the same protein concentration
351 (15%, w/w), the gel formed with larger whey protein aggregates (diameter of 62.7 nm) was
352 weaker compared with the gel formed with smaller whey protein aggregates (diameter of 49.3
353 nm), while the gel formed with native WPI showed the strongest gel. The strength of the
354 protein gel highly depends on surface interactions (e.g., hydrophobic interactions) between
355 WPA (Andoyo, Guyomarc'h, & Famelart, 2016; Purwanti et al., 2011). Therefore, the size
356 and the number of particles, as well as the number of connections and the distance between
357 the particles, are key elements for the gel formation. These interactions have been suggested
358 to be hydrophobic patches on the aggregates surface (Andoyo et al., 2016). In the current
359 study, the larger particles in PFA have a more branched and expanded structure, as reported
360 by Loiseleux et al. (2018) and Mahmoudi et al. (2007). The presence of these larger
361 aggregates will lead to an increased surface, but probably with less hydrophobic patches, as

362 the increased steric hindrance will restrict the access to reactive sites. This can substantially
363 limit hydrophobic interactions, thus resulting in a lower gel strength.

364 The study of the yield stress (τ_0) allowed further characterisation of the yoghurts. As
365 shown in Table 2, for the same protein concentration, yoghurts enriched with WPI exhibited a
366 significantly higher yield stress than yoghurts enriched with both MFA and PFA. The yield
367 stress is the point where the network structure starts to be disrupted, causing the yoghurt to
368 flow. Therefore, it could be related to in-mouth sensations and texture perception. The model
369 parameters calculated from the viscosity measurements (K and n) also indicated a difference
370 between yoghurts enriched with WPI and yoghurts enriched with FA. Yoghurts with the
371 lowest flow behaviour indexes corresponded to yoghurts enriched with WPI (WPI10 and
372 WPI15) and to yoghurts enriched with high concentrations of MFA or PFA (MFA15 and
373 PFA15) (Table 2). This means that, for yoghurts with added WPI, the higher the amount of
374 added protein, the more shear-thinning the behaviour and the lower the viscosity at high
375 shear. The other yoghurts enriched with MFA and PFA were grouped together regardless of
376 the concentration added. A 2-way ANOVA on protein concentration and protein type
377 confirmed these results by highlighting a significant difference between yoghurts enriched
378 with WPI and yoghurts enriched with both MFA and PFA (results not shown). This
379 significant difference in flow behaviour could explain differences in texture perception (Daget
380 & Joerg, 1991).

381

382 3.4. *Effect of fractal aggregates on the textural characteristics of yoghurts*

383

384 The penetrometry test was used to assess the impact of FA and their polydispersity on
385 mechanical properties of yoghurts at large deformations (Table 3). The aim was to relate the
386 results with the sensory perception of texture.

387 Firstly, the results of the texture analysis confirmed the differences evidenced in
388 rheology between yoghurts enriched with WPI and yoghurts enriched with FA. WPI-enriched
389 yoghurts were always significantly firmer and thicker than the MFA enriched ones. At 1%
390 added protein, firmness was 25% higher in yoghurts enriched with WPI than in yoghurts
391 enriched with MFA. The same trend was found for thickness. Less work was necessary to
392 disrupt the yoghurts enriched with FA. This could imply that, at the same protein content, the
393 yoghurts enriched with MFA or PFA could be perceived as less stiff or less compact than
394 yoghurts enriched with WPI. This property may be interesting to provide protein-enriched
395 yoghurts with a softer texture, which could be more desirable for consumers.

396 The firmness of MFA-enriched yoghurts increased significantly with increasing
397 concentrations of MFA, which indicated their texturising properties. MFA15 had the highest
398 firmness, which was about 3 times higher than that of MFA02. The two other parameters,
399 thickness and adhesiveness, also increased with the increase of MFA concentration. These
400 results are consistent with rheological measurements which showed a gel reinforcement with
401 MFA addition. A contribution of whey protein aggregates to gel hardness has been previously
402 reported by Vasbinder, van de Velde, and de Kruif (2004), who showed that the addition of
403 whey protein aggregates (diameter of 62 nm) to a whey protein-free skim milk led to an
404 increase of gel hardness thanks to the formation of disulphide bonds between aggregates and
405 casein micelles.

406 Finally, where 1.0% or 1.5% of FA was added to the yoghurts, a significant difference
407 was found between MFA and PFA. Yoghurts enriched with MFA were significantly firmer
408 and thicker than yoghurts enriched with PFA. Liu et al. (2017) compared microparticulated
409 and nanoparticulated and showed that the firmness of yoghurts enriched with nanoparticulated
410 whey proteins was always higher than that of yoghurts enriched with microparticulated whey
411 proteins. It is interesting to note that changing the polydispersity of whey protein aggregates

412 in a smaller size range ($\leq 1 \mu\text{m}$) led to comparable results. It can be assumed that the reduction
413 of the ability of large aggregates to interact with the protein network formed during
414 acidification induced a reduction of firmness and thickness.

415 These results at high deformation suggested that it is possible to modulate texture
416 perception of yoghurts by adding FA, and more precisely by adding MFA or PFA depending
417 on the desired level of firmness and thickness.

418

419 3.5. *Syneresis*

420

421 Syneresis measurements are shown in Table 3. As expected, the rise of protein
422 concentration in yoghurts led to a reduction of whey separation. Syneresis showed an opposite
423 tendency to firmness; the percentage of syneresis was 40% lower when the % of added
424 proteins was increased from 0.2% to 1.0%. No more decrease of whey drainage was observed
425 when more than 1% WPI and MFA were added to the yoghurts. This suggested that a
426 minimum level of expelled whey had been reached by increasing the protein concentration.
427 Interestingly, independently of their polydispersity, FA had the same impact on whey
428 separation as native WPI for the same concentration. While the presence of a population of
429 big aggregates in the FA led to yoghurts that were significantly less firm and less thick, this
430 population has the same water-holding capacity as WPI and MFA. It can be hypothesised that,
431 thanks to their large expanded structure, the population of large FA is able to bind more water
432 than smaller FA. However, as seen before, these larger aggregates exhibit less interactions
433 with the protein network, thus inducing a lower gel firmness. The protein concentration seems
434 to be the only factor influencing whey separation. This result differs from previous studies
435 where yoghurts with the lowest G' and the lowest firmness are usually linked to highest

436 values of whey drainage (Andoyo et al., 2014). Liu et al. (2017) also reported that the addition
437 of MicroWPA in a model gel significantly increased syneresis compared with NanoWPA.

438

439 3.6. *Effect of FA on the microstructure of yoghurts.*

440

441 The confocal laser scanning microscopy (CLSM) images were used to visualise the
442 protein microstructural network of the yoghurts enriched with different types of whey proteins
443 (Fig. 4). The micrographs showed that the three samples (WPI10, MFA10 and PFA10) had a
444 continuous and homogeneous protein network. However, MFA10 seemed to have a denser
445 network in comparison with PFA10 and WPI10, which had a more open structure with larger
446 pores. To confirm these small differences, image analysis was performed on a series of
447 micrographs to quantify the porosity of the network. Porosity is defined as the division of the
448 void area by the total image area and it is a way to characterise the protein network by
449 quantifying the geometrical properties of the yoghurt structure (Silva & O'Mahony, 2018;
450 Silva, Legland, Cauty, Kolotuev, & Flourey, 2015). MFA10 had a significantly lower porosity
451 than PFA10. But, surprisingly, WPI did not have a denser network than PFA (Table 3).

452 Although porosity was significantly different between MFA and PFA samples, it was
453 difficult to make a visual difference at this scale of observation. However, the difference in
454 porosity could explain the lower G' , K , firmness and thickness obtained in the rheological and
455 textural analysis. Previous studies reported the existence of large serum channels in the
456 protein network of yoghurts enriched with MicroWPA (Silva & O'Mahony, 2018; Torres et
457 al., 2011). The MicroWPA belonged to a larger size range particle (from 6 to 60 μm), leading
458 to clear differences between samples. According to Liu et al. (2016), the increase in the size
459 of whey protein aggregates goes hand in hand with a lower connectivity and inability to be
460 integrated in the protein network. The increased polydispersity of FA and the presence of a

461 population of large FA could thus disturb the protein network formation, causing the
462 development of larger pores. The open structure of the acid milk gels has been linked to a
463 decreased water-holding capacity (Torres et al., 2011). The moderate impact of the change in
464 the polydispersity of FA on the protein network structure might be the reason why no
465 detrimental whey release was measured in yoghurts enriched with PFA.

466

467 3.7. *Sensory analysis*

468

469 Sensory tests were performed to determine if the observed impact of FA on
470 mechanical properties, texture and microstructure were perceived by panellists.

471 Two sets of triangle tests were performed to determine if the differences between
472 yoghurts enriched with MFA and WPI were perceived, and secondly to find out if the
473 polydispersity of FA had an impact on texture perception (Table 4). For an enrichment of
474 0.2% of either WPI or MFA, the test was non-conclusive. For this low level of proteins, the
475 panellists did not perceive differences between samples even if WPI02 had significantly
476 higher G', K, firmness and thickness values than MFA02. However, differences were
477 perceived by panellists for samples with higher added protein concentrations (MFA05 versus
478 WPI05) (Table 4). This result showed the existence of a concentration threshold above which
479 the addition of MFA has an impact on the sensory perception of yoghurts.

480 The impact of the polydispersity of the FA on texture perception was assessed by
481 comparing yoghurts enriched with 0.5%, 1.0% and 1.5% of PFA and MFA. For yoghurts
482 enriched with 0.5% or 1.0% of FA, no differences were perceived, whereas a significant
483 difference was perceived between PFA15 and MFA15. The result for PFA05 and MFA05 was
484 consistent with instrumental measurements, because no significant difference in firmness or
485 thickness was detected. In contrast, for 1.0% of added protein, panellists did not perceive

486 differences, even if significant differences were measured both in texture analysis and in the
487 CLSM micrographs. Considering together the results of the triangle tests and the instrumental
488 data, it can be hypothesised that yoghurts enriched with PFA were less firm than yoghurts
489 enriched with MFA. Torres et al. (2011) studied the impact of two populations of MicroWPA
490 using quantitative descriptive analysis (QDA) in low-fat yoghurts. For the same protein level,
491 yoghurts enriched with the bigger WPA (30-58 μm) were characterised by a smoother but
492 grainier texture than yoghurts enriched with smaller WPA. Using NanoWPA such as MFA
493 and PFA could be relevant to tackle the problem of graininess while having a positive impact
494 on the texture of yoghurts.

495 In the triangle tests, panellists were asked to choose the criteria (appearance, flavour,
496 odour, texture) on which they based their choice to make a difference. For all the triangle
497 tests, more than 80% of the panellists who gave the right answer based their choice on texture
498 (results not shown). This suggests that the addition of native WP or FA was not responsible
499 for the appearance of aromatic defaults or aftertastes.

500 Finally, a ranking test was performed to confirm the differences in texture perceived
501 between yoghurts enriched with WPI and MFA by the panellists. Panellists were asked to
502 order the yoghurts from least to firmest (Fig. 5). Firmness perceived by panellists increased
503 with the quantity of added proteins, and the MFA-enriched yoghurts were always perceived
504 slightly less firm than the native WPI-enriched yoghurts. These results corroborated the
505 previous rheological and texture measurements.

506 Future work will focus on sensory descriptive tests to assess the impact of FA on other
507 modalities. It would be interesting to evaluate if MFA, besides their texturising properties,
508 have a positive impact on other desirable descriptors such as smoothness.

509

510 **4. Conclusions**

511

512 The results showed that the addition of FA modified the texture properties of fat-free
513 set-type yoghurts compared with the conventionally used native WPI. The differences
514 measured instrumentally were also perceived by panellists, who found yoghurts enriched with
515 FA less firm than yoghurts enriched with WPI. These results obtained for fat-free yoghurts
516 confirmed and expanded prior work done on NanoWPA in model systems (Andoyo et al.,
517 2014; Purwanti et al., 2011).

518 Yoghurts enriched with PFA clearly differed from yoghurts enriched with MFA and
519 WPI regarding the mechanical properties of the gel and the texture properties. The presence of
520 a population of large aggregates might be the reason why yoghurts enriched with PFA had a
521 lower storage modulus, yield stress, firmness and thickness. On the contrary, yoghurts
522 enriched with MFA had a denser protein network, which helps to explain their higher
523 firmness. Interestingly MFA and PFA samples had identical properties to WPI regarding
524 whey retention. This study confirms previous results in a complex food matrix. By using both
525 instrumental and sensory analyses, the impacts of structure and morphology modifications of
526 WPA on texture properties and texture perception of fat-free set-type yoghurts were
527 highlighted. These results are relevant to obtain desirable texture properties while developing
528 fat-free dairy products using natural dairy ingredients.

529

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531

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539

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643

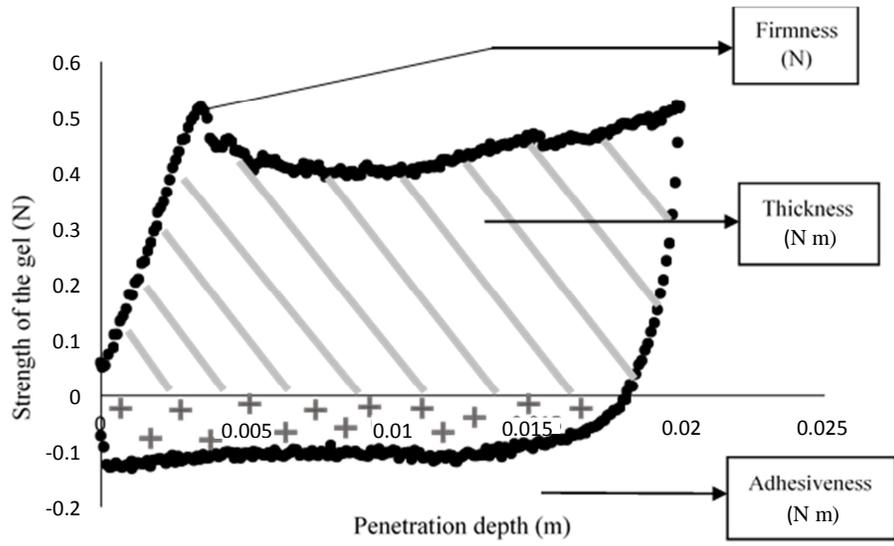


Figure 1

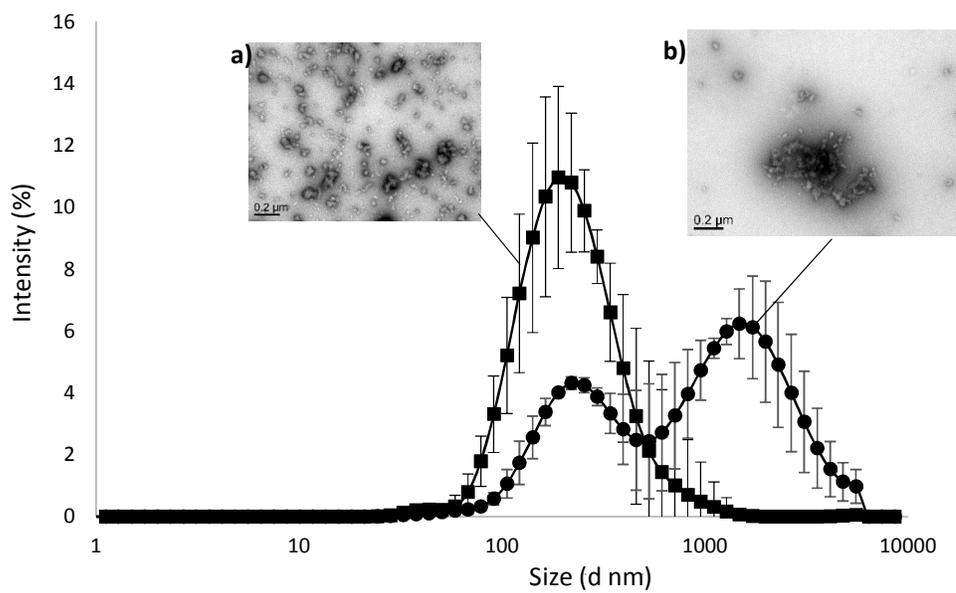


Figure 2

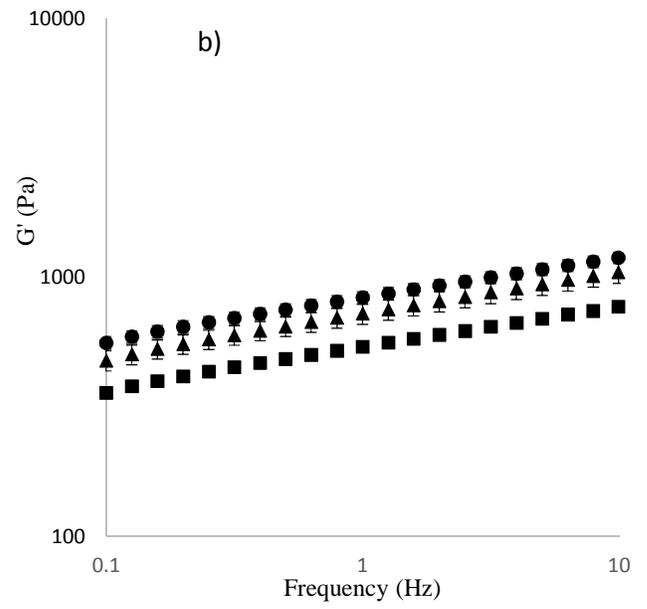
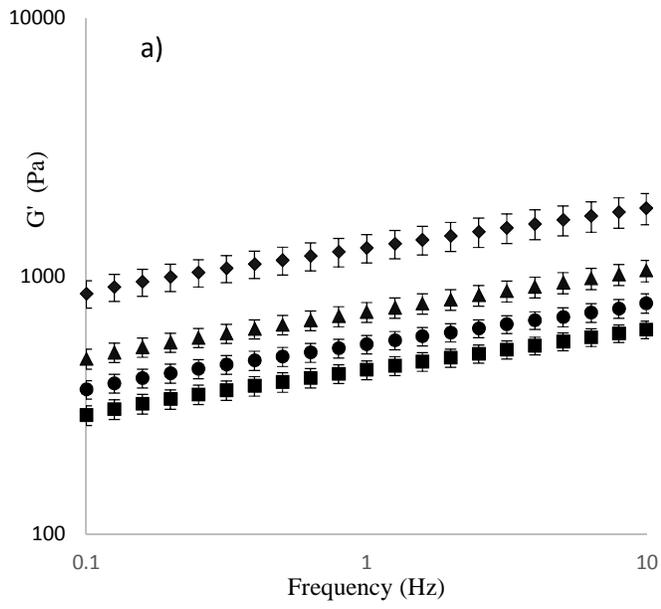


Figure 3

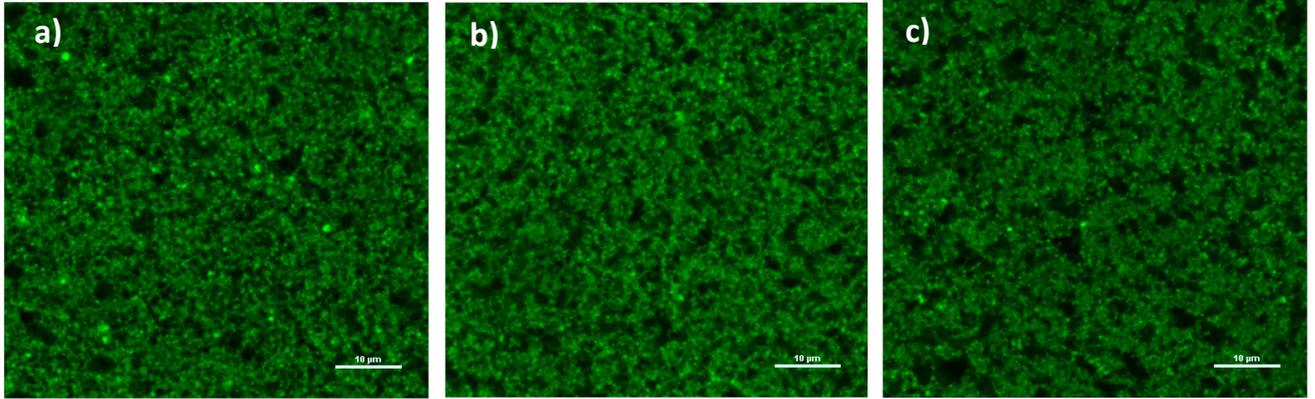


Figure 4

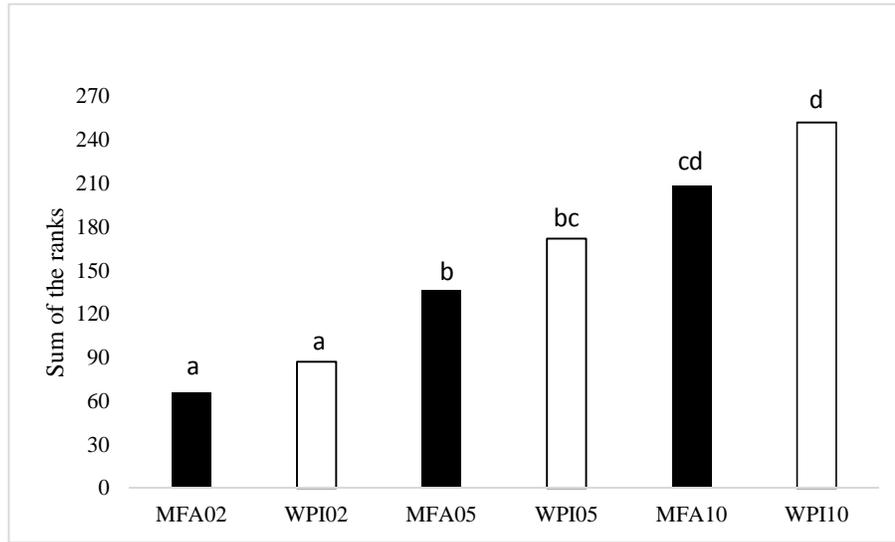


Figure 5

Table 1

Overview of the experimental design used. ^a

Yoghurt sample codes	Protein content (% w/w)		Type of protein added (% w/w)	
	Total protein level	Total whey protein	Native WPI	Fractal aggregates
Control	3.40	0.68	-	-
WPI02	3.60	0.75	0.2	-
MFA02	3.60	0.75	-	0.2
PFA02	3.60	0.75	-	0.2
WPI05	3.90	1.02	0.5	-
MFA05	3.90	1.02	-	0.5
PFA05	3.90	1.02	-	0.5
WPI10	4.40	1.46	1.0	-
MFA10	4.40	1.46	-	1.0
PFA10	4.40	1.46	-	1.0
MFA15	4.90	1.90	-	1.5
PFA15	4.90	1.90	-	1.5

^a Two factors were varied: the total protein content (theoretical values in %, w/w), and the type of protein (WPI, MFA and PFA) added to the yoghurts.

Table 2Rheological properties of yoghurts. ^a

Yoghurt sample codes	Frequency sweep		Stress sweep	Fitting of power law model	
	G' (Pa) at 1 Hz	G'' (Pa) at 1 Hz	τ_0 (Pa)	K (Pa s)	n
Control	342.8±38 ^h	91.5±10.2 ^h	56.51±1.34 ^g	46.69±2.47 ⁱ	0.12±0.01 ^{cba}
WPI02	572.7 ±60.42 ^{fed}	151.6 ±14.97 ^{def}	82.28±8.68 ^{def}	72.40±7.66 ^{fe}	0.11±0.03 ^{cb}
MFA02	443.24 ±30.01 ^{hg}	116.6 ±7.87 ^{gh}	65.66±8.81 ^{fg}	59.96±5.98 ^{hg}	0.15±0.01 ^a
PFA02	363.3±32.5 ^h	99.3±6.33 ^h	69.9±11 ^{fg}	54.02±7.99 ^{ih}	0.12±0.02 ^{ba}
WPI05	662.06 ±95.46 ^d	174.0 ±22.70 ^d	102.8±10.9 ^{cd}	83.97±11.24 ^d	0.07±0.02 ^d
MFA05	549.2 ±46.84 ^{fe}	143.3 ±12.02 ^{efg}	77.44±14.9 ^{ef}	68.28±9.31 ^{gf}	0.12±0.02 ^{cba}
PFA05	523.3 ±100.33 ^{gf}	134.6 ±22.74 ^{fg}	67.484±18 ^{fg}	63.18±14.34 ^{hgf}	0.14±0.02 ^a
WPI10	825.53 ±56.90 ^c	203.0 ±16.79 ^c	127.76±6.74 ^b	107.61±6.86 ^{cb}	0.04±0.01 ^c
MFA10	883.9 ±93.89 ^c	224.5 ±32.13 ^b	106.0±23.1 ^c	103.66±11.55 ^c	0.11±0.02 ^c
PFA10	642.5 ±45.86 ^{de}	163.2 ±9.97 ^{de}	90.7±6.23 ^{cde}	83.77±13.10 ^{cd}	0.13±0.03 ^{ba}
MFA15	1319.75 ±167.06 ^a	335.9 ±49.85 ^a	157.8±17 ^a	110.51±13.15 ^a	0.09±0.02 ^d
PFA15	989.62 ±97.01 ^b	245.4 ±22.18 ^b	146.3±5.24 ^{ab}	132.15±6.29 ^b	0.08±0.02 ^d

^a Values within a column not sharing a common superscript are significantly different ($p < 0.05$) according to the LSD method. The values of G', G'' and $\tan\delta$ are taken at 1Hz. Sample codes are: Control, yoghurt without any protein addition; WPI, yoghurt enriched with WPI; MFA, yoghurt enriched with monodisperse fractal aggregate; PFA, yoghurt enriched with polydisperse fractal aggregates.

Table 3

Results of the texture analysis from the penetrometry tests and the syneresis measurements of yoghurts enriched in different types of whey proteins. ^a

Yoghurt samples codes	Penetrometry test			Syneresis	CSLM
	Firmness (N)	Adhesiveness (N mm)	Thickness (N mm)	Syneresis (%)	Porosity (%)
Control	0.15±0.01 ^h	0.52±0.07 ^f	3.50±0.14 ^h	29.39±0.2 ^{ab}	-
WPI02	0.22±0.03 ^f	1.84±0.24 ^{cd}	4.83±0.47 ^f	29.85±3.0 ^{ab}	-
MFA02	0.16 ±0.01 ^h	1.19±0.17 ^{def}	4.01±0.35 ^h	30.98±2.3 ^a	-
PFA02	0.16±0.02 ^{gh}	0.81±0.09 ^{ef}	3.82±0.34 ^{gh}	30.61±2.7 ^a	-
WPI05	0.34±0.04 ^{cd}	2.14±0.79 ^{bc}	7.09±0.99 ^{cd}	26.05±1.8 ^c	-
MFA05	0.20±0.02 ^{fg}	1.44±0.23 ^{de}	4.77±0.40 ^{fg}	25.54±0.6 ^c	-
PFA05	0.17±0.03 ^{gh}	1.16±0.41 ^{def}	4.20±0.81 ^{gh}	27.07±1.2 ^{bc}	-
WPI10	0.40±0.02 ^b	2.58±0.47 ^b	8.35±0.19 ^b	18.83±2.5 ^{de}	12.86±0.4 ^a
MFA10	0.32±0.02 ^d	2.31±0.40 ^b	7.44±0.65 ^d	18.52±2.0 ^{de}	7.86±2.4 ^b
PFA10	0.26±0.01 ^e	1.43±0.23 ^{de}	6.12±0.36 ^e	21.49±1.5 ^d	14.39±1.9 ^a
MFA15	0.47±0.03 ^a	4.25±1.21 ^a	10.32±0.85 ^a	16.58±0.7 ^e	-
PFA15	0.35±0.01 ^c	2.33±0.24 ^{bc}	8.21±0.41 ^c	16.73±0.4 ^e	-

^a Values within a column not sharing a common superscript are significantly different ($p < 0.05$) according to the LSD method. Sample codes are: Control, yoghurt without any protein addition; WPI, yoghurt enriched with WPI; MFA, yoghurt enriched with monodisperse fractal aggregate; PFA, yoghurt enriched with polydisperse fractal aggregates.

Table 4

Results of the triangle tests performed on the yoghurts enriched with different protein types.

Test	Total number of panellists	Number of correct answers	Significant difference between samples
WPI02 versus MFA02	40	17	No
WPI05 versus MFA05	40	24	Yes ($p = 0.00049$)
MFA05 versus PFA05	36	14	No
MFA10 versus PFA10	36	16	No
MFA15 versus PFA15	36	20	Yes ($p = 0.0047$)