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27 ABSTRACT

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29 Hydrophobisation of whey proteins, followed by erythritol addition into the hydrophobised protein dispersion, enables formation of water-in-water (W/W) emulsions of the proteins and 30 alginate. In this study, we investigated the interfacial and gel rheology of whey proteins as 31 32 affected by hydrophobisation, heat denaturation and erythritol addition. Additionally, the gel rheology of the resultant W/W emulsion was assessed. Hydrophobisation shortened the linear 33 viscoelastic region and decreased the surface storage modulus (G'_s) , the flow stress and the 34 35 flow strain of protein layer at the air-liquid interface. Erythritol addition into the 36 hydrophobised protein caused a further reduction of G'_s . In accordance with interfacial rheology, protein hydrophobisation and erythritol addition decreased the dynamic moduli of 37 acid-induced protein gels. Frequency sweep tests indicated that the gelled emulsion had 38 higher dynamic moduli than all WPI gels. The higher firmness of the emulsion gel was 39 40 ascribed, based on microstructural images, to micro-phase separation of alginate droplets. 41

43 1. Introduction

44

Different types of whey proteins, such as β -lactoglobulin (β -lg) and bovine serum 45 albumin, as well as whey protein products such as whey protein isolate (WPI) and whey 46 protein concentrate (WPC), are widely used in various fields of food technology, including 47 foam and emulsion stabilisation (Jaishankar, Sharma, & McKinley, 2011), fat replacement, 48 49 and gel formation (Zhang, Arrighi, Campbell, Lonchamp, & Euston, 2018). Each application may require modification of one or several functional properties of whey proteins. Enzymatic 50 hydrolysis, heat-induced polymerisation (Foegeding, Davis, Doucet, & McGuffey, 2002), 51 52 high pressure processing (Liu, Powers, Swanson, Hill, & Clark, 2005), and chemical 53 functionalisation (Zhao, Ma, Yuen, & Phillips, 2004) are examples of whey protein modifications. 54

55 Recently, WPI was hydrophobised through a process consisting of two consecutive steps including a pre-acetylation stage followed by a combined acetylation/heat treatment 56 period. The process resulted in acetylation of $\geq 90\%$ of available amino groups of whey 57 proteins. Details about the structural characterisation of the hydrophobised WPI have been 58 provided elsewhere (Madadlou, Floury, & Dupont, 2018). The modified product was then 59 60 used to form a water-in-water (W/W) emulsion of alginate and WPI, which was attributed to the adsorption of protein particles to the liquid-liquid interface. The interfacial tension 61 between the two incompatible biopolymer phases was further modulated by addition of 62 erythritol, a low-calorie sugar alcohol (Madadlou, Saint-Jalmes, Guyomarc'h, Floury, & 63 Dupont, 2019). 64

Adsorption of macromolecules and particles to the liquid/liquid interface during
emulsification forms a viscoelastic layer, which can be assessed by shear rheology
components (elasticity and viscosity). Shear rheology characterises the response of the

(particle-laden) interface to a shape deformation caused by an external mechanical 68 69 perturbation, in a similar way done for bulk rheology (Mendoza et al., 2014). Inter-particle attractive and repulsive forces such as electrostatic and hydrophobic interactions govern the 70 71 interface rheology. Indeed, interfacial rheology is very sensitive to the structure and composition of a protein layer formed at the interface, as well as to the nature of interactions 72 within the layer (Dickinson & Matsumura, 1991). Therefore, it was presumed that probing 73 74 interfacial rheology is advantageous for assessing the emulsification behaviour of whey proteins as influenced by hydrophobisation. 75

Rheological analysis is also helpful to study the gelation properties of whey proteins. 76 77 Cold-set gelation of whey proteins is of great value in forming particles that can modify viscosity (Zhang et al., 2018), and encapsulate heat-labile cargos (Farjami, Madadlou, & 78 Labbafi, 2016; Hashemi, Madadlou, & Salami, 2017). This property can also be used to form 79 80 whey protein-stabilised emulsion gels for fortification and technological purposes. For example, such emulsions can be incorporated into cultured milk products like yoghurt to 81 82 improve texture (Boutin, Giroux, Paquin, & Britten, 2007). The cold-set gelation process takes place by a two-step consecutive procedure. Initially, the proteins are heat-denatured at 83 non-gelling conditions (i.e., at low ionic strength, low protein content and at pH values far 84 85 from the proteins' isoelectric point, pI), which causes formation of whey protein soluble aggregates. Then the protein dispersion is gelled at ambient temperature by adding salts 86 and/or adjusting pH toward the pI (Ju & Kilara, 1998; Meydani, Vahedifar, Askari, & 87 Madadlou, 2019). The rheology of such gels is governed by the interaction between the 88 aggregating protein particles, which is highly dependent on the aggregate chemistry (Alting, 89 Hamer, De Kruif, & Visschers, 2003). Therefore, the modification of whey proteins via 90 hydrophobisation is expected to alter the cold-set gelation behaviour and the gel rheology of 91 WPI. 92

93		The first objective of the present study was to analyse the interfacial rheology of WPI
94	at the	air-liquid interface as influenced by heat denaturation and hydrophobisation treatments,
95	as wel	l as, erythritol (E) addition into the hydrophobised WPI. Subsequently, bulk rheological
96	experi	ments were carried out to assess the acid-induced cold-set gelation process and the gel
97	proper	ties of the hydrophobised WPI (with and without E) and the alginate-supplemented E-
98	added	hydrophobised WPI (i.e., W/W emulsion).
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100	2.	Materials and methods
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102	2.1.	Materials
103		
104		WPI was received as a gift from Lactalis ingredients (Bourgbarré, France). It
105	contai	ned, according to the information provided by the manufacturer, 90% protein, 5.1%
106	water,	3.0% lactose, and 1.97% ash. Reversed-phase high-performance liquid
107	chrom	atography (RP-HPLC) analysis indicated that the α -lactalbumin and β -lactoglobulin
108	conter	nt of the WPI was 16% and 56% of total protein, respectively. Erythritol was purchased
109	from V	Vita-World GmbH (Taunusstein, Germany). Alginic acid sodium salt from brown algae
110	was p	rocured from Sigma-Aldrich (SKU W201502, Sigma-Aldrich Chemie S.a.r.l., Saint-
111	Quent	in Fallavier, France). Sodium hydroxide and hydrochloric acid were bought from
112	Sigma	-Aldrich. Ultrapure, 15 m Ω -resistivity water prepared by reverse osmosis was used
113	throug	shout the study and all chemicals were analytical grade.
114		
115	2.2.	Protein modification, emulsion preparation and gelation
116		

117	WPI was hydrophobised following the method described in our previous report
118	(Madadlou et al., 2018). Briefly, WPI powder was hydrated (65 mg mL ⁻¹) for 18 h and
119	supplemented with acetic anhydride at a ratio of 0.18 g g ⁻¹ protein. After 30 min acetylation
120	while the pH was kept over 8.0 through successive additions of 1 M NaOH, the WPI
121	dispersion was heated at 85 °C for 20 min. A non-acetylated but heat-denatured WPI sample
122	was also prepared by sequentially adding 1 ${\rm M}$ HCl (200 ${\mu}L),$ and 1 ${\rm M}$ NaOH (300 ${\mu}L)$ to a
123	native WPI dispersion (9.5 mL, pH 6.7) to set pH to 8.25 ± 0.05 , and subsequent heat
124	treatment at 85 °C for 20 min. The sequential addition of the acid and alkali was carried out to
125	mimic the fall and rise of pH during the hydrophobisation process. After heat treatment, the
126	pH value of the hydrophobised and heat-denatured WPI samples was adjusted to 7.50 ± 0.05
127	using approximately 20 µL of either 1 M HCl or 1 M NaOH.
128	The alginate-in-WPI W/W emulsions were prepared according to the method
129	described elsewhere (Madadlou et al., 2019) with minor changes. At first, erythritol was
130	added (100 mg mL ⁻¹) into the hydrophobised WPI dispersion and stirred, followed by addition
131	of sodium alginate solution (30 mg mL ⁻¹) into WPI-erythritol mixture at a volume ratio of 1:9.
132	The mixture was vortexed at a rotating frequency of 40 Hz (Bioblock Top Mix 11118, Fisher
133	Scientific, Waltham, MA, USA) for 90 s to obtain the W/W emulsion.
134	The hydrophobised (with and without added erythritol) and heat-denatured WPI
135	samples, as well as the alginate-in-WPI mixed (i.e., W/W emulsion) system (pH 7.50 ± 0.1)
136	were gelled at 37 °C by acidification. Glucono- δ -lactone (GDL) was added (5, 10 and 20 mg
137	mL ⁻¹) into the samples to gradually hydrolyse into gluconic acid, causing acid gel formation.
138	The samples were stored at 37 °C for 24 h before analyses. The pH value of the samples
139	during gelation was monitored using a digital pH meter (CyberScan Series pH 110, Eutech
140	Instrument, Singapore). The accuracy of the pH measurement was ±0.02 pH unit.
141	

A stress-controlled rheometer (Discovery Series Hybrid Rheometer (DHR) HR-2, 144 New Castle, DE, USA) using the double wall ring (DWR) geometry was used to perform the 145 interfacial shear analyses including strain amplitude sweep and creep tests. The WPI sample 146 was poured into a round trough and an annular ring made of a platinum-iridium alloy was 147 positioned at the liquid-air interface. The DWR geometry has been thoroughly described 148 (Vandebril, Franck, Fuller, Moldenaers, & Vermant, 2010) and provides certainty that high 149 values of the Boussinesq number are attained. Therefore, the contribution of the induced sub-150 151 phase flow to torque is negligible and the interfacial torque dominates (Jaishankar & McKinley, 2013). Based on time sweep tests at a strain amplitude of 0.1% and an angular 152 frequency of 10 rad s⁻¹ and on the literature review (Jaishankar et al., 2011), all steady state 153 154 experiments were carried out after waiting for 3 h at 20 °C. WPI dispersions (56 mg mL⁻¹) were loaded into the trough, the annular ring was positioned at the interface and after the rest 155 156 period, the experiments were carried out. It is noteworthy that in the current study, the interfacial shear rheology of WPI 157 dispersions was analysed at the same concentration as emulsion preparation was done. 158 159 Commonly, the interfacial rheology tests are performed at much lower concentrations of amphiphilic compounds to make sure that a monolayer of the compounds is present at the 160 interface. However, as noted earlier (Lexis & Willenbacher, 2014), the structure of the 161

interfacial layers of proteins depends largely on concentration; therefore, a correlation
between properties such as foaming and emulsification, and interfacial rheology is achieved
only when the interfacial layers at both conditions (i.e., emulsion formation and interfacial
rheology test) are comparable. Nonetheless, as the thickness of the interfacial layer may not
be negligible anymore, the measured moduli are treated as apparent values. Similar

167 concentrations have already been used for the interfacial rheological analysis of bovine serum
168 albumin (Jaishankar & McKinley, 2013; Jaishankar et al., 2011; Sharma, Jaishankar, Wang,
169 & McKinley, 2011).

After amplitude sweep tests, creep tests were performed on WPI interfacial layers over 170 short durations. In stress-controlled rheometry, for sufficiently viscoelastic fluids, even if they 171 are simple Newtonian fluids, a ringing event (i.e., damped oscillations) is observed at the start 172 173 of a creep test (Ewoldt & McKinley, 2007). The coupling of fluid elasticity and instrument inertia causes periodic oscillations which decay exponentially due to viscous dissipation 174 (Jaishankar & McKinley, 2013). The creep ringing logarithmic decrement, Δ and ringing 175 frequency, ω_* (Nagarkar et al., 2009) of WPI interfacial layers were calculated (where 176 applicable) using the following equations: 177

178
$$\Delta = 2ln \left[\frac{(J_1 - J_2)}{(J_3 - J_2)} \right]$$
 [Eq. 1]

179
$$\omega_* = \frac{1}{(t_3 - t_1)}$$
 [Eq. 2]

180 where J_1 , J_2 , and J_3 are the compliance values of the 1st peak, 1st valley and 2nd peak of the 181 creep ringing data, respectively (Nagarkar et al., 2009) and t_3 and t_1 are the times of the 2nd 182 peak and 1st peak, respectively.

183

184 2.4. Particle size and ζ -potential of WPI dispersions during gelation

185

186 The hydrodynamic diameter and ζ -potential of protein particles during gelation were 187 approximated by dynamic light scattering (DLS) following the methods of Ju and Kilara 188 (1998) and de Jong, Klok, and van de Velde (2009a) for acid-induced gelation of whey 189 proteins. A Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) was used for 190 this purpose. Protein samples (at 37 °C) were supplemented with GDL (20 mg mL⁻¹ for 191 hydrophobised WPI and 5 mg mL⁻¹ for heat-treated WPI), and stored at 37 °C (de Jong, Klok,

192	& van de Velde, 2009b) for different durations, i.e., 130 min for the hydrophobised WPI
193	samples and 35 min for the heat-denatured WPI. At various time intervals, the samples were
194	diluted 100 fold with deionised water and subjected to DLS analysis. Dilution did not have a
195	remarkable influence (less than 0.2 unit) on the pH value of the samples. The refractive index
196	of dispersant (water) was 1.33, and the dispersant viscosity was set to 0.69 mPa s. A laser-
197	backscattering angle of 173° and a wavelength of 633 nm were applied in size measurements.
198	
199	2.5. Rheological analyses of WPI and alginate-WPI gel samples
200	
201	The gelation time (Tig) and rheological properties of WPI and emulsion samples were
202	determined using an Anton Paar Physica MCR 301 rheometer (Anton Paar GmbH, Graz,
203	Austria) equipped with a cone-plate geometry (diameter 49.96 mm and cone angle 1.99°).
204	Samples supplemented with GDL (20 mg mL ⁻¹ for the hydrophobised WPI and the emulsion
205	and 5, 10, and 20 mg mL ^{-1} for the heat-denatured WPI) were poured onto the plate, followed
206	by introducing the cone. Temperature was kept at 37 °C. Low-density paraffin was added
207	around the samples to prevent evaporation. Changes in storage modulus (G'), loss modulus
208	(G"), and damping factor (tan δ) during gelation of the samples were measured at an angular
209	frequency of 1 rad s ⁻¹ and 0.1% strain. Gelation time (Ti _g) was arbitrarily defined as $G' = 1$ Pa
210	(Andoyo, Guyomarc'h, & Famelart, 2016). Gels were oscillated in situ for 24 h to obtain a
211	fully developed network. Then, a frequency sweep test was performed to determine the effect
212	of time scale of deformation on the rheological properties when the samples were formed
213	(Lee & Lucey, 2003). Frequency was increased from 0.0628 to 628 rad s ⁻¹ at 0.1% strain and
214	37 °C.
215	

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218	Confocal laser scanning microscopy (CLSM) observation of gel microstructure was
219	performed using the ZEISS LSM 880 inverted confocal microscope (Carl Zeiss AG,
220	Oberkochen, Germany) set at magnification 63× (Plan Apochromat objective, oil immersion,
221	NA 1.4) using the Airyscan detection unit. The dispersion of WPI and emulsion containing
222	GDL were mixed with a 1% (w/v) solution of Fast Green at a ratio of 9:1. Each sample (1
223	mL) was injected into the chamber of a gel cassette® system (Brocklehurst, Mackie, Steer, &
224	Wilson, 1998) and vertically incubated at 37 °C for 24 h. After gelification, Airyscan images
225	were acquired using a He/Ne laser with a wavelength of 633 nm, using with a main beam
226	splitter MBS488/561/633 and no additional emission filter. The zoom was set at 4 for gel
227	imaging and 1.8 for W/W emulsion sample. For further image analysis of W/W emulsion, ten
228	micrographs of 1336×1336 pixels (1 μ m = 18.17 pixels) were taken on different regions in a
229	constant z-position. Zen Black 2.1 (Version 13.0.0.0) software was used to process the
230	acquired datasets using the 2D mode at default settings of the Airyscan processing function,
231	including a Wiener filter deconvolution.
232	Airyscan confocal micrographs of the W/W emulsions were converted to binary
233	images using the "Default" thresholding algorithm of Fiji-win64 software. The segmentation
234	procedure was validated by visual comparison of the resulted binary image with its original
235	image. The particle areas were measured using the Analyze Particles measurements tool (in
236	μ m ²). Corresponding diameters were calculated, based on the hypothesis that all particles
237	were spherical.
238	
239	2.7. Statistical analysis

241	Samples were prepared in triplicate and the experiments were done at least two times.
242	The results were analysed by one-way ANOVA with SPSS software version 16 (IBM
243	software, New York, NY, USA) using Duncan's test at a significance level of $P < 0.05$.
244	
245	3. Results and discussion
246	
247	3.1. Interfacial rheology
248	
249	3.1.1. Amplitude sweep
250	The dynamic moduli of air-WPI interfaces were first measured over time at a fixed
251	strain amplitude and oscillation frequency. The interfacial shear storage (G'_s) and loss (G''_s)
252	moduli reached equilibrium 2–3 h after sample loading, which indicates that the structure of
253	interface reached steady state (Jaishankar & McKinley, 2013). Then, small amplitude
254	oscillatory strain sweeps were carried out to find out the onset of non-linearity of the samples
255	viscoelastic response. Typical curves of the results of strain sweep tests are illustrated in Fig.
256	1. In agreement with the literature on native β -lactoglobulin (Ridout, Mackie, & Wilde, 2004)
257	the G'_s of the air-liquid interfacial layer of native WPI had a value of about 13 mN m ⁻¹ (Table
258	1). Heat denaturation and hydrophobisation decreased the G'_s value of the air-liquid interfacial
259	layer of WPI. Native β -lactoglobulin, as the major whey protein, when adsorbed at interface

polymerises over time, concomitant with lack of any appreciable exchange between adsorbed 260

polymers and non-adsorbed monomers. The polymerisation takes place by the gradual 261 exposure of the free –SH groups embedded in the interior of the molecule, triggering

progressive formation of disulphide crosslinks. It develops a remarkable extent of β-263

lactoglobulin oligomers at interface 2 h after interfacial adsorption (Dickinson & Matsumura, 264

1991). 265

In contrast to native proteins, extensive conformational changes and disulphide bond 266 formation take place before interfacial adsorption in heat-processed proteins (Meydani et al., 267 2019), i.e., the heat-denatured and hydrophobised WPI samples. This reduces the extent of 268 permanent crosslinks formation after interfacial adsorption. Furthermore, the greater absolute 269 values of negatively signed ζ -potentials of heat-denatured and hydrophobised WPIs in 270 comparison with native WPI at comparable pH values (Madadlou et al., 2018) suggest a 271 272 stronger electrostatic repulsion between protein particles at the interfacial layer, reducing the stiffness and hence the G'_s of the layer. Likewise, a smaller extent of crosslinking between 273 protein molecules was argued to explain the lower interfacial viscosity of β-lactoglobulin at 274 275 the air-water interface when compared with oil-water interface (Roth, Murray, & Dickinson, 276 2000).

The point at which G'_s deviated by more than 10% from a constant value was considered as the onset of non-linearity (TA Instruments, New Castle, DE, USA: http://www.tainstruments.com/pdf/literature/RS23.pdf), which took place at a strain amplitude of 1.8%, 1.6%, and 1.2% for the air-liquid interfacial layers of native, heatdenatured and hydrophobised WPI samples, respectively. For these three samples, a shorter linear region coincided a lower G'_s value (Table 1).

Although erythritol addition slightly decreased the G'_s and G''_s of the hydrophobised 283 WPI interfacial layer, the reduction was not statistically significant (Fig. 1; Table 1). 284 Likewise, the phase-shift angle, δ of the layer was not influenced by erythritol addition (Table 285 1). Furthermore, the δ values of the air-liquid interfacial layers of the hydrophobised WPI 286 287 samples (either E-free or E-added) were not different from that of the heat-denatured sample. In contrast, native WPI had a remarkably smaller δ in the linear region. Within the linear 288 region, G'_s was larger than G''_s for all samples, indicating that the interfacial layers were 289 predominately elastic (Langevin, 2000; Sharma et al., 2011). 290

291	All interfacial layers underwent a yielding process at high strain amplitudes. The
292	process is shown by a steep collapse in the magnitude of G'_s and the domination of G''_s beyond
293	a certain amplitude (Jaishankar et al., 2011). The values of oscillation flow stress (τ_f) and
294	flow strain (γ_f) of the WPI interfacial layers were affected by heat denaturation and
295	hydrophobisation. The crossovers of G'_s and G''_s , which indicate the flow points of the
296	interfacial layers, are specified by the circles in Fig. 1, and the corresponding τ_f and γ_f values
297	are reported in Table 1. In accordance with the influence on G'_s , heat-denaturation and
298	hydrophobisation decreased the values of τ_f and γ_f of the interfacial layer, and erythritol
299	addition into the hydrophobised WPI did not influence the flow-point criteria (i.e., τ_f and γ_f)
300	of the air-liquid interfacial layer. Again, hydrophobisation caused a greater reduction of the
301	flow-point criteria than heat denaturation.

303 *3.1.2.* Creep ringing

The creep compliance of the interfacial layers as a function of time at different 304 magnitudes of interfacial stress, τ_s over a few seconds is illustrated in Fig. 1. A typical creep 305 ringing behaviour, i.e., damping free oscillation, is observed for the interfacial film of native 306 WPI. In the linear regime, strain is a linear function of stress; hence, the compliance curves 307 308 should overlap for an ideal linear viscoelastic material. This happened at times shorter than 2 s for the interfacial layer of native WPI and indicates that the elastic component of the sample 309 controls the first instant response, causing comparable ringing behaviours irrespective of the 310 value of applied τ_s (Goudoulas & Germann, 2016). Likewise, the creep compliance curves of 311 polyacrylamide solutions (Goudoulas & Germann, 2016) and bovine serum albumin 312 313 interfacial films (Jaishankar et al., 2011) deviated at periods longer than 2 s and 5 s, respectively, indicating the onset of non-linear behaviour and the domination of viscous 314 component. 315

Creep ringing was weakly observed for the air-liquid interfacial film of the heat-316 317 denatured WPI. In addition, the compliance curves of this sample (heat-denatured WPI) commenced to deviate only after 0.8 s. The interfacial films of the hydrophobised WPI and 318 the E-added hydrophobised WPI did not show any creep ringing property. Indeed, to observe 319 creep ringing, the sample must have sufficient elasticity, so that $G_I > G_{criticial}$, where G_I is the 320 modulus of spring in the Jeffreys model (Ewoldt & McKinley, 2007). Therefore, the absence 321 322 of the ringing property in the air-water interfacial layers of the hydrophobised WPIs (with and without added erythritol) agrees with the results of the strain amplitude oscillatory test. 323 324 Δ and ω_* values of the interfacial layer of native WPI-air were 0.125 \pm 0.010 and 1.17 \pm 0.06 Hz, respectively. The short time creep response is quadratic in time and according to Struick's 325 326 formula, the interfacial elastic modulus is proportional to the square of ω_* (Jaishankar et al., 2011): 327

328
$$G'_s \approx \alpha_s \omega_*^2 \left[1 + \left(\frac{\Delta}{2\pi}\right)^2 \right]$$
 [Eq. 3]

The parameter $\alpha_s = \frac{I}{b}$ is an instrumentation parameter and remains unchanged for the samples because the total moment of inertia (*I*) of the system and the factor *b* which is determined by the geometry are identical. The ratio of the square values of ω_* of native WPIair layer was 2.25, which is very close to the ratio of the samples G'_s values in the linear region, i.e., 2.35. This indicates that the linear elastic modulus can be directly determined using the data from the creep ringing test.

335

336 *3.2.* Buffering capacity and particle size of WPI during acid gelation

337

Interfacial rheology analyses indicated that hydrophobisation decreased the dynamicmoduli of air-liquid interfacial layers of WPI. Next, we assessed the influence of

hydrophobisation on the acid-induced cold-set gelation properties of WPI using bulk 340 rheology. The WPI samples and the W/W emulsion produced were gelled with GDL. The pH 341 value of the heat-denatured WPI supplemented with GDL at a level of 20 mg mL⁻¹ decreased 342 to \approx 3.8, after acidification for 130 min, whereas its value for hydrophobised WPI 343 supplemented with a comparable GDL concentration (20 mg mL⁻¹) decreased only to 5.07 344 after an identical period of time (results not shown). On the other hand, the acidification 345 346 profiles (pH versus time) of the heat-denatured and hydrophobised WPI samples supplemented with GDL at the respective concentration of 5 mg mL⁻¹ and 20 mg mL⁻¹ were 347 very close. Therefore, the buffering capacity (i.e., resistance to pH changes) of whey proteins 348 349 increased due to hydrophobisation.

The carboxylic acid group of amino acids has a pKa value of approximately 2.3, which 350 is significantly lower than the pK_a value (4.8) of acetic acid due to negative inductive 351 (electron withdrawing) effect on the carboxylic acid group by the $-NH_3^+$ group. Accordingly, 352 the carboxyl acid group of α -amino acids is about 300 times stronger an acid than acetic acid. 353 In proteins and peptides, the pK_a of terminal α -carboxyl groups is 3.1–3.8 (intermediate 354 between the carboxylic acid groups of acetic acid and glycine) because of the lower negative 355 inductive effect of proximal peptide bond rather than the $-NH_3^+$ group in an α -amino acid. It is 356 noteworthy that the β and γ carboxylic acid groups of aspartic (pK_a = 3.9) and glutamic (pK_a 357 = 4.3) acids are somewhat insulated from these electronic effects by intervening saturated 358 359 carbons (Barrett & Elmore, 1998). Hydrophobisation, which occurred through the acetylation of a great majority (>90%) of free amino groups of whey proteins (Madadlou et al., 2018), 360 decreased the inductive effect and increased the pKa values of terminal carboxylic acid 361 362 groups. A large amount of acid or base is needed to change pH near the pK_a, so that buffer solutions are stronger at pH levels near their pKa values (Shimadzu Corporation, 2015). The 363 hydrophobised WPI that had a higher pK_a value was a stronger buffer and required a 4-fold 364

higher amount of GDL to undergo an acidification profile comparable with that of the heat-denatured WPI.

During formation of acid-induced protein gels, reduction of pH towards the pI 367 decreases the net negative charge on protein particles. Then, the particles begin to crosslink, 368 forming clusters of different but finite size. At a certain threshold of interconnection, the final 369 370 gel network is formed (Madadlou, Emam-Djomeh, Mousavi, Mohamadifar, & Ehsani, 2010). 371 The particle size of WPI dispersions was measured during acidification immediately after GDL addition. The hydrophobised protein particles had a mean hydrodynamic diameter of 372 approximately 82 nm, 3 min after GDL addition. Fig. 2A indicates that during the 373 374 acidification, the particle size of hydrophobised WPI initially decreased, followed by a 375 progressive increase over the rest of acidification. Plotting the mean hydrodynamic diameter 376 of protein particles versus mean pH during acidification until gel formation time (Fig. 2B) 377 indicated that the size of hydrophobised protein particles decreased with decreasing pH to approximately 5.5 (identified by an arrow in Fig. 2B). This pH value corresponded to 30 min 378 379 after GDL addition. Likewise, the particle diameter of heat-denatured WPI showed a small reduction in the beginning of acidification, followed by a remarkable increase (Fig. 2A). As 380 soon as GDL was added, the pH value of WPI dispersions sharply decreased (results not 381 382 shown), and then reached a plateau after gel formation over long durations. The initial reduction and the subsequent increase of protein particle size during acid gelation, which were 383 observed for both heat-denatured and hydrophobised WPI dispersions are attributed 384 respectively to protein particles shrinkage due to the decrease of intra-particle electrostatic 385 repulsion and succeeding aggregation of the particles. 386

Erythritol addition did not influence the initial particle size of hydrophobised WPI dispersion, which suggests that erythritol did not interact electrostatically with WPI particles (Madadlou et al., 2019). It did not also affect the aggregate size as a function of pH reduction

(Fig. 2B). However, erythritol addition postponed the onset of aggregation of protein particles
to a lower pH value (approximately 5.4) and caused formation of comparatively smaller
aggregates just before the gelation point (Fig. 2A).

393

394 *3.4. Gelation time*

395

Different WPI samples supplemented with GDL (20 mg mL⁻¹ for the hydrophobised 396 WPI samples, and 5, 10, and 20 mg mL⁻¹ for the heat-denatured WPI) were gelled at 37 °C on 397 a rheometer and the corresponding gelation times are illustrated in Fig. 3A. The heat-398 399 denatured WPI gelled approximately 5, 13, and 42 min after adding GDL at respective concentrations of 20, 10, and 5 mg mL⁻¹. The G' values of the heat-denatured WPI gels 400 prepared using GDL at 20 and 10 mg mL⁻¹ levels decreased after a peak value (Fig. 3B). The 401 reduction of G' was substantial for the heat-denatured WPI supplemented with 20 mg mL⁻¹ 402 GDL, and is attributed to gel disintegration due to over-acidification, which reduced pH to 403 404 values well below the proteins pI.

Gelation time (t_g) of whey proteins was influenced by hydrophobisation, as well as by 405 erythritol and alginate addition (Fig. 3A). Hydrophobised WPI required about 125 min to gel 406 (pH 5.07 \pm 0.01) after adding GDL at 20 mg mL⁻¹. Erythritol addition, which did not alter the 407 pH value of the hydrophobised WPI, postponed the t_q (Fig. 3A). The E-added hydrophobised 408 409 WPI gelled approximately 134 min (pH 5.06 ± 0.02) after GDL supplementation (20 mg mL⁻ ¹), which is 9 min longer compared with the counterpart without erythritol. On the contrary, 410 alginate addition into the E-added hydrophobised WPI resulted in a shorter t_g (94 \pm 2 min) in 411 comparison with both E-added and E-free hydrophobised WPIs. 412

413

414 3.5. Dynamic rheology of the WPI gels

416 The gels developed in situ on the rheometer were subjected to frequency sweep test to 417 gain an insight into their time-dependent behaviour. This rheological analysis is useful to gather information on the internal structure of samples (polymer solutions, gels, emulsions, 418 419 etc.) and the stability of dispersions during storage. The results of the frequency sweep test are illustrated in Fig. 4. The values of G' and G'' of all samples increased with increasing 420 frequency and G' was greater than G'' at any frequency for all samples, which suggests 421 significant protein-protein interactions at the gels (Zhang et al., 2018) and domination of the 422 423 elastic component to the viscous one (Madadlou, Khosroshahi, & Mousavi, 2005). However, the difference between the G' and G'' of all gels was smaller than 1 log cycle, indicating that 424 the gels were rather weak (Kamal, Foukani, & Karoui, 2017). 425 426 In a manner similar to that of the interfacial rheology results, the hydrophobised WPI gel had lower G' values than the heat-denatured WPI gel throughout the whole frequency 427 range. Moreover, erythritol addition decreased the maximum G' value of the hydrophobised 428 WPI gel in the frequency sweep analysis from approximately 5 kPa to 4 kPa. The G'' of the 429 430 WPI gel decreased at a comparable extent (≈ 1.27 fold) due to erythritol addition (Fig. 4). Both E-free and E-added hydrophobised gels had a final (i.e., after 24 h) pH value of 4.75 ± 0.1 . 431 Erythritol might interrupt gel network development (Cai, Feng, Regenstein, Lv, & Li, 2017; 432 Torres, Raymundo, & Sousa, 2013). We hypothesise that erythritol increased the hydration of 433 protein particles, reducing the formation of non-covalent bonds (He, Azuma, Hagiwara, & 434 Kanno, 2006) between the particles, impairing gel development. This may explain why the E-435 added hydrophobised WPI had relatively smaller aggregates than E-free sample just before 436 437 the gelation point. Although erythritol did not interact with whey proteins (Madadlou et al., 438 2019), it could increase protein hydration because of excluded volume effect, a phenomenon that weakly depends on the native or denatured state of proteins (Ebel, Eisenberg, & 439

Ghirlando, 2000). Addition of carbohydrates such as sugar alcohols to native protein solutions
is known to delay the heat-induced aggregation and gel formation (Wijayanti, Bansal, &
Deeth, 2014).

In accordance with the influence on gelation time, alginate addition increased the G'443 and G'' values of the E-added hydrophobised WPI gel. One may assume that alginate gelation 444 along with whey proteins accelerated the gelation and increased gel firmness. Alginate can gel 445 without the aid of calcium (and other multivalent cations) when pH is gradually lowered (for 446 example by GDL) because the fraction of unionised carboxyl groups on the uronic acid 447 448 residues increases and water is squeezed out (Yotsuyanagi, Yoshioka, Segi, & Ikeda, 1991). Gradual pH reduction is requisite as an abrupt acidification leads to precipitation of alginic 449 450 acid rather than gel formation. It has been estimated that a minimum alginate concentration of 3.5 mg mL⁻¹ is needed to obtain a GDL-induced gel (Draget, Skjåk Bræk, & Smidsrød, 1994), 451 which is remarkably lower than the alginate concentration employed in the current study (30 452 mg mL⁻¹), although alginate would partition between the protein-rich and alginate-rich phases 453 454 of the emulsion (Madadlou et al., 2019).

To assess alginate gelation, we added different concentrations of GDL to the alginate 455 solution (30 mg mL⁻¹) and found that a GDL concentration of 1.1 mg mL⁻¹ decreased pH after 456 about 90 min (similar to the emulsion t_a) to 5.15 ± 0.04, which is comparable with the 457 458 emulsion pH at gelation point. Nevertheless, the alginate solution did not gel. The alginateadded WPI gel had a final (after 24 h) pH of approximately 4.75, similar to that of heat-459 denatured and hydrophobised WPI gels, associated with the protein buffering capacity 460 (Léonard, Husson, Langella, Châtel, & Saurel, 2016). Also, de Jong et al. (2009a) observed a 461 constant pH, independent of polysaccharide type and amount for the acid-induced gels of 462 WPI/polysaccharide mixtures. It was presumed that the continuous and disperse phases of the 463 W/W emulsion had equal pH values (Singh, Medronho, Miguel, & Esquena, 2018). Adding 464

higher concentrations of GDL, namely 1.4 and 1.7 mg mL⁻¹, decreased pH (after 90 min) to approximately 4.85 and 4.75, respectively; however, gelation did not happen. Indeed, acid gelation of alginate happens at pH values below the pK_a of the uronic acid residues which is approximately 3.7 (Bu, Kjøniksen, & Nyström, 2005).

Although the WPI powder used in the current study contained a small amount of 469 minerals, which could cause alginate hardening/gelation when pH was reduced, the shorter tg 470 and higher dynamic moduli of alginate-added gel compared with the alginate-free WPI 471 systems is mainly attributed to the microstructural organisation of the composite gel. 472 Likewise, a higher G' of an alginate/caseinate aqueous two-phase system gel than individual 473 gels of either alginate or caseinate was ascribed to the development of a more organised and 474 475 interconnected structure in the mixed gel (Léonard et al., 2016). Hence, the microstructural 476 features of the samples were assessed to explain the difference between the dynamic moduli of the alginate-added gel and the alginate-free WPI gels. 477

478

479 3.6. Microstructure of the WPI gels

480

The microstructures of well developed (i.e., stored for 24 h) gel samples were imaged 481 by CLSM and the typical images are depicted in Fig. 5. The polysaccharide-free WPI gels had 482 homogenous microstructures, composed of a protein-rich phase (green) and a serum phase. 483 484 The latter is observed as black micro-channels interconnected throughout the gel matrix. Microscopic images (Fig. 5) showed that alginate was dispersed as droplets in a continuous 485 matrix of proteins. Micro-phase separation of protein-polysaccharide pairs is usual and known 486 487 to increase the firmness of the subsequently formed protein gels (Zand-Rajabi & Madadlou, 2016). Micro-phase separation concentrates the incompatible polymers in separated domains, 488 causing higher effective concentrations than nominal (Beaulieu, Turgeon, & Doublier, 2001). 489

The diameter of approximately 1700 droplets were measured and the result is illustrated in Fig. 5. The emulsion gel droplet size was highly inhomogeneous and droplets as large as 20 µm were present. It is also observed in Fig. 5D that some adjacent alginate droplets were in contact and partially coalesced. Modification of the gelation method might be helpful to confine the droplet disproportionation kinetics.

495

496 5. Conclusions

497

Hydrophobisation of whey proteins through acetylation decreased the liquid-air 498 499 interfacial viscoelasticity of WPI. Air is a very hydrophobic medium. Hence, concurrent reduction of the interfacial elasticity (observed in the current study) and interfacial tension 500 (observed in a previous study; Madadlou et al., 2019) of hydrophobised WPI at the liquid-air 501 502 interface indicates that more hydrophobic protein particles which accumulated at the interface were less elastic. An opposite process took place in the alginate-protein emulsions, i.e. more 503 504 elastic but less hydrophobic protein particles stabilised the all-aqueous interfaces. This conclusion relies on the assumption that at least two populations of particles with different 505 hydrophobicities were present at the protein phase. It is noteworthy that macroscopic phase 506 507 separation of non-gelled W/W emulsions is conventionally slowed down by accumulation of third component particles at the water-water interface. However, as communicated in an 508 earlier report (Madadlou et al., 2019), stable W/W emulsions can be made using a pair 509 consisting of only hydrophobised protein and a co-charged polysaccharide without a third 510 511 component. The fabrication of the two-component stable W/W emulsions is thrilling. Should the above-mentioned conclusion be correct, one may design a wide range of two-component 512 (protein and polysaccharide only) stable and fluid (not gelled) W/W emulsions by masterful 513 manipulation of the shape, size, and surface hydrophobicity of protein particles. Acidification 514

515	of the emulsion produced soft gels which might be used for encapsulation of drugs,
516	nutraceuticals and enzymes as two-phase oil-free systems. In addition, the emulsion could be
517	utilised to perform reactions at the interface of two discrete, but interfacially touching,
518	aqueous phases. Addition of erythritol to the hydrophobised WPI dispersion, which facilitated
519	emulsification, resulted in an inferior interfacial and gel viscoelasticity. Therefore, it is
520	possible to tune the interfacial and gel viscoelasticity of hydrophobised WPI and the W/W
521	emulsions using a non-caloric sweetener.
522	
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524	
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Figure legends

Fig. 1. Interfacial strain sweep (A, C, E, G) and creep (B, D, F, H) tests of whey protein isolate (WPI) samples: A and B, native WPI; C and D, heat denatured WPI; E and F, hydrophobised WPI; G and H, erythritol-added hydrophobised WPI. The interfacial shear storage (G'_s) and loss (G''_s) moduli are shown by \bullet and \blacksquare , respectively. The dashed line (strain sweep results) indicates the onset of non-linear viscoelasticity and the dashed circle specifies the crossover of the dynamic moduli. The creep tests were performed at three different interfacial stress, τ_s values of 0.0649, 0.324, and 0.649 µPa m.

Fig. 2. The particle diameter, as a function of (A) time, and (B) pH, and ζ -potential (C) of WPI samples (\blacktriangle , heat-denatured; \bullet , hydrophobised; \blacksquare , erythritol-added hydrophobised) during acidification with glucono δ -lactone (GDL) at 37 °C. The heat-denatured and hydrophobised WPI samples were gelled by the addition of GDL at a level of 5 and 20 mg mL⁻¹, respectively. The arrow specifies the onset of protein particles growth in the hydrophobised WPI solution as pH decreases. Error bars represent standard deviation.

Fig. 3. Evolution of the storage modulus (G') of whey protein isolate (WPI) solutions during acidification with glucono δ -lactone (GDL) for (A) up to 150 min and (B) over 1500 min. Symbols in panel A are: \blacktriangle , \blacklozenge , and \times , heat-denatured WPI at 5, 10 and 20 mg GDL mL⁻¹, respectively; \bullet , \blacksquare and \bullet , hydrophobised WPI, erythritol-added hydrophobised WPI and emulsion, respectively, at 20 mg GDL mL⁻¹ (error bars represent standard deviation). Symbols in panel B are: \diamondsuit and \times , heat-denatured WPI at 10 and 20 mg GDL mL⁻¹, respectively.

Fig. 4. Mean dynamic moduli of different whey protein isolate (WPI) samples: \blacktriangle and \triangle , heat-denatured WPI; \blacklozenge and \bigcirc , erythritol-free hydrophobised WPI; \blacklozenge and \diamondsuit , erythritol-added hydrophobised WPI (diamond); \blacksquare and \Box , alginate-in-erythritol-added hydrophobised emulsion. Solid symbols indicate storage modulus (G') and the hollow symbols indicate loss modulus (G'').

Fig. 5. CLSM images of acid-induced WPI gels and alginate-in-WPI emulsion gel: A, heatdenatured WPI; B, hydrophobised WPI; C, erythritol-added hydrophobised WPI; D, alginatein-erythritol-added hydrophobised WPI. Panel E presents the results of droplet size analysis of the emulsion gel.







Figure 1



Figure 2



Figure 3



Figure 4





Figure 5

Table 1

Interface	G'_s	δ_l	$ au_f$	γ_f
	$(mN m^{-1})$	(°)	(µN m ⁻¹)	(%)
Native WPI-air	$12.95^{a} \pm 0.36$	$10.9^{b} \pm 0.6$	3.8	8.0
Heat-denatured WPI-air	$4.40^{b} \pm 1.3$	30.3 ^a ±2.8	1.2	3.1
Hydrophobised WPI-air	0.86 ^c ±0.13	30.9 ^a ±2.7	0.2	2.6
Erythritol-added hydrophobised WPI-air	$0.63^{\circ} \pm 0.11$	30.5 ^a ±2.1	0.2	2.6

Interfacial properties of whey protein isolate (WPI) samples.^a

^a Abbreviations are: G'_s , storage modulus in the linear viscoelastic region; δ_l , phase-shift angle in the linear viscoelastic region; τ_f , flow stress; γ_f , flow strain of the interfacial layer. The τ_f and γ_f values were acquired from Fig. 1, which shows typical curves of the strain sweep test at the liquid-air interface of different WPI samples; means with different superscript letters within a column differ significantly ($P \le 0.05$).