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► To cite this version:

Maroua Ellouze, Christophe Vial, Hamadi Attia, Mohamed Ali Ayadi. Camel α -lactalbumin at the oil–water interface: Effect of pH and heat treatment on the structure, surface characteristics and emulsifying properties. *LWT - Food Science and Technology*, 2019, 116, pp.108550 -. 10.1016/j.lwt.2019.108550 . hal-03488164

HAL Id: hal-03488164

<https://hal.science/hal-03488164>

Submitted on 20 Dec 2021

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1 **Camel α -lactalbumin at the oil–water interface: Effect of**
2 **pH and heat treatment on the structure, surface**
3 **characteristics and emulsifying properties**

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34 **Abstract**

35 The effects of pH (3.0, 6.0, 9.0) and heat treatment (25°C, 65°C, 95°C) on the emulsifying
36 properties of α -lactalbumin extracted from camel milk were investigated and compared to
37 bovine α -lactalbumin. **The results show that** both studied proteins displayed a rather different
38 evolution of their emulsifying activity as a function of the main factors (**pH and temperature**);
39 however, they exhibited very close maximum values, between 100–110 m².g⁻¹. The stability of
40 camel α -lactalbumin stabilised emulsions at pH 3.0 and 9.0 was attributed to the associated
41 electrostatic forces (ζ -potential ~ -30 mV). **The emulsifying properties of camel α -lactalbumin**
42 **were less sensitive to heat treatment (95°C), due to the higher conformational flexibility, as**
43 **surface hydrophobicity increased with temperature (from 16 A.U to 51 A.U);** conversely, bovine
44 α -lactalbumin enhanced emulsion stability vs. pH and heat treatment, due to hydrophobic
45 interactions and a more rigid molecular structure. Statistical tests also showed that the adsorption
46 of camel α -lactalbumin at the oil–water interface was significantly affected by pH change.

47

48 **Keywords:** camel milk, emulsion, Alpha-lactalbumin, hydrophobicity, surface pressure.

49 **1. Introduction**

50 Milk proteins are commonly used as surface active molecules, in particular whey proteins
51 due to their nutritional importance and their wide range of functionality in food and
52 pharmaceutical products including thickening, gelling, foaming and emulsification. The
53 ability of milk proteins to adsorb at the oil–water interface and to stabilise emulsions is a
54 very important functionality which has been exploited by many industries of food (cream
55 liqueurs and dairy desserts), cosmetics (cleansers, body lotions) as well as of
56 pharmaceutical products (encapsulation of active ingredients).

57 Camel milk (*Camelus dromedarius*) is considered as the main dairy product in certain
58 regions of the world, particularly in Africa and Asia, and is well known for its exceptional
59 therapeutic characteristics. First, studies involving camel milk mainly focused on the
60 differences in composition with cow's milk. More recently, some functional properties of
61 camel milk were explored, in particular their foaming activity (Hu, Ting, Hu, & Hsieh,
62 2017; Lajnaf, Picart-Palmade, Attia, Marchesseau, & Ayadi, 2017a; Lajnaf et al., 2018). A
63 recent study using camel whey proteins in a model beverage emulsion (Momen, Salami,
64 Alavi, Emam-Djomeh, & Moosavi-Movahedi, 2019) also showed their significant
65 emulsifying properties compared to bovine whey in specific ranges of pH (3.3) and
66 temperature (85°C). Understanding the mechanism of camel milk protein adsorption at the
67 oil–water interface at larger ranges of pH and temperature is, therefore, of great interest,
68 especially for the food and the pharmaceutical industries.

69 Camel milk is marked by the absence of β -lactoglobulin (β -Lg) compared to cow milk;
70 α -lactalbumin is thus known to be the most abundant whey protein of camel milk,
71 exceeding 40% of total whey protein content (Lajnaf et al., 2017a). Camel α -lactalbumin
72 (Ala-C) is a globular protein with a molecular mass of 14.6 kDa and an isoelectric point
73 (pI) of 5.1–5.3 (Conti et al., 1985). Ala-C consists of 123 amino acid residues forming a
74 compact globular structure stabilised by four disulphide bonds; Ala-C exhibits a high
75 affinity for metal ions, calcium in particular. Sequence similarity and identity between
76 camel and bovine α -lactalbumin are estimated at 82.9% and 69.1%, respectively (Atri et
77 al., 2010). **However, heating of Ala-C results in very little protein**
78 **polymerization/aggregation due to the lack of free thiol groups in comparison to bovine**
79 **α -lactalbumin (Ala-B) (Lajnaf, Picart-Palmade, Attia, Marchesseau, & Ayadi, 2017b).**

80 Structurally, α -lactalbumin is an amphiphilic protein elected to be a good emulsifier agent
81 to produce protein-stabilised emulsions (Lam & Nickerson, 2013). When added to
82 oil–water interface, the bovine α -lactalbumin proteins form a cohesive interfacial protein
83 layer around oil droplets, providing steric and either electrostatic repulsive or attractive
84 forces between emulsion droplets. The relative stability of emulsions against coalescence is
85 governed by the balance between these forces. Major advances have been made to better
86 understand the adsorption process of cow milk proteins at the oil–water interface, the
87 structure and the composition of the adsorbed layer, and the influence of the physical and
88 chemical environment on their emulsifying properties (Dickinson, 2001; Singh, 2011).

89 In this respect, understanding the emulsifying properties of camel's milk α -lactalbumin
90 protein under different treatments is of great industrial and scientific interest promoting
91 novel and interesting substitute to existing emulsifier. The main objective of this work is,
92 therefore, to study the behaviour of camel milk α -lactalbumin protein (Ala–C) at the
93 oil–water interface in a wide range of heat treatment temperature and pH, and to compare it
94 to cow's α -lactalbumin protein in a low-fat emulsion model.

95 **2. Materials and methods**

96 **2.1. Materials**

97 Camel milk used in this study was purchased from a local farm in the region of Tozeur in south
98 Tunisia. Just after milking, 0.2 g/L of sodium azide (NaN_3) was added to stop bacterial
99 proliferation; then, milk was stored at 4°C.

100 Cow's milk α -lactalbumin (Ala–B) was purchased from Sigma–Aldrich (USP Reference
101 Standard). Protein content was measured to be 898.8 g/kg and this protein was used without
102 further purification.

103 Local produced rapeseed oil for alimentary use was purchased from a local supplier and used
104 without further purification. Water was produced using a Millipore Milli–Q™ water purification
105 system (Millipore Corp., Milford, MA, USA). All other chemicals used in this study are of
106 reagent grade and purchased from Sigma–Aldrich (USA).

107 **2.2. Camel α -lactalbumin purification**

108 Once the camel milk was skimmed by centrifugation (3,800 ×g, 20 min, 7°C), milk casein was
109 removed by acidic aggregation at pH 4.2 using hydrochloric acid (HCl, 1 mol/L), followed by

110 centrifugation at 5,000 \times g for 15 min (Lajnaf et al., 2018). Supernatant purification was then
111 carried out using a 50 kDa ultrafiltration membrane (VivaFlow 200, Sigma–Aldrich, USA). A
112 pH adjusted water at 6.7 was used to refilter the retentate of the purification. The washing
113 process was carried out six times. Between each cycle, the initial flow of ultrafiltration
114 membrane was restored using a concentrated NaOH solution (10 mol/L) and rinsed with
115 Milli–Q™ water. Once the permeate was collected, it was concentrated using a 5 kDa
116 ultrafiltration membrane (VivaFlow 200, Sigma–Aldrich, USA), and then dialysed against
117 Milli–Q™ water at 4°C and steady stirring. Water was replaced every 12 h for 4 days (Salami et
118 al., 2018). The protein content was 760 ± 2 g/kg, measured using a total nitrogen analyser
119 (TNM–1, Shimadzu Corp., Japan) and a nitrogen conversion factor of 6.38. A protein profile
120 (sodium dodecyl sulphate polyacrylamide gel (12% SDS–PAGE)) was achieved at each step of
121 the purification process to test the Ala–C purity. The purified protein was freeze–dried and stored
122 at 20°C for further usage.

123 **2.3. Sample and emulsion preparation**

124 The α –lactalbumin stock solutions (2 g/L, corrected for protein content) were prepared by
125 dispersing Ala–C and Ala–B lyophilised powders in Milli–Q™ water. pH was adjusted to 3.0,
126 6.0 and 9.0 using either 0.5 mol/L HCl or 0.5 mol/L NaOH, followed by mechanical stirring (550
127 rpm) at room temperature (23–25°C) for 90 min. Protein solutions were then poured into
128 screw–capped 15 mL centrifuge tubes to prevent moisture losses and held in a water bath for 15
129 min at 25°C (room temperature), 65°C and 95°C. Tubes were, then, cooled down in an ice bath
130 to room temperature prior to further analysis.

131 Finally, emulsions were prepared by mixing 5 g of the α –lactalbumin stock solution with 20%
132 (w/w) of rapeseed oil within a 50 mL plastic centrifuge tube, followed by homogenisation at
133 21,500 rpm for 3 min using an Ultraturrax T25 homogenizer (Ika–Werke GmbH, Germany)
134 equipped with a SN25–10G ST tool.

135 **2.4. Emulsion properties**

136 Emulsion stability and activity indexes (ESI and EAI, respectively) were measured according to
137 the modified method of Lam and Nickerson (2015a). Immediately after homogenisation, aliquots
138 of 50 μ L were transferred into 10 mL of a pH–adjusted aqueous solution containing 1 g/kg
139 sodium dodecyl sulphate (SDS) and vortexed for 10 s. Optical density was read at 500 nm using
140 an ultraviolet–visible spectrophotometer (Biomate 2S, ThermoFisher Sci., USA) and plastic

141 cuvettes (1 cm path length). After 10 min, a second aliquot of the same emulsion was taken,
 142 following the previous procedure. The EAI and ESI were then calculated using Eqs. (1) and (2),
 143 respectively:

$$144 \quad \text{EAI (m}^2 \cdot \text{g}^{-1}) = \frac{2 \times 2.203 \times N \cdot A_0}{10^5 \times C \cdot \varphi} \quad (1)$$

$$145 \quad \text{ESI (min)} = \frac{A_0}{\Delta A} \cdot t \quad (2)$$

146 where A_0 is the absorbance of the diluted emulsion immediately after homogenisation, N the
 147 dilution factor (250), C the weight of protein per volume (g/ml), φ the oil volume fraction in the
 148 emulsion, ΔA the difference of the absorbance between at time 0 and time 10 min ($A_0 - A_{10}$), and t
 149 the time interval (10 min).

150 **2.5. Emulsion ζ -potential**

151 ζ -potential is a key property of emulsions (McClements, 2004), was measured using a Zetasizer
 152 Delsa Nano HC (Beckman Coulter, USA). The emulsion samples were diluted 1:100 (v/v) using
 153 Milli-Q™ water. Samples were equilibrated for 120 s before collecting data. The sampling time
 154 was fixed at 400 μ s. Data were accumulated from 10 sequential readings at 25°C, and the
 155 mathematical model of Smoluchowski (Sze, Erickson, Ren, & Li, 2003) was selected to convert
 156 the electrophoretic mobility measurements into ζ -potential values using Eq. (3):

$$157 \quad \zeta = \frac{3\eta}{2\varepsilon} U \quad (3)$$

158 where ζ is the measured ζ -potential (mV), η the viscosity of the emulsion (Pa.s), ε the
 159 permittivity of water, and U the electrophoretic mobility ($\text{m}^2/\text{V}\cdot\text{s}$).

160 **2.6. Droplet size and microscopic observation**

161 The droplet size distribution was determined using a laser scattering technique (Mastersizer
 162 3000E, Malvern PANAnalytical, UK). Just after homogenisation, 1 ml aliquot of each emulsion
 163 was gently blended to an equal volume of pH-adjusted water containing 10 g/L sodium dodecyl
 164 sulphate (SDS) to avoid multi-scattering effect and prevent emulsion flocculation. Emulsion
 165 droplet sizes were measured under steady agitation (1,500 rpm) and reported as the Sauter mean
 166 diameter, d_{32} , defined as:

$$d_{32} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2} \quad (4)$$

168 where n_i is the number of particles of diameter d_i .

169 Microscopic observations were carried out using an Axiovert 25 inverted microscope (Carl Zeiss
170 GmbH, Germany) equipped with a monochrome Pulnix camera (JAI, Japan, 640×480 pixels).
171 Emulsion aliquots of 20 μ L were placed onto a microscope slide and carefully covered with a
172 coverslip, avoiding any bubble formation. Micrographs were recorded at $\times 100$ magnification.

173 **2.7. Surface hydrophobicity and interfacial tension**

174 Surface hydrophobicity of Ala-C and Ala-B solutions treated under different conditions of pH
175 and temperature were measured according to the modified method of Alizadeh-Pasdar and Li-
176 Chan (2000) using a spectrofluorimeter Flx (SAFAS, Monaco). An
177 8-anilino-1-naphthalenesulfonate (ANS) probe was added to interact with hydrophobic moieties
178 on the protein surface to give a fluorescent signal. Each protein solution was diluted to a
179 concentration of 0.8 g/L. 20 μ L of ANS ($8 \cdot 10^{-3}$ mol/L) solution dissolved in a phosphate buffer
180 ($50 \cdot 10^{-3}$ mol/L, pH 7.0) was added to 4 mL of each protein solution. The solution was excited at
181 390 nm, and the emission spectrum was measured from 400 to 600 nm. The emission and
182 excitation slits were set to 5 nm, and the measurements were performed at 25°C. The maximum
183 area of the fluorescence spectrum was corrected with the buffer area. **Surface hydrophobicity is**
184 **reported as the maximum of the obtained spectrum.**

185 The interfacial tension for each protein solution was measured using a K12 tensiometer (Krüss
186 GmbH, Germany) equipped with a platinum Wilhelmy's plate to achieve complete wetting
187 (contact angle θ is 0, *i.e.* $\cos(\theta)=1$). Within a 20 mm diameter glass sample cup, 5 mL of protein
188 solution (1 g/L of protein content) was added, followed by the immersion of Wilhelmy's plate;
189 then, an upper layer of rapeseed oil (10 mL) was poured over it. The measurement time was
190 fixed to 2,000 s. The interfacial tension was obtained by correlating the force F (mN) applied on
191 the immersed plate to the wetted length of the plate L (mm) between the plate and the liquid as
192 expressed by Eq. (5):

$$193 \quad \sigma \left(\frac{\text{mN}}{\text{m}} \right) = \frac{F}{L} \cos(\theta) = \frac{F}{L} \quad (5)$$

194 The resulted data was displayed by the change in interfacial tension from the pure fluid value/log
195 time (Eq. (6)), which gives access to an easy comparison between systems with different σ_0 (the

196 interfacial tension of pure fluids), and helps visualize the diffusion rate of proteins at the
197 oil/water interface (Beverung, Radke, & Blanch, 1999).

$$198 \quad \Pi(t) = \sigma_0 - \sigma_t \quad (6)$$

199 where Π is the surface pressure, and σ_t the measured interfacial tension at time t .

200 **2.8. Rheological behaviour**

201 Rheological measurements were conducted at 25.0°C using an AR-G2 rheometer (TA
202 Instruments, USA) equipped with a 40 mm standard steel parallel plate. 2-ml aliquots of freshly
203 prepared emulsions were used per measurement. An up and down shear rate sweep test was
204 carried out between 0.1 s⁻¹ to 1,000 s⁻¹ in order to measure emulsion viscosity. For all
205 measurements, the gap distance was fixed at 1,000 μm.

206 **2.9. Statistics**

207 All analyses were performed in triplicate and reported as the mean ± standard deviation. A
208 two-way analysis of variance (ANOVA) was applied to test for significance of the main effects,
209 *i.e.* pH (3.0, 6.0, 9.0) and heat treatment (25.0, 65.0, 95.0°C), along with their associated
210 interactions, on the physicochemical and emulsifying properties of Ala-C and Ala-B proteins. A
211 quadratic model with a second-order interaction term was assumed to correlate by **linear**
212 **regression** the physicochemical and emulsifying properties of proteins to the main factors, as
213 expressed in Eq. (7):

$$214 \quad Y_i = a_0 + a_1.pH + a_2.T + a_3.pH^2 + a_4.T^2 + a_5.pH.T \quad (7)$$

215 where Y_i is the tested response, a_i are the observed effects, and pH and T are the main factors.
216 The significance of the effects is expressed in terms of the p-value (p) **which tests the null**
217 **hypothesis that the coefficient is equal to zero (no effect)**. Statistical analyses were carried out
218 using IBM SPSS Statistics (Ver. 20, IBM, USA).

219 **3. Results and discussion**

220 **3.1. Surface properties of camel and bovine α-lactalbumin**

221 **3.1.1. Surface hydrophobicity**

222 Figure 1 shows the evolution of surface hydrophobicity of Ala-C and Ala-B as a function of pH
223 (3.0, 6.0 and 9.0) and heat treatment (25°C, 65°C and 95°C). Globally, the surface
224 hydrophobicity of Ala-C proteins is lower than Ala-B proteins at most treatments. Figure (1B)

225 illustrates that Ala-B protein's hydrophobicity decreases when the temperature of heat treatment
 226 increases at pH 3.0 and 9.0, while it remains nearly constant for pH 6.0. Contrary to Ala-B,
 227 surface hydrophobicity of Ala-C proteins (Fig. 1A) increases only when the temperature of heat
 228 treatment is high (95°C) for all pH values. However, it must be pointed out that the effect of pH
 229 is the same for both proteins: surface hydrophobicity is significantly higher at pH 3.0, while at
 230 pH 6.0 and 9.0, differences emerge only after heat treatment above 65°C, as described by
 231 ANOVA in Table 1. Ala-B proteins probably aggregate through hydrophobic interactions at
 232 these conditions (pH 3.0 and pH 9.0), resulting in a reduced hydrophobicity. The stable
 233 behaviour at pH 6.0 might be explained by its globular rigid conformation and its enhanced
 234 resistance to heat treatment close to pI (Lam & Nickerson, 2015b; Zhai et al., 2012). For camel
 235 milk proteins, this evolution is explained by the exposure of hydrophobic moieties at high heat
 236 treatment, which highlights partial denaturation enhanced at acid pH (3.0). Several researchers
 237 had already investigated the denaturation and aggregation behaviour of bovine milk proteins.
 238 Irreversible denaturation was reported at a temperature above 90°C. This is due to the
 239 susceptibility of cow whey protein, in particular Ala-B, to high heat treatment, as mentioned
 240 previously in several previous works (Elagamy, 2000; Laleye et al., 2008, Felfoul, Jardin,
 241 Gaucheron, Attia, & Ayadi, 2017). These results show that the flexible molecular structure
 242 observed for Ala-C is affected by pH variation and heating temperature and leads to a
 243 conformational reorganisation to possibly align at the oil-water interface.

244 3.1.2. Interfacial properties

245 Interfacial tension expressed as surface pressure for Ala-C and Ala-B treated proteins is plotted
 246 in Figure 2. For Ala-B proteins (Fig. 2B), the surface pressure reaches the highest value (1.7
 247 mN/m) at alkaline pH, particularly after heat treatment at 65°C. Ala-B treated proteins reduce
 248 less rapidly and less efficiently the interfacial tension between the two immiscible phases than
 249 Ala-C proteins; the induction time of Ala-B (i.e. the time from the start of the measurement until
 250 the first change of the curve slope) which reflects the first changes of protein's conformation to
 251 adsorb at the contact between the low and high density phases (oil and water, respectively) is
 252 estimated at 300 s. At higher pH, the decrease in interfacial tension is favoured by a simultaneous
 253 increase in temperature and pH, which is corroborated by the significant effect of the interaction
 254 terms ($pH \times T$) of the ANOVA table (Tab. 1). This is attributed to the ability of adsorbed
 255 proteins to change their conformation and unfold in response to hydrophilic/hydrophobic side
 256 chains and the enhanced interactions with the oil phase (Beverung et al., 1999; Miller et al.,
 257 2000). Contrary to Ala-B, the surface pressure of Ala-C proteins (Fig. 2A) reaches 2.1 mN/m at

258 pH 3.0 mainly at low-temperature heat treatment. For most treatments, the induction time is
259 estimated to 80 s for Ala-C. At pH 3.0 where the degree of denaturation was higher (95°C), the
260 second diffusion regime of proteins at the interface (which starts from the first change of curve
261 slope until the second change, it reflects, therefore, the diffusion-controlled adsorption of the first
262 layer of proteins at the interface) was observed instead. ANOVA (Tab.1) shows a very
263 significant effect of pH ($p < 0.001$). This suggests that the diffusional regime of the Ala-C
264 proteins at oil-water interface is mainly governed by electrostatic forces between the charged
265 moieties of both phases. Thus, unheated Ala-C proteins present an adequate molecular
266 conformation to interact with the fatty acids of rapeseed oil, resulting in a very significant
267 reduction in interfacial tension.

268 **3.2. Emulsifying properties**

269 **3.2.1. Emulsifying activity and stability indices**

270 Figure 3 represents the emulsifying activity and stability indices (EAI and ESI respectively) of
271 Ala-C and Ala-B stabilised emulsions as a function of pH and heat treatment. EAI reflects the
272 relative surface coverage of the protein on the oil droplet within a dilute emulsion and the droplet
273 size. Conversely, ESI is an estimation of the relative stability of the emulsion after a
274 predetermined time (Lam & Nickerson, 2015a).

275 Regarding EAI (Fig. 3A and B), both proteins display a different behaviour as a function of pH
276 and treatment temperature, but they exhibit comparable maximum values, between 100–110
277 $\text{m}^2\cdot\text{g}^{-1}$. EAI of Ala-B stabilised emulsions shifts limitedly between 84 and 110 $\text{m}^2\cdot\text{g}^{-1}$ (Fig. 3B),
278 while EAI of Ala-C stabilised emulsions (Fig. 3A) declines to 22 $\text{m}^2\cdot\text{g}^{-1}$ at pH 6.0, where only
279 the effect of pH is significant ($p < 0.001$).

280 Similar trends had already been observed for cow proteins (Lam & Nickerson, 2015a; Zhai et al.,
281 2012): actually, it depends on the native state of the protein (apo or holo). For example, Ala-B in
282 the holo-form (calcium binding) was found to be more resistant to conformational changes in
283 response to pH and temperature pre-treatments than the apo-form. As a result, higher droplet
284 surface coverage (EAI) was obtained for the apo than the holo-form of Ala-B. **Even though both**
285 **proteins, Ala-C and Ala-B, are in their apo-form in this work (no calcium present or added in**
286 **the commercial and prepared samples), structure flexibility is, therefore, widely different.**

287 Besides, Voutsinas et al. (1983) reported that the change in emulsifying properties of whey
288 proteins was reported to remain small against heat treatment, as in this work. However,

289 Dissanayake and Vasiljevic (2009) stated, in contrast, that the EAI of bovine whey proteins
290 increased with thermal treatment and denaturation due to chemical composition changes.

291 At pH 6.0, the adsorption of the Ala-C proteins at the oil interface seems to be ineffective,
292 mainly due to the reduction of electrostatic repulsion between proteins close to their pI (5.1–5.2);
293 thus, the proteins keep their globular conformation and cannot adhere to the oil–water interface
294 under the dynamic conditions of emulsion preparation. In contrast, when EAI was greater (pH
295 3.0 and pH 9.0), Ala-C proteins are at their charged state and even exhibit conformational
296 changes, especially at 95°C, as previously observed by higher surface hydrophobicity in section
297 3.1.

298 As for emulsifying stability, Fig. 3D shows that ESI values of Ala-B stabilised emulsions are
299 relatively higher than Ala-C (Fig. 3C), where maximum stability is estimated at 53 min and 35
300 min, respectively. The stability of protein-stabilised emulsions has been abundantly discussed in
301 the literature (Dickinson, 2001; Tcholakova et al., 2006; Zhai et al., 2012; Lam and Nickerson,
302 2015b) to be associated with electrostatic repulsion forces between droplets, steric stabilization
303 by protein tails or loops on the oil droplet surface, and/or high viscosity of the continuous phase.
304 It must be pointed out that opposite results have been reported on the effect of heat treatment on
305 ESI for whey proteins (Dissanayake & Vasiljevic, 2009; Voutsinas et al., 1983). While Voutsinas
306 et al., (1983) described an increase in ESI with heat treatment, Dissanayake and Vasiljevic,
307 (2009) advocated that no change in ESI was observed with increasing heat treatment
308 temperature. However, (Lam & Nickerson, 2015b) reported that the increased ability of the
309 apo-form of Ala-B to adhere to the interface also allowed for greater emulsion stability. Stability
310 was greater at pH 7.0 than pH 5.0 due to the presence of electrostatic repulsive forces between
311 protein coated droplets. Furthermore, a pre-treatment at a temperature of 65 °C on Ala-B
312 apo-form resulted in an increase in its secondary structure, leading to the greatest emulsion
313 stability, presumably due to the partial denaturation and refolding at this temperature.

314 Finally, the stability of Ala-C stabilised emulsions at pH 3.0 and 9.0, exhibiting high EAI, can
315 also be attributed to electrostatic forces. This conclusion is strengthened by the negligible effect
316 of heat treatment on camel milk proteins as previously reported (Atri et al., 2010; Lajnaf et al.,
317 2017b).

318 **3.2.2. Surface charge (ζ -potential)**

319 The stability of Ala-C and Ala-B stabilised emulsions after heat treatment and pH change was
 320 further assessed through ζ -potential measurements (Fig. 4). For both protein stabilised
 321 emulsions, the ζ -potential remains relatively constant regardless of the temperature. No
 322 significant effect of heat treatment ($p > 0.05$) was confirmed by ANOVA (Tab.1). The
 323 ζ -potential of Ala-B stabilised emulsions (Fig. 4B) is relatively higher than Ala-C for which it
 324 remains between -25 mV and -40 mV and between -15 mV and -37 mV, respectively.
 325 Bhattacharjee (2016) reported that highly stable emulsion could be observed when ζ -potential
 326 departed from zero by about at least ± 30 mV. In this work, ζ -potential exceeds -35 mV for both
 327 emulsions at specific values of pH and temperature (*i.e.* pH 9.0–25°C/95°C, and pH
 328 6.0–65°C/95°C, pH 9.0–65°C for Ala-C and Ala-B respectively), which highlights that
 329 electrostatic repulsions are involved in the emulsion stability by reference to ESI in section 3.2.1.
 330 The low net charge of emulsions at pH 3.0 is associated with positively charged Ala proteins
 331 below pI (5.2 and 4.2 for Ala-C and Ala-B, respectively). The significant effect of pH was
 332 higher for Ala-C than Ala-B stabilised emulsions according to ANOVA analysis (Tab. 1).
 333 Changes in surface charge lead the conformation structures of Ala-C to be more affected by pH
 334 change.

335 3.2.3. Droplet size analysis

336 The variation of droplet size diameter (d_{32}) from image analysis and volume-based size
 337 distribution, as well as microscopic observations for Ala-C and Ala-B stabilised emulsion, as a
 338 function of the different pH and temperature treatment are presented in Table 2 and Fig. 5,
 339 respectively.

340 Overall, d_{32} for Ala-B and Ala-C stabilised emulsions, is ranging between 3.34 ± 0.01 μm and
 341 9.8 ± 0.2 μm , and between 1.4 ± 0.04 μm and 14.2 ± 0.4 μm , respectively. It must, however, be
 342 pointed out that the smallest values of 1.4 and 2 μm , are observed for Ala-C stabilised emulsions
 343 when pH is 6.0, which corresponds to very low EAI values (Fig. 3A), *i.e.* large or flocculated
 344 droplets; thus, these emulsions had been subjected to creaming before high pressure
 345 homogenization, and only a fraction of the oil phase was in the homogenized emulsions, which
 346 lead to a small number of very fine droplets. This result is supported by microscopic observations
 347 (Fig. 5) which highlight higher droplet density (*i.e.* number of droplets per picture) both at pH
 348 3.0 and 9.0 with a similar diameter (Tab. 2), while a very low droplet density is observed at pH
 349 6.0. Consequently, only the smallest droplets could be formed and stabilised at a pH close to pI.
 350 ANOVA indicates that the pH and the treatment temperature, as well as the interaction ($pH \times T$)

351 for Ala-C proteins, are significant (Tab. 1). After heat treatment at 95°C, all the d_{32} values of
 352 Ala-C stabilised emulsions reach their maximum values, which corresponds to high EAI (Fig.
 353 3A). Larger droplets are formed and stabilised through hydrophobic interactions. Indeed, high
 354 surface hydrophobicity (Fig. 1A) was observed at this treatment temperature; equally, low
 355 interfacial tension was reported (Fig. 2A). It seems that, increasing the temperature of heat
 356 treatment at 95°C usually increases significantly the Sauter diameter (d_{32}) of the droplets (Tab.2)
 357 as in the case of the Ala-C stabilised emulsions, which also agrees with literature data on Ala-B
 358 reported after heat treatments at 80°C and 90°C (Zhai et al., 2012). However, for Ala-B
 359 stabilised emulsions, this increase in d_{32} at 95°C of heat treatment could clearly be related to the
 360 decrease in ESI observed in Fig. 3D. Thus, protein denaturation induced by heat treatment shows
 361 a reduced capacity to form emulsified droplets close to pI when EAI remains low.

362 **3.3. Emulsion rheology**

363 The apparent viscosity (η) was measured vs. shear rate between 0.1 to 1,000 s^{-1} and plotted in
 364 Fig. 6. The evolution of viscosity of Ala-C and Ala-B stabilised emulsions at a fixed shear rate
 365 ($10 s^{-1}$) is presented in terms of pH (3.0, 6.0 and 9.0) and temperature treatment (25 °C, 65°C and
 366 95°C). First, all the studied emulsions exhibit a nearly Newtonian behaviour at a high shear rate,
 367 and non-Newtonian trends at low shear rate. The emulsion viscosity is lower for Ala-B (Fig. 6C)
 368 than Ala-C (Fig.6A), and ranges between 2 mPa.s and 40 mPa.s, and between 1.5 mPa.s and 87
 369 mPa.s, respectively. For Ala-B stabilised emulsions, the change in viscosity due to pH remains
 370 weak. Thus, the effect of the heat treatment is higher than the pH effect (Tab. 1). This relative
 371 invariability of the viscosity of Ala-B stabilised emulsions is correlated to EAI and ESI data
 372 presented in section 3.2. For Ala-C stabilised emulsions, only the effect of pH is significant ($p <$
 373 0.001). The emulsion viscosity is higher at pH 3.0 than at pH 6.0 and 9.0, regardless of heat
 374 treatment. Some trends remain, however, similar for both proteins: pH 3.0 provided in general
 375 the highest values, and pH 9.0 the lowest ones. In terms of temperature, heat treatments at 95°C
 376 and 65°C lead to the lowest viscosity, while the highest is reported for unheated proteins (25°C).
 377 For Ala-B stabilised emulsions, the effect of treatment temperature could be explained by
 378 changes in the conformational structure at the oil-water interface. At 25°C, proteins adsorb in
 379 their native form and then unfold at the interface, mainly by hydrophobic interactions (Fig. 1B).
 380 A viscoelastic film is, therefore, formed around the oil droplets, resulting in high viscosity
 381 values. Conversely, after heat treatment at 65°C and 95°C of Ala-B proteins, their structure has
 382 already been modified by the temperature effect mainly through hydrophobic interaction, as

383 shown previously, explaining the decrease in surface hydrophobicity at this temperature (Fig.
384 1B). Moreover, a slight shear-thickening region is observed between 20 and 100 s⁻¹ (Fig. 6C).

385 As for Ala-C stabilised emulsions, the decrease in viscosity between pH 3.0 and 6.0 results from
386 friction reduction caused by low droplet density, as shown by microscopic observations in Fig.
387 5A, as well as by the low surface coverage (EAI) as previously discussed (Fig. 3A). High
388 viscosity at acidic pH is, therefore, consequent to attractive electrostatic forces between the two
389 phases according to the low ζ -potential of camel protein emulsions previously reported (Fig. 4A)
390 as well as to a reduced interfacial tension (Fig. 2A). Besides, an intermediate plateau emerged
391 between 20 and 100 s⁻¹, included between the shear-thinning behaviour observed at low and vert
392 high shear, i.e. before the high-shear Newtonian plateau; this can be due to the elastic behaviour
393 of protein layers when compressed due to shear (Amin, Barnett, Pathak, Roberts, & Sarangapani,
394 2014). Several authors (Dickinson, 2001; Liang et al., 2013; Alvarez-Sabatel et al., 2018) had
395 already reported that elastic interfacial layers, which is the case of adsorbed protein layers, could
396 lead to shear-thickening trends. It was reported that interfacial tension plays also an important
397 role in controlling the viscosity of dilute emulsions where high shear rate causes internal
398 circulation inside the drops and drop deformation, and the interfacial tension minimizes the
399 movement of the flow pattern outside the drops (Otsubo & Prud'homme, 1994).

400 **Conclusions**

401 Two different types of α -lactalbumin proteins from camel and cow milks have been compared as
402 a function of pH change and heat treatment conditions; their behaviours at the oil-water interface
403 have been deduced through direct and indirect analysis of their structures and their emulsifying
404 properties. It has been identified that the major factor governing the camel α -lactalbumin
405 emulsion stability is its flexibility enabling conformational rearrangement, which is affected by
406 protein heat treatment, while electrostatic repulsion is still a key factor to explain the effect of pH
407 change. Camel α -lactalbumin is significantly affected by pH variation while cow α -lactalbumin
408 is more sensitive to heat treatment. High heat treatment enhances the camel α -lactalbumin
409 surface properties, which maintains the emulsifying properties of this protein. Ala-C proteins
410 decrease the interfacial tension by exposing its hydrophobic groups, especially at pH 3.0 after
411 heat treatment at 95°C. Emulsion activity and stability are affected by electrostatic repulsion for
412 both proteins, especially for Ala-C. Thus, it is more difficult to prepare and stabilise emulsions
413 close to the pI, *i.e.* when pH is about 6.0, with Ala-C than with Ala-B, due to reduced

414 hydrophobic interaction. Compared to cow α -lactalbumin, the major factor that differs from
 415 camel α -lactalbumin is, therefore, steric hindrance which behaves differently as a function of
 416 environmental changes. These facts suggest an unbalance in the two dominant forces (the
 417 electrostatic effect and configurational entropy) that drives conformational rearrangement at
 418 emulsion interfaces toward the electrostatic repulsion. These results thus broaden our
 419 understanding of the factors controlling protein structural change at emulsion interfaces and how
 420 this affects emulsion stability. **Depending on the desired application (high/low stability, high/low**
 421 **emulsifying activity, high/low viscosity...), it is possible to adjust the main factors (i.e. pH,**
 422 **temperature and protein type) to obtain desired functionality.**

423

424 **Acknowledgements**

425 This project was financially supported by the higher education and scientific research ministry of
 426 Tunisia. Experiments were conducted at Institut Pascal (UMR CNRS-UCA-Sigma Clermont
 427 6602), GePEB group in Clermont-Fd (France). The author wants to thank Dr. Roua LAJNAF and
 428 Dr. Ahmed ZOUARI for proofreading.

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432 **References**

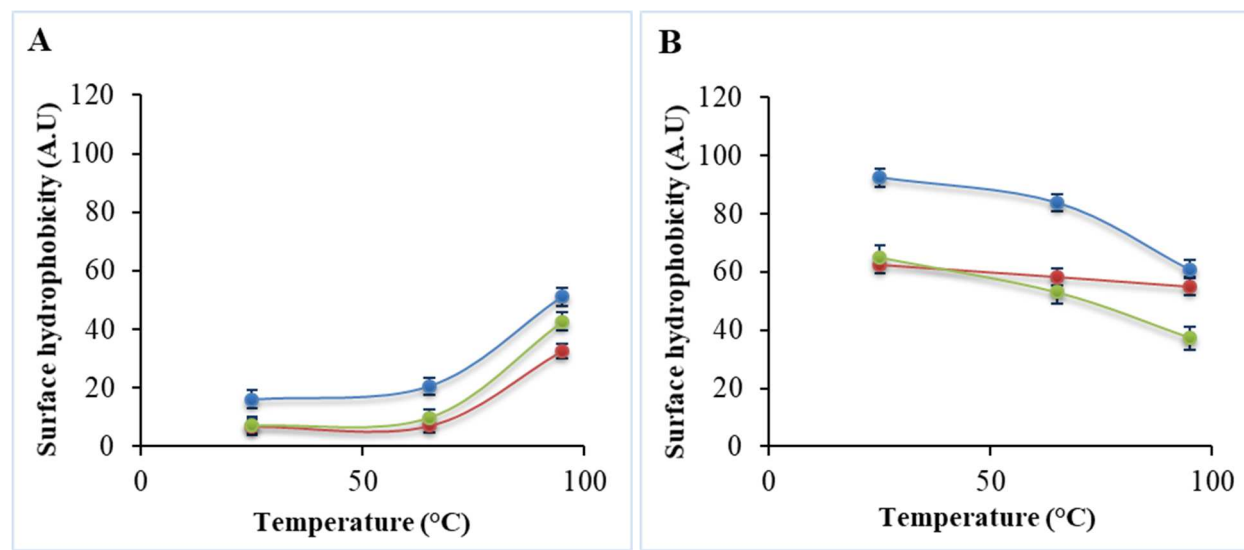
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531 **Figures**

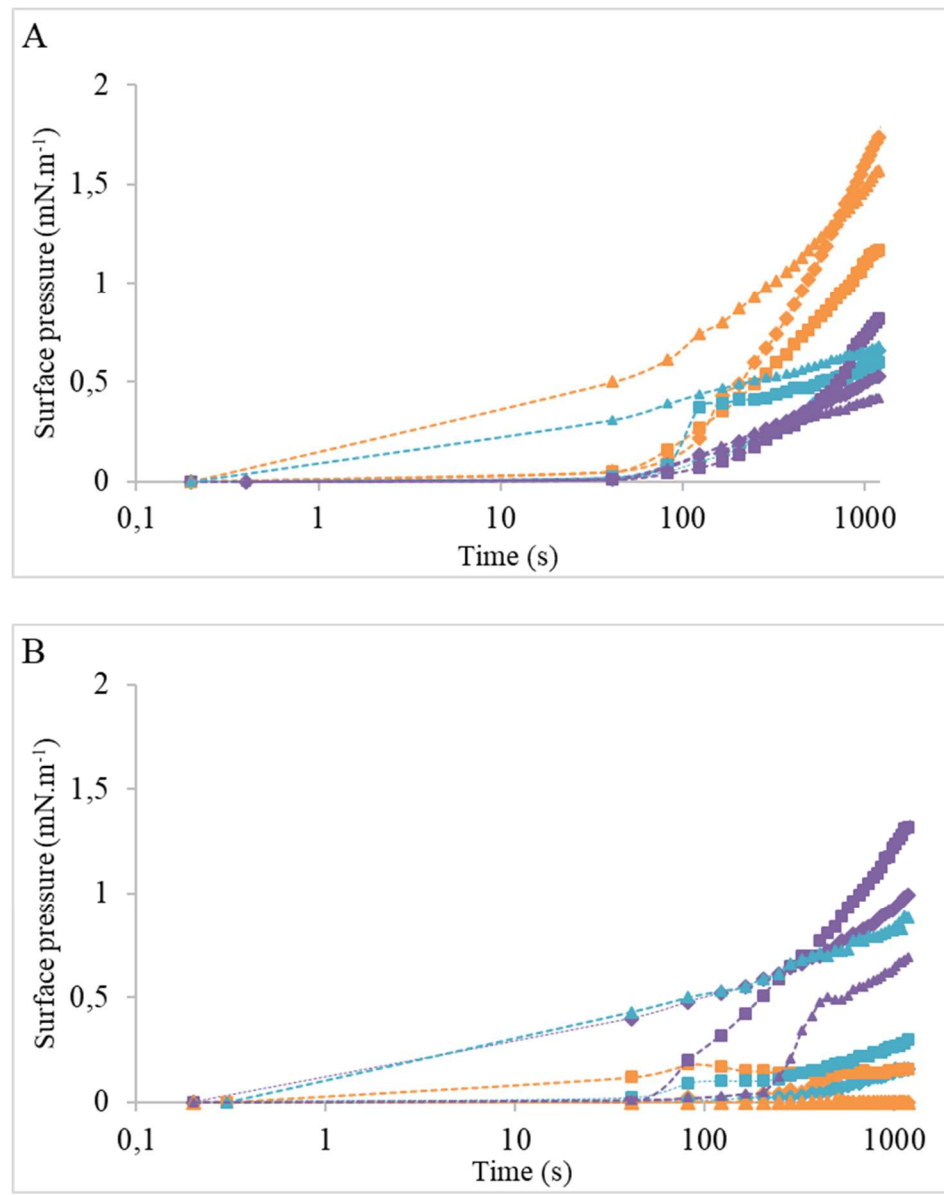
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Fig. 1

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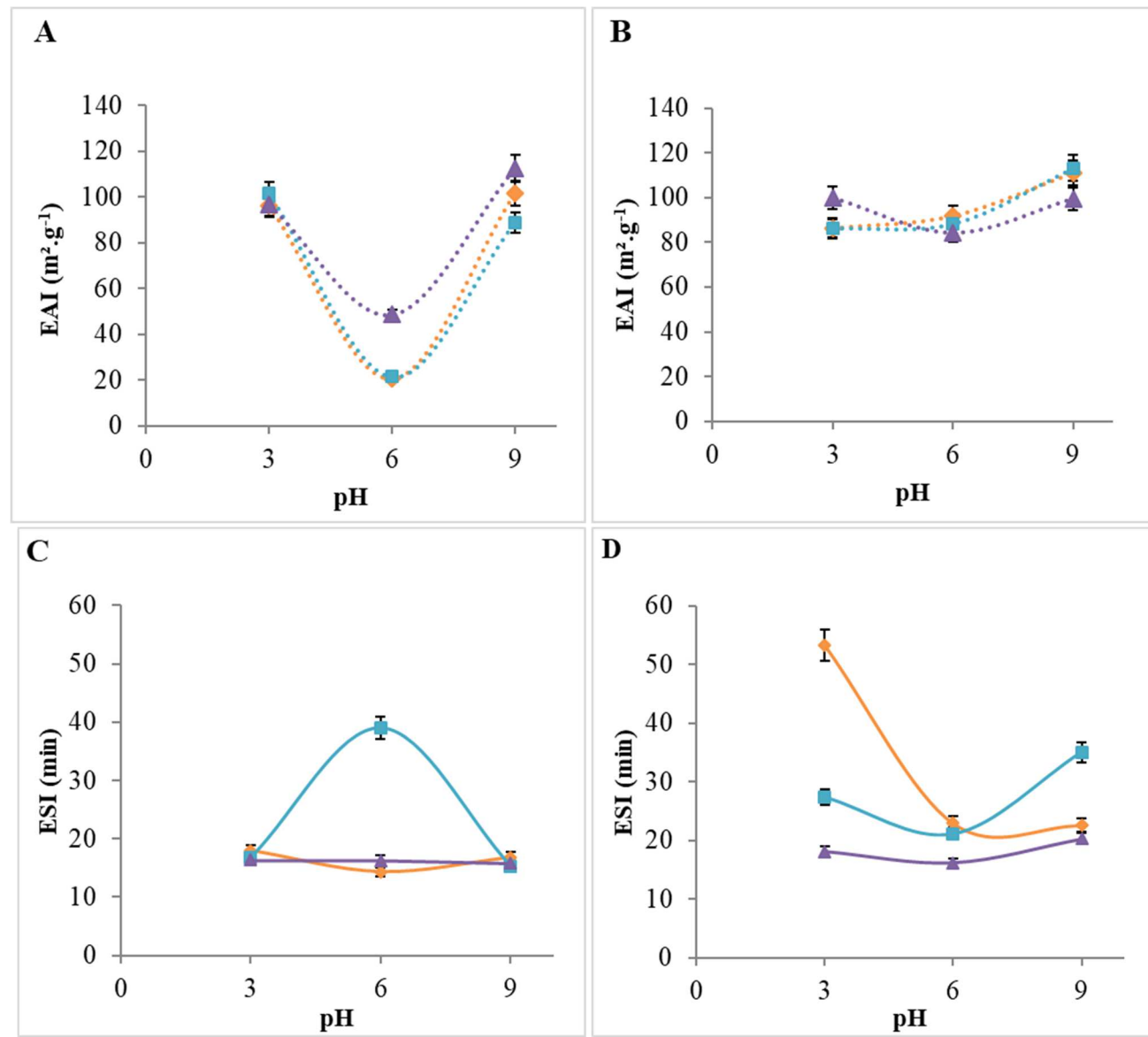
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Fig. 2



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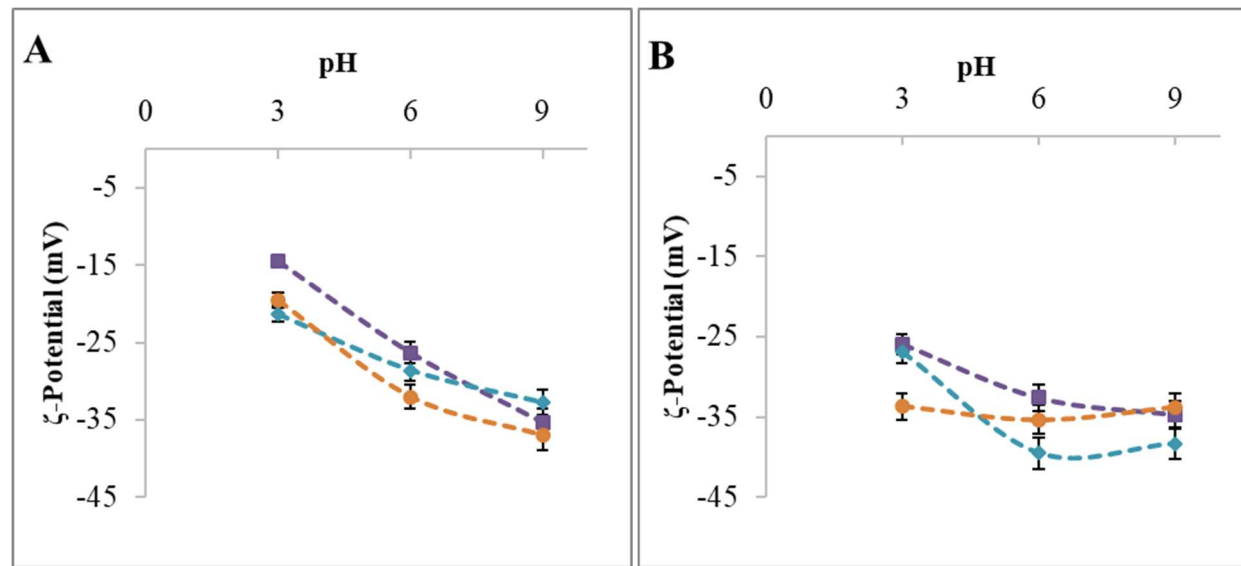
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Fig. 3



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Fig. 4

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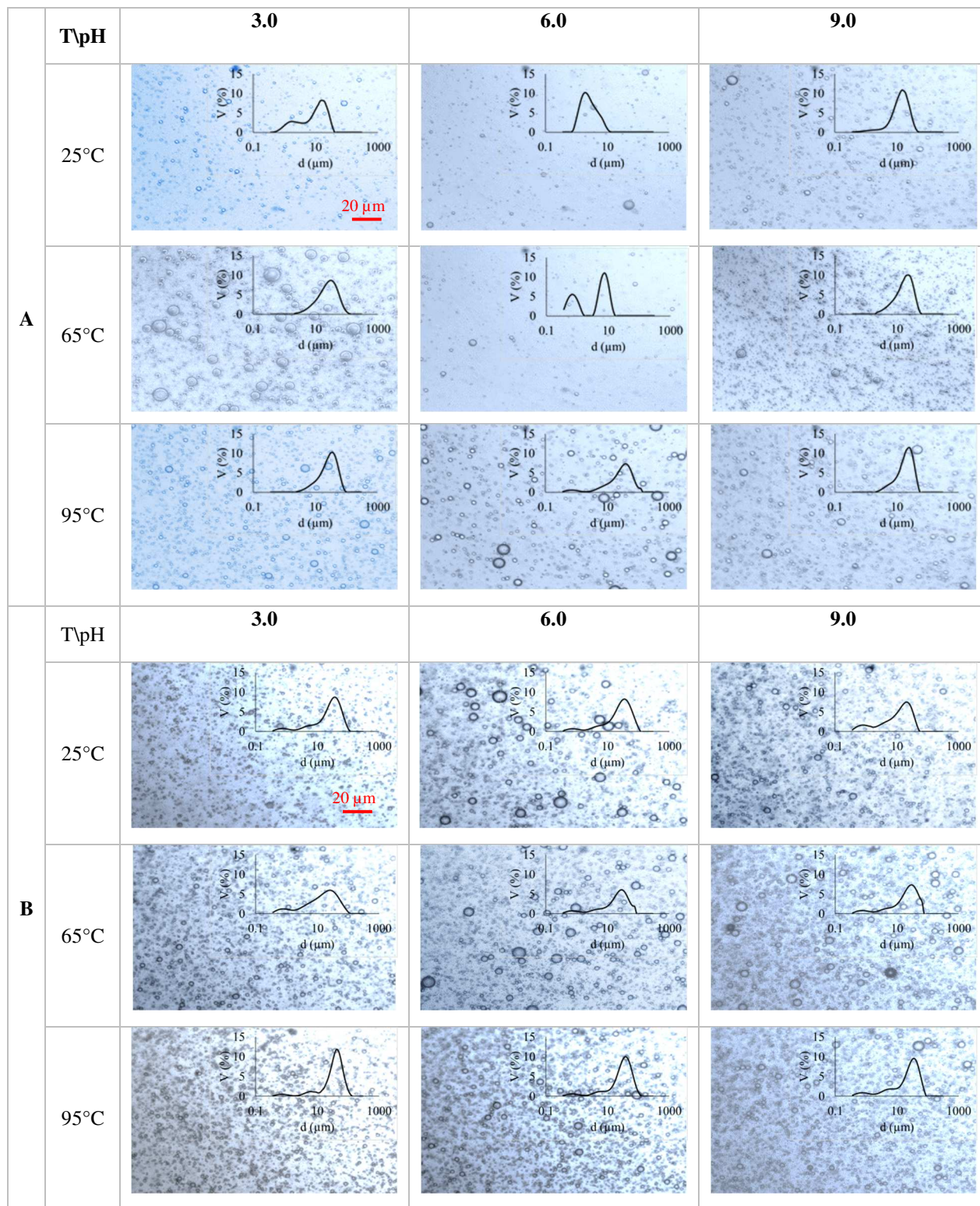


Fig. 5

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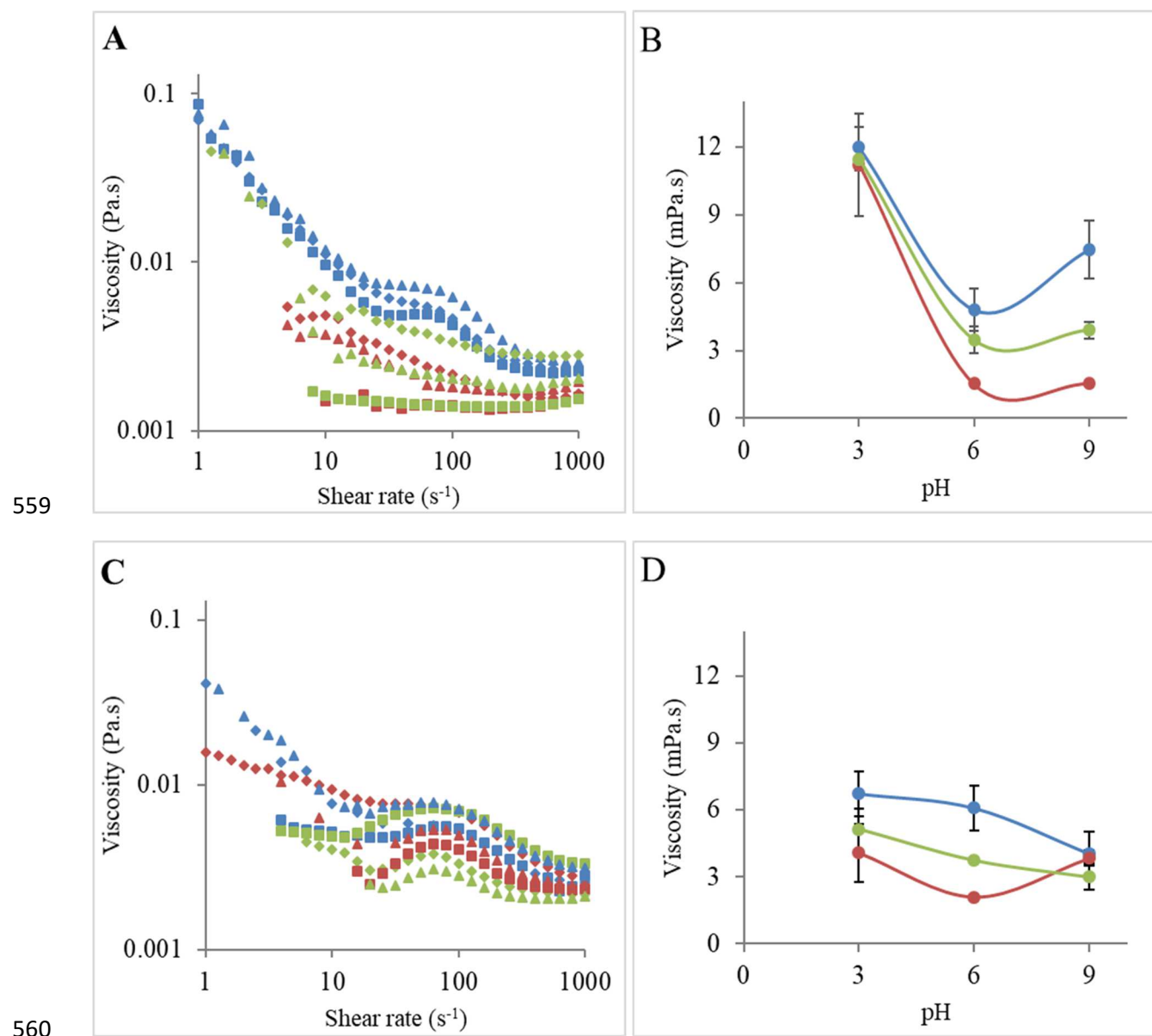


Fig. 6

570 **Table 1.** Statistical results from a 2-way ANOVA describing the physicochemical and
 571 emulsifying properties of Ala-C and Ala-B proteins as a function of pH and temperature
 572 treatments.

Factors		<i>pH</i>	<i>T</i>	<i>pH</i> ²	<i>T</i> ²	<i>pH</i> × <i>T</i>
<i>EAI</i>	Ala-C	***	NS	***	NS	NS
	Ala-B	***	NS	***	NS	***
<i>ESI</i>	Ala-C	*	NS	NS	NS	NS
	Ala-B	**	NS	**	NS	NS
<i>ζ-Potential</i>	Ala-C	**	NS	NS	NS	NS
	Ala-B	*	NS	NS	NS	NS
<i>Surface hydrophobicity</i>	Ala-C	**	***	*	***	*
	Ala-B	***	***	***	***	***
<i>d₃₂</i>	Ala-C	***	*	***	NS	**
	Ala-B	NS	**	**	***	NS
<i>Surface pressure</i>	Ala-C	***	NS	***	NS	NS
	Ala-B	NS	***	*	**	*
<i>Viscosity</i>	Ala-C	***	NS	***	NS	NS
	Ala-B	*	**	NS	**	NS

Note that NS means 'not significant', referring to a $p > 0.05$, (*) means $0.01 < p \leq 0.05$, (**) $0.001 < p \leq 0.01$, (***) $p \leq 0.001$.

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580 **Table 2.** Sauter diameter d_{32} (μm) for Ala-C and Ala-B stabilised emulsions as a function of pH
 581 (3.0, 6.0, 9.0) and treatment temperature (25, 65, 95°C). The letters "a", "b" and "c" represent
 582 homogenous subsets of different classes.

	Temperature	25°C	65°C	95°C
pH 3.0	Ala-C	3.95 ^{ba}	13.67 ^b	14.15 ^{bc}
	Ala-B	6.87 ^{ba}	4.89 ^{ba}	9.82 ^b
pH 6.0	Ala-C	2.00 ^a	1.43 ^{ab}	9.88 ^{ac}
	Ala-B	7.24 ^{ba}	6.53 ^{ba}	8.98 ^b
pH 9.0	Ala-C	8.93 ^{ba}	11.38 ^b	12.12 ^{bc}
	Ala-B	3.34 ^a	6.08 ^a	7.31 ^{ab}

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588 **Figures captions**

589 **Fig. 1.** Surface hydrophobicity (A.U.) of Ala-C (A) and Ala-B (B) proteins in terms of
 590 temperature of heat treatment for different pH (●) 3.0, (●) 6.0, (●) 9.0. Data represent the mean ±
 591 standard deviation (n=3).

592 **Fig. 2.** Surface pressure ($\text{mN}\cdot\text{m}^{-1}$) over time (s) between rapeseed oil and Ala-C (A) and Ala-B
 593 (B) proteins treated at different pH (—) 3.0, (—) 6.0, (—) 9.0 and temperature of heat
 594 treatment (◆) 25°C, (■) 65°C, (▲) 95°C for 15 min. Data represent the mean of 3 measurements
 595 and standard deviation vary between 0.02 and 0.46.

596 **Fig. 3.** Emulsifying Activity ($\text{m}^2\cdot\text{g}^{-1}$) and Stability (min) Indices of Ala-C (A, C) and Ala-B (B,
 597 D) emulsions as a function of pH (3.0, 6.0 and 9.0) and temperature of heat treatment (◆) 25°C,
 598 (■) 65°C, (▲) 95°C. Data represent the mean ± standard deviation (n=3).

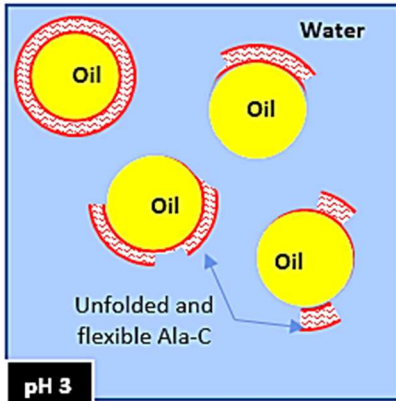
599 **Fig. 4.** ζ -Potential (mV) of Ala-C (A) and Ala-B (B) stabilised emulsions as a function of pH
 600 for different temperature of heat treatment (■) 25°C, (◆) 65°C, and (●) 95°C. Data represent the
 601 mean ± standard deviation (n=3).

602 **Fig. 5.** Volume-size distribution (%) of oil droplet diameter (μm) and micrographs of emulsions
 603 stabilised by Ala-C (A) and Ala-B (B) proteins treated at different pH (3.0, 6.0, 9.0) and
 604 temperature (25, 65 and 95°C for 15 min)

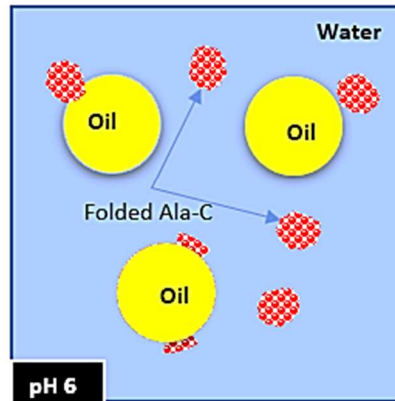
605 **Fig. 6.** Apparent viscosity (Pa.s) vs. shear rate (A, C) respectively for Ala-C and Ala-B
 606 stabilised emulsions in terms of pH (●) 3.0, (●) 6.0, (●) 9.0 and temperature of heat treatment (◆)
 607 25°C, (■) 65°C and (▲) 95°C and at a fixed shear rate (B, D) respectively for Ala-C and Ala-B
 608 stabilised emulsions in terms of pH for different treatment temperature (—) 25°C, (—) 65°C,
 609 (—) 95°C. Data represent the mean ± standard deviation (n=3).

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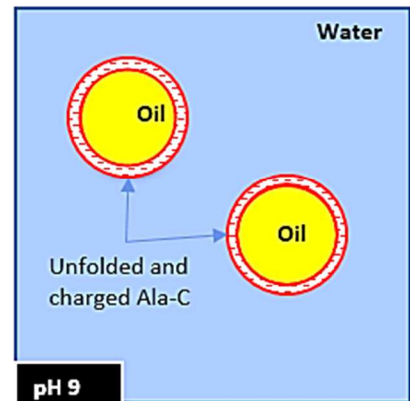
→ Without heat treatment (25°C)



Good emulsifying activity due to Hydrophobic forces

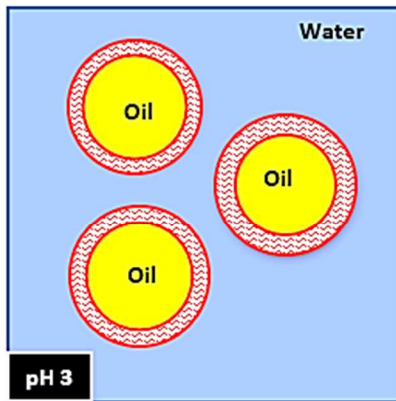


Poor emulsifying activity close to pI

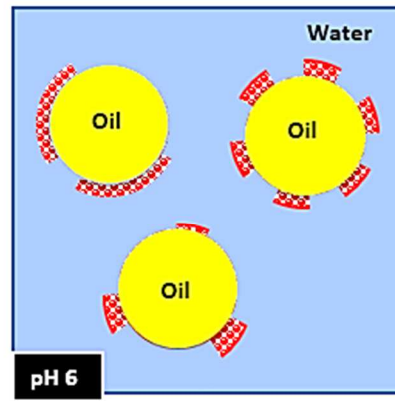


Good emulsifying activity due to electrostatic forces

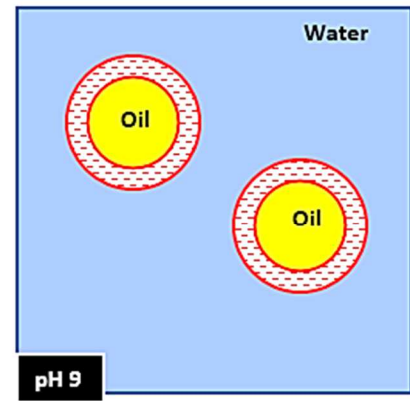
→ After heat treatment (95°C)



Improved emulsifying activity after heat treatment



Onset of emulsifying activity after heat treatment



Improved emulsifying activity after heat treatment