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1	Camel α -lactalbumin at the oil-water interface: Effect of
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34 Abstract

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

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

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

80 Structurally, α -lactalbumin is an amphiphilic protein elected to be a good emulsifier agent to produce protein-stabilised emulsions (Lam & Nickerson, 2013). When added to 81 82 oil-water interface, the bovine α -lactalbumin proteins form a cohesive interfacial protein layer around oil droplets, providing steric and either electrostatic repulsive or attractive 83 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 understand the adsorption process of cow milk proteins at the oil-water interface, the 86 87 structure and the composition of the adsorbed layer, and the influence of the physical and chemical environment on their emulsifying properties (Dickinson, 2001; Singh, 2011). 88

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

95 **2.** Materials and methods

96 **2.1.Materials**

Camel milk used in this study was purchased from a local farm in the region of Tozeur in south
Tunisia. Just after milking, 0.2 g/L of sodium azide (NaN₃) was added to stop bacterial
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.

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

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

108 Once the camel milk was skimmed by centrifugation $(3,800 \times g, 20 \text{ min}, 7^{\circ}\text{C})$, 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 ×g for 15 min (Lajnaf et al., 2018). Supernatant purification was then carried out using a 50 kDa ultrafiltration membrane (VivaFlow 200, Sigma-Aldrich, USA). A 111 pH adjusted water at 6.7 was used to refilter the retentate of the purification. The washing 112 process was carried out six times. Between each cycle, the initial flow of ultrafiltration 113 membrane was restored using a concentrated NaOH solution (10 mol/L) and rinsed with 114 115 Milli– Q^{TM} water. Once the permeate was collected, it was concentrated using a 5 kDa ultrafiltration membrane (VivaFlow 200, Sigma-Aldrich, USA), and then dialysed against 116 Milli–QTM water at 4°C and steady stirring. Water was replaced every 12 h for 4 days (Salami et 117 al., 2018). The protein content was 760 ± 2 g/kg, measured using a total nitrogen analyser 118 119 (TNM-1, Shimadzu Corp., Japan) and a nitrogen conversion factor of 6.38. A protein profile (sodium dodecyl sulphate polyacrylamide gel (12% SDS-PAGE)) was achieved at each step of 120 121 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**

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

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

135 **2.4. Emulsion properties**

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

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

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

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

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

150 **2.5. Emulsion** ζ–potential

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

157
$$\zeta = \frac{3 \eta}{2 \varepsilon} U \tag{3}$$

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 (m²/V.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:

167
$$d_{32} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2}$$
(4)

7

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

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

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

Surface hydrophobicity of Ala-C and Ala-B solutions treated under different conditions of pH 174 and temperature were measured according to the modified method of Alizadeh-Pasdar and Li-175 (2000)176 Chan using а spectrofluorimeter Flx (SAFAS, Monaco). An 8-anilino-1-naphthalenesulfonate (ANS) probe was added to interact with hydrophobic moieties 177 on the protein surface to give a fluorescent signal. Each protein solution was diluted to a 178 concentration of 0.8 g/L. 20 μ L of ANS (8.10⁻³ mol/L) solution dissolved in a phosphate buffer 179 (50.10⁻³ mol/L, pH 7.0) was added to 4 mL of each protein solution. The solution was excited at 180 390 nm, and the emission spectrum was measured from 400 to 600 nm. The emission and 181 182 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 reported as the maximum of the obtained spectrum. 184

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 solution (1 g/L of protein content) was added, followed by the immersion of Wilhelmy's plate; 188 then, an upper layer of rapeseed oil (10 mL) was poured over it. The measurement time was 189 190 fixed to 2,000 s. The interfacial tension was obtained by correlating the force F (mN) applied on the immersed plate to the wetted length of the plate L (mm) between the plate and the liquid as 191 expressed by Eq. (5): 192

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

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

198
$$\Pi(t) = \sigma_0 - \sigma_t \tag{6}$$

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

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

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

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

219 **3. Results and discussion**

3.1. Surface properties of camel and bovine α–lactalbumin

221 **3.1.1. Surface hydrophobicity**

Figure 1 shows the evolution of surface hydrophobicity of Ala–C and Ala–B as a function of pH (3.0, 6.0 and 9.0) and heat treatment (25°C, 65°C and 95°C). Globally, the surface 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 increases at pH 3.0 and 9.0, while it remains nearly constant for pH 6.0. Contrary to Ala–B, 226 surface hydrophobicity of Ala–C proteins (Fig. 1A) increases only when the temperature of heat 227 treatment is high (95°C) for all pH values. However, it must be pointed out that the effect of pH 228 is the same for both proteins: surface hydrophobicity is significantly higher at pH 3.0, while at 229 230 pH 6.0 and 9.0, differences emerge only after heat treatment above 65°C, as described by ANOVA in Table 1. Ala–B proteins probably aggregate through hydrophobic interactions at 231 232 these conditions (pH 3.0 and pH 9.0), resulting in a reduced hydrophobicity. The stable behaviour at pH 6.0 might be explained by its globular rigid conformation and its enhanced 233 234 resistance to heat treatment close to pI (Lam & Nickerson, 2015b; Zhai et al., 2012). For camel milk proteins, this evolution is explained by the exposure of hydrophobic moieties at high heat 235 236 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 susceptibility of cow whey protein, in particular Ala-B, to high heat treatment, as mentioned 239 240 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 observed for Ala-C is affected by pH variation and heating temperature and leads to a 242 conformational reorganisation to possibly align at the oil-water interface. 243

244 **3.1.2.** Interfacial properties

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

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 slope until the second change, it reflects, therefore, the diffusion-controlled adsorption of the first 261 layer of proteins at the interface) was observed instead. ANOVA (Tab.1) shows a very 262 263 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 conformation to interact with the fatty acids of rapeseed oil, resulting in a very significant 266 reduction in interfacial tension. 267

3.2. Emulsifying properties

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

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

Regarding EAI (Fig. 3A and B), both proteins display a different behaviour as a function of pH and treatment temperature, but they exhibit comparable maximum values, between 100–110 $m^2.g^{-1}$. EAI of Ala–B stabilised emulsions shifts limitedly between 84 and 110 m².g⁻¹ (Fig. 3B), while EAI of Ala–C stabilised emulsions (Fig. 3A) declines to 22 m².g⁻¹ at pH 6.0, where only the effect of pH is significant (*p* <0.001).

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

Besides, Voutsinas et al. (1983) reported that the change in emulsifying properties of whey 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

increased with thermal treatment and denaturation due to chemical composition changes.

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

As for emulsifying stability, Fig. 3D shows that ESI values of Ala-B stabilised emulsions are 298 relatively higher than Ala-C (Fig. 3C), where maximum stability is estimated at 53 min and 35 299 min, respectively. The stability of protein-stabilised emulsions has been abundantly discussed in 300 301 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 ESI for whey proteins (Dissanayake & Vasiljevic, 2009; Voutsinas et al., 1983). While Voutsinas 305 306 et al., (1983) described an increase in ESI with heat treatment, Dissanayake and Vasiljevic, (2009) advocated that no change in ESI was observed with increasing heat treatment 307 temperature. However, (Lam & Nickerson, 2015b) reported that the increased ability of the 308 309 apo-form of Ala-B to adhere to the interface also allowed for greater emulsion stability. Stability was greater at pH 7.0 than pH 5.0 due to the presence of electrostatic repulsive forces between 310 311 protein coated droplets. Furthermore, a pre-treatment at a temperature of 65 °C on Ala-B apo-form resulted in an increase in its secondary structure, leading to the greatest emulsion 312 stability, presumably due to the partial denaturation and refolding at this temperature. 313

Finally, the stability of Ala–C stabilised emulsions at pH 3.0 and 9.0, exhibiting high EAI, can also be attributed to electrostatic forces. This conclusion is strengthened by the negligible effect of heat treatment on camel milk proteins as previously reported (Atri et al., 2010; Lajnaf et al., 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 emulsions, the ζ -potential remains relatively constant regardless of the temperature. No 321 significant effect of heat treatment (p > 0.05) was confirmed by ANOVA (Tab.1). The 322 323 ζ -potential of Ala-B stabilised emulsions (Fig. 4B) is relatively higher than Ala-C for which it remains between -25 mV and -40 mV and between -15 mV and -37 mV, respectively. 324 Bhattacharjee (2016) reported that highly stable emulsion could be observed when ζ -potential 325 departed from zero by about at least ± 30 mV. In this work, ζ -potential exceeds -35 mV for both 326 emulsions at specific values of pH and temperature (i.e. pH 9.0-25°C/95°C, and pH 327 328 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 higher for Ala–C than Ala–B stabilised emulsions according to ANOVA analysis (Tab. 1). 332 333 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

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

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

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

362 3.3. Emulsion rheology

The apparent viscosity (η) was measured vs. shear rate between 0.1 to 1,000 s⁻¹ and plotted in 363 Fig. 6. The evolution of viscosity of Ala–C and Ala–B stabilised emulsions at a fixed shear rate 364 (10 s⁻¹) is presented in terms of pH (3.0, 6.0 and 9.0) and temperature treatment (25 °C, 65°C and 365 95°C). First, all the studied emulsions exhibit a nearly Newtonian behaviour at a high shear rate, 366 and non-Newtonian trends at low shear rate. The emulsion viscosity is lower for Ala–B (Fig. 6C) 367 368 than Ala–C (Fig.6A), and ranges between 2 mPa.s and 40 mPa.s, and between 1.5 mPa.s and 87 mPa.s, respectively. For Ala-B stabilised emulsions, the change in viscosity due to pH remains 369 weak. Thus, the effect of the heat treatment is higher than the pH effect (Tab. 1). This relative 370 371 invariability of the viscosity of Ala-B stabilised emulsions is correlated to EAI and ESI data presented in section 3.2. For Ala–C stabilised emulsions, only the effect of pH is significant (p < p372 0.001). The emulsion viscosity is higher at pH 3.0 than at pH 6.0 and 9.0, regardless of heat 373 treatment. Some trends remain, however, similar for both proteins: pH 3.0 provided in general 374 375 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 changes in the conformational structure at the oil-water interface. At 25°C, proteins adsorb in 378 their native form and then unfold at the interface, mainly by hydrophobic interactions (Fig. 1B). 379 A viscoelastic film is, therefore, formed around the oil droplets, resulting in high viscosity 380 values. Conversely, after heat treatment at 65°C and 95°C of Ala-B proteins, their structure has 381 382 already been modified by the temperature effect mainly through hydrophobic interaction, as

shown previously, explaining the decrease in surface hydrophobicity at this temperature (Fig.

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 friction reduction caused by low droplet density, as shown by microscopic observations in Fig. 386 387 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 phases according to the low ζ -potential of camel protein emulsions previously reported (Fig. 4A) 389 390 as well as to a reduced interfacial tension (Fig. 2A). Besides, an intermediate plateau emerged between 20 and 100 s⁻¹, included between the shear-thinning behaviour observed at low and vert 391 392 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 already reported that elastic interfacial layers, which is the case of adsorbed protein layers, could 395 396 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 circulation inside the drops and drop deformation, and the interfacial tension minimizes the 398 movement of the flow pattern outside the drops (Otsubo & Prud'homme, 1994). 399

400 **Conclusions**

401 Two different types of α -lactalbumin proteins from camel and cow milks have been compared as a function of pH change and heat treatment conditions; their behaviours at the oil-water interface 402 have been deduced through direct and indirect analysis of their structures and their emulsifying 403 404 properties. It has been identified that the major factor governing the camel α -lactalbumin emulsion stability is its flexibility enabling conformational rearrangement, which is affected by 405 406 protein heat treatment, while electrostatic repulsion is still a key factor to explain the effect of pH change. Camel α -lactalbumin is significantly affected by pH variation while cow α -lactalbumin 407 is more sensitive to heat treatment. High heat treatment enhances the camel α -lactalbumin 408 surface properties, which maintains the emulsifying properties of this protein. Ala–C proteins 409 410 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 camel α -lactalbumin is, therefore, steric hindrance which behaves differently as a function of 415 416 environmental changes. These facts suggest an unbalance in the two dominant forces (the electrostatic effect and configurational entropy) that drives conformational rearrangement at 417 418 emulsion interfaces toward the electrostatic repulsion. These results thus broaden our understanding of the factors controlling protein structural change at emulsion interfaces and how 419 this affects emulsion stability. Depending on the desired application (high/low stability, high/low 420 421 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

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570	Table I. 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		рН	Τ	pH^2	T^2	pH imes T
EAI	Ala–C	***	NS	***	NS	NS
	Ala–B	***	NS	***	NS	***
ESI	Ala–C	*	NS	NS	NS	NS
	Ala–B	**	NS	**	NS	NS
ζ–Potential	Ala–C	**	NS	NS	NS	NS
	Ala–B	*	NS	NS	NS	NS
Surface hydrophobicity	Ala–C	**	***	*	***	*
	Ala–B	***	***	***	***	***
d 32	Ala–C	***	*	***	NS	**
	Ala–B	NS	**	**	***	NS
Surface pressure	Ala–C	***	NS	***	NS	NS
	Ala–B	NS	***	*	**	*
Viscosity	Ala–C	***	NS	***	NS	NS
	Ala–B	*	**	NS	**	NS

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

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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.
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	Temperature	25°C	65°C	95°C
nU 2 0	Ala–C	3.95 ^{ba}	13.67 ^b	14.15 ^{bc}
рп 5.0	Ala–B	6.87 ^{ba}	4.89 ^{ba}	9.82 ^b
рЦ 6 0	Ala–C	2.00 ^a	1.43 ^{ab}	9.88 ^{ac}
рп 0.0	Ala–B	7.24 ^{ba}	6.53 ^{ba}	8.98 ^b
	Ala–C	8.93 ^{ba}	11.38 ^b	12.12 ^{bc}
рп 9.0	Ala–B	3.34 ^a	6.08 ^a	7.31 ^{ab}

588 **Figures captions**

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

- **Fig. 2.** Surface pressure (mN.m⁻¹) over time (s) between rapeseed oil and Ala–C (A) and Ala–B
- (B) proteins treated at different pH (-) 3.0, (-) 6.0, (-) 9.0 and temperature of heat treatment (\blacklozenge) 25°C, (\blacksquare) 65°C, (\blacktriangle) 95°C for 15 min. Data represent the mean of 3 measurements and standard deviation vary between 0.02 and 0.46.
- **Fig. 3.** Emulsifying Activity (m².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 (\diamond) 25°C, 598 (**a**) 65°C, (**a**) 95°C. Data represent the mean ± standard deviation (n=3).
- **Fig. 4.** ζ -Potential (mV) of Ala–C (A) and Ala–B (B) stabilised emulsions as a function of pH for different temperature of heat treatment (**■**) 25°C, (**♦**) 65°C, and (**●**) 95°C. Data represent the mean ± standard deviation (n=3).
- **Fig. 5.** Volume-size distribution (%) of oil droplet diameter (μ m) and micrographs of emulsions stabilised by Ala–C (A) and Ala–B (B) proteins treated at different pH (3.0, 6.0, 9.0) and temperature (25, 65 and 95°C for 15 min)
- **Fig. 6.** Apparent viscosity (Pa.s) vs. shear rate (A, C) respectively for Ala–C and Ala–B stabilised emulsions in terms of pH (\bullet) 3.0, (\bullet) 6.0, (\bullet) 9.0 and temperature of heat treatment (\bullet)
- 607 25° C, (**a**) 65° C and (**b**) 95° C and at a fixed shear rate (B, D) respectively for Ala–C and Ala–B
- stabilised emulsions in terms of pH for different treatment temperature (----) 25° C, (----) 65° C,
- 609 (---) 95°C. Data represent the mean \pm standard deviation (n=3).
- 610

→ Without heat treatment (25°C)



Good emulsifying activity due to Hydrophobic forces

→ After heat treatment (95°C)



Poor emulsifying activity close to pl



Good emulsifying activity due to electrostatic forces



Improved emulsifying activity after heat treatment



Onset of emulsifying activity after heat treatment



Improved emulsifying activity after heat treatment