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1 **Effect of pH and heat treatment on structure, surface**
2 **characteristics and emulsifying properties of purified**
3 **camel β -casein**

4
5
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12 **Abstract**

13 Oil-in-water emulsions (20%/80%, w/w) were stabilised by two types of β -caseins (1 g/L, w/w)
14 extracted by rennet coagulation from camel and cow's milk, respectively. Both extracts were
15 treated under different ranges of pH (3.0, 6.0 and 9.0) and temperature (25, 65 and 95°C for 15
16 min) before emulsification. The emulsifying properties of the proteins were studied by surface
17 and interfacial measurements. Results show that the emulsifying activity (EAI) of camel
18 β -casein is higher than the bovine protein. Yet, both proteins exhibited heat stability and no
19 significant effect of temperature was reported. Conversely, a significant effect of pH on camel
20 β -casein was recorded: at pH 6.0, the lowest values of EAI were measured and explained by
21 the formation of micellar protein structure. Under such conditions, camel β -casein is therefore
22 a novel emulsifying protein with high potential to stabilise oil-in-water interfaces which
23 provides numerous applications for the food chemistry field.

24

25 **Key words**

26 Camel milk, β -casein, emulsion, surface hydrophobicity, interfacial tension, granulometry,
27 dairy protein.

28 **1. Introduction**

29 Camel milk plays an important role in human nutrition; its production has evolved on a broad
30 commercial scale in modern camel farms (FAOSTAT, 2018; Hailu et al., 2016). Its consumption
31 is increasingly common in many countries in Asia, Africa and Europe for its nutritious and
32 medicinal properties due to the presence of essential nutrients (protein, fat, lactose, minerals),
33 bioactive molecules, such as lactoferrin and lysozyme (Al Haj & Al Kanhal, 2010; Elagamy,
34 2000; Figliola et al. 2021; Hailu et al., 2016), and especially for the absence of β -lactoglobulin
35 (Maqsood et al., 2019; Swelum et al., 2021).

36 Cow's milk proteins, in particular caseins are good emulsifiers that have been widely studied
37 (Dickinson, 2001; Huck-Iriart et al., 2016; Jahaniaval et al., 2000; Liang et al., 2016;
38 McSweeney et al., 2004) and often used as emulsifiers in many food, pharmaceutical and
39 cosmetic applications due to their amphiphilic character and their ability to change their
40 molecular conformation according to pH and temperature operating conditions. The most
41 abundant, β -casein, is found in molecular or aggregated form in solution, based on
42 physiochemical parameters. Its content reaches 36% in cow's milk casein and up to 65% in

43 camel milk casein. Thus, our interest is particularly oriented to explore the potential emulsifying
44 properties of camel β -casein protein.

45 Many studies have characterised the molecular structure of this protein in comparison with
46 the bovine one and showed that the molecular weight of camel β -casein (24.65 kDa) is higher
47 compared to that of bovine β -casein (23.58 kDa). The numbers of amino acid residues in camel
48 and bovine β -caseins are 217 and 209, respectively, as reported by Barzegar et al. (2008) and
49 Mohamed et al. (2021). Sequence alignment of bovine and camel β -caseins shows that
50 sequence similarity and identity between these two caseins are 84.5% and 67.2%, respectively
51 (Barzegar et al., 2008). The β -casein has a flexible linear disordered secondary structure and
52 no intramolecular crosslinks. It has also a hydrophilic region at the N-terminal and a
53 hydrophobic region of zero net charge at the C-terminal of the molecule (Li et al., 2016;
54 McCarthy et al., 2014). The amphiphilic molecular nature of these proteins provides very
55 interesting emulsifying properties, as mentioned by several researches (Dalglish, 2006; Li et
56 al., 2016; Yahimi Yazdi et al., 2014). However, the techno-functional properties of camel milk
57 proteins are poorly understood and hardly studied as in the case of the camel β -casein. In a
58 recent work, Lajnaf et al. (2020) investigated various features of camel β -casein, such as their
59 antioxidant, antimicrobial, emulsifying and physico-chemical properties. Their experimental
60 results displayed an increase in emulsion activity and stability with pH from 5.0 to 9.0 and
61 improved properties in comparison to bovine β -casein.

62 In the light of these homologies and differences, the aim of this paper is to focus on the
63 emulsifying properties of camel β -casein in a larger range of pH (3.0–9.0) and as a function of
64 heat treatment (up to 95°C), then to compare these properties to those of the bovine β -casein
65 at the oil–water interface, and finally to correlate emulsifying properties to physicochemical
66 properties and molecular structure in order to promote new applications of camel β -casein to
67 the food chemistry field.

68 **2. Materials and methods**

69 *2.1 Materials*

70 Camel (*Camelus dromedaries*) milk and cow (*Bos taurus*) milk used in this study were
71 purchased from a local farm in the region of Touser in south Tunisia and in the region of
72 Clermont-Ferrand in the centre of France, respectively. Just after milking, 0.02% of sodium
73 azide (NaN₃) was added to stop bacterial proliferation; then, milk was stored at 4°C. The

74 β -casein proteins of camel and cow's milk were obtained according to the method described
75 below (section 2.2).

76 Rapeseed oil for alimentary use was purchased from a local supplier and used without further
77 purification. Water was produced using a Millipore Milli-Q™ water purification system
78 (Millipore Corp., Milford, MA, USA). All other chemicals used in this study are of reagent
79 grade and purchased from Sigma-Aldrich (USA).

80 2.2 Methods

81 2.2.1 β -Casein extraction

82 The extraction of β -casein from camel and cow's milk was carried out following the
83 modified method of (Huppertz et al., 2006). Briefly, caseins of both types of milk were
84 precipitated by rennet coagulation (active chymosin ≥ 520 mg/l, C.P.F., France) at 35°C for 90
85 and 60 min for camel and cow's milk, respectively. A first centrifugation at 5,000 g for 15 min
86 followed by inactivation of the enzyme at hot water (80°C for 5 min) were carried out. The
87 resulting casein curd was then washed and suspended in cold water at an equivalent volume to
88 the discarded whey. The curd-cold water mixture thus obtained was kept at 5°C for 24 hours at
89 steady stirring, then centrifuged at 5,000 g for 15 min at 5°C. The β -casein recovered after
90 filtration of the supernatant was freeze-dried and stored for further use.

91 2.2.2 Chemical characterization of the β -casein extracts

92 The protein content was evaluated using a total nitrogen analyzer (TNM-1, Shimadzu Corp.,
93 Japan). This allows the specific detection of nitrogen by chemiluminescence according to the
94 EN 12260 standard. The protein solutions were previously diluted to a total nitrogen content
95 lower than 100 ppm (optimal detection range). The protein content is given by Eq. (1):

$$96 \quad C \text{ (mg. L}^{-1}\text{)} = TN \times f \times d \quad (1)$$

97 where C is the protein mass concentration (mg/L), TN is the measured total nitrogen content
98 (ppm), f is the conversion factor equal to 6.38 and d is the dilution factor.

99 Lactose content was measured using the modified Dubois method. Briefly, 500 μ L of
100 previously diluted sample was added to an equal volume of 5% (w/v) phenol and 2.5 mL of
101 pure sulfuric acid. The mixture was incubated for 10 min without stirring, then a second
102 incubation was performed for 30 min at 35°C after rigorous stirring. The optical density was

103 measured at a wavelength of 438 nm. The lactose content was determined after projection on a
104 0.1g/L glucose standard prepared under the same conditions.

105 Protein profile was performed according to the modified method of Laemmli (1970). 20 μ L
106 of each fraction was mixed with an equal volume of buffer solution (10% (w/w) SDS, 0.5 M
107 Tris-HCl (pH 6.8), 2% (w/w) glycerol, 0.5 M β -mercaptoethanol, and 0.1% (w/w) bromophenol
108 blue). The mixture was then heated at 95°C for 5 min, and then 20 μ l of sample was loaded into
109 the concentration gel wells. Electrophoretic migration was performed with a Bio-Rad apparatus
110 (Mini-Protean Tetra Cell, BioRad Laboratories, USA). The migration gel was composed of 4%
111 acrylamide concentration gel and 15% separation gel (Ereifej et al., 2011), was then subjected
112 to 120 V electric current for 2 hours. The gels were stained under agitation for 20 minutes with
113 a solution containing 0.1% (w/w) Brilliant Blue R-250 Coomassie Blue in a 10:40:50 solution
114 of acetic acid, ethanol, and water (v:v:v) and then decolorized for 4 hours in a solution
115 containing 14:10:76 ratio of acetic acid, ethanol, and distilled water (v:v:v). The molecular
116 weights of the different proteins were obtained using protein markers (Promega Corporation,
117 USA) with molecular weights ranging from 10 to 200 kDa, prepared under the same conditions
118 as the samples. Quantification was performed using an appropriate densitometric software,
119 provided by GelQuant.NET (biochemlabsolutions.com).

120 *2.2.3 Sample and emulsion preparation*

121 The β -casein stock solutions (0.1% w/w, corrected for protein content) were prepared by
122 dispersing lyophilized powders in Milli-Q™ water by mechanical stirring (550 rpm) at cold
123 temperature (5-7°C) for 90 min. pH was adjusted to 3.0, 6.0 and 9.0 using either 0.5 M HCl or
124 0.5 M NaOH. Heat treatment was followed at 65 and 95°C for 15 min; then, treatment was
125 stopped by ice bath to room temperature (23-25°C). Other samples of β -casein solutions were
126 kept at room temperature without heat treatment.

127 Emulsions were prepared by mixing 5 g of β -casein solutions with 20% w/w of rapeseed oil
128 within plastic centrifuge tube, followed by homogenisation at 21,500 rpm for 3 min using an
129 Ultraturrax T25 homogenizer (Ika-Werke GmbH, Germany) equipped with a SN25-10G ST
130 tool.

131 *2.2.4 Emulsion properties*

132 Emulsion stability and activity indexes (ESI and EAI, respectively) were measured
133 according to the method used in our previous work (Ellouze et al., 2020). EAI and ESI were
134 then calculated using Eqs. (2) and (3), respectively

135
$$\text{EAI (m}^2 \cdot \text{g}^{-1}) = (2 \times 2.203 \times N \cdot A_0) / (10^5 \times C \cdot \varphi) \quad (2)$$

136
$$\text{ESI (min)} = (A_0 / \Delta A) \times t \quad (3)$$

137 where A_0 is the absorbance of the diluted emulsion immediately after homogenisation, N the
138 dilution factor (250), C the weight of protein per volume (g/ml), φ the oil volume fraction in
139 the emulsion (20%), ΔA the difference of the absorbance between time 0 and 10 min ($A_0 - A_{10}$),
140 and t the time interval (10 min).

141 *2.2.5 Emulsion ζ -potential*

142 The ζ -potential was measured using a Zetasizer Nano ZS (Malvern Pananalytical, UK). The
143 emulsion samples were diluted at a ratio of 1:100 (v/v). Samples were equilibrated for 120 s
144 before collecting data and the sampling time was fixed at 400 μ s. Data was accumulated from
145 10 sequential readings at 25°C, and the mathematical model of Smoluchowski was selected
146 (Sze et al., 2003) to convert the electrophoretic mobility measurements into ζ -potential values

147 *2.2.6 Droplet size and microscopic observation*

148 The droplet size distribution was determined using a laser scattering technique (Mastersizer
149 3000E, Malvern Pananalytical, UK). Just after homogenisation, 1 ml aliquot of each emulsion
150 was gently blended to an equal volume of pH-adjusted buffer containing 1% sodium dodecyl
151 sulphate (SDS) to avoid multi-scattering effect and prevent emulsion flocculation. The droplet
152 size distribution of each emulsion was measured at steady agitation (1,500 rpm). The Sauter
153 diameter, d_{32} , was used to describe the mean diameter of droplets; this is defined as:

154
$$d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2 \quad (4)$$

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

156 Microscopic observations were carried out using an Axiovert 25 inverted microscope (Carl
157 Zeiss GmbH, Germany) equipped with a monochrome Pulnix camera (JAI, Japan, 640×480
158 pixels). Emulsion aliquots of 20 μ L were placed onto a microscope slide and carefully covered

159 by a cover slip, avoiding any bubble formation. Micrographs were recorded at $\times 100$
160 magnification.

161 2.2.7 Surface properties

162 Surface hydrophobicity of β -casein proteins treated at different pH and temperature was
163 measured according to the modified method of Alizadeh-Pasdar & Li-Chan (Alizadeh-Pasdar
164 & Li-Chan, 2000), which uses an 8-anilino-1-naphthalenesulfonate (ANS) probe to interact
165 with hydrophobic moieties on protein surface to give a fluorescent signal. Each protein solution
166 was diluted to a final protein concentration of 0.08%. 20 μ L of ANS (8 mM) solution dissolved
167 in a phosphate buffer (50 mM, pH 7.0) was added to 4 mL of each protein solution. The solution
168 was excited at 390 nm, and the emission spectrum was measured from 400 to 600 nm using a
169 spectrofluorimeter Flx (SAFAS, Monaco). The emission and excitation slits were set to 5 nm,
170 and the measurements were performed at 25°C. The maximum area of the fluorescence
171 spectrum was corrected with the area of the buffer.

172 The interfacial tension for each protein solution was measured using a K12 tensiometer
173 (Krüss GmbH, Germany) equipped with a platinum Wilhelmy's plate to achieve complete
174 wetting (contact angle θ is 0, i.e. $\cos(\theta)=1$). Within glass sample cup (40 mm diameter), 5 mL
175 of protein solution were added, followed by the immersion of Wilhelmy's plate; then, an upper
176 layer of rapeseed oil (10 mL) was poured over it. The measurement time was fixed to 2000 s.
177 The interfacial tension was obtained by correlating the force F (mN) applied on the immersed
178 plate to the wetted length of the plate L (mm) between the plate and the liquid as expressed by
179 Eq. (5):

$$180 \quad \sigma \left(\frac{\text{mN}}{\text{m}} \right) = \frac{F}{L} \cos(\theta) = \frac{F}{L} \quad (5)$$

181 The resulted data was displayed by the change in tension from the pure fluid value vs. log
182 time (Eq. 6), which allows an easy comparison of systems of different σ_0 and the visualization
183 of the diffusion rate of proteins at the oil-water interface (Beverung et al., 1999; Wu et al.,
184 1999).

$$185 \quad \Pi(t) = \sigma_0 - \sigma_t \quad (6)$$

186 In this equation, Π is the surface pressure, σ_t the measured interfacial tension at time t , and σ_0
187 the interfacial tension of pure fluids.

188

2.2.8 Rheological behaviour

189 Rheological measurements were conducted at 25°C using an AR-G2 rheometer (TA
190 Instruments, USA) equipped with a 40 mm standard steel parallel plate. 2-ml aliquots of freshly
191 prepared emulsion were used per measurement. A flow test was carried out between 0.1 s⁻¹ and
192 1,000 s⁻¹ of shear rate. For all measurements, a gap distance was fixed at 1000 µm.

193

2.2.9 Statistics

194 All experiments were performed in triplicate and reported as the mean ± standard deviation.
195 A two-way analysis of variance (ANOVA) was applied to test for significance of the main
196 effects, i.e. pH (3.0, 6.0, 9.0) and temperature treatment (25°C, 65°C, 95°C), along with their
197 associated interactions, on the physicochemical and emulsifying properties of β-CNC and
198 β-CNB proteins. A quadratic model with a second-order interaction term was assumed to
199 correlate the physicochemical and emulsifying properties of proteins to the main factors, as
200 expressed in Eq. (7):

$$201 \quad Y_i = a_0 + a_1 \cdot pH + a_2 \cdot T + a_3 \cdot pH^2 + a_4 \cdot T^2 + a_5 \cdot pH \cdot T \quad (7)$$

202 where Y_i is the tested response, a_i are the observed effects, and pH and T are the main factors.
203 The significance of the effects is expressed in terms of p-value (p). Statistical analyses were
204 carried out using IBM SPSS Statistics (Ver. 20, IBM, USA).

205 The significance of the effects is expressed in terms of the p-value (p) and is represented in
206 supplementary materials.

207 3 Results and discussion

208

3.1 Chemical characterization of the β-casein extracts

209 Protein extracts of β-CN from cow's milk and camel milk (β-CNB and β-CNC,
210 respectively) obtained according to the described protocol of (Huppertz et al., 2006) were
211 characterized and results are presented. The chemical composition (Table 1) and the protein
212 profile (Fig. 1) of the two obtained extracts (C1 and C4) as well as milk caseins before
213 extraction (C2 and C3) show that the protein contents of the two β-CN extracts are 73% and
214 51% for camel and cow's milk, respectively. The mineral and the lactose contents (Table 1) are
215 higher for β-CNB than for β-CNC. The extraction yield of β-CN is therefore higher for camel
216 milk considering its high β-CN content compared to cow's milk.

217 The protein profile (Fig. 1) displays a large band of β -CN protein in both extracts (molar
218 mass of 23~25 kDa). Besides, a slight band appears at the range of 18~19 kDa and is identified
219 to the κ -CN (columns C1 and C4). Table 2 summarizes the different proportions of the
220 extracted proteins (β -CN and κ -CN) which are in order of 87% and 12% for bovine extract
221 (C1) and of 93% and 6% for cameline extract (C4), respectively. Similar results were obtained
222 by Huppertz et al. (2006). The extraction of the bovine β -CN with the described method is,
223 therefore, accompanied by a small percentage of κ -CN. However, at low concentrations of
224 proteins (as in the case of this study: 0.1%), the presence of κ -CN is thus considered negligible.

225 *3.2 Emulsifying properties of the β -casein*

226 The emulsifying activity and stability indexes (EAI and ESI) of the prepared oil-in-water
227 emulsions stabilized by the β -CN extracts of cow's and camel milk at the concentration of 1
228 g/L are represented in terms of pH and temperature in Fig. 2.

229 **The EAI (Fig. 2A) of β -CN depends on pH and treatment temperature** as well as on the
230 milk type. According to ANOVA, **only the effect of pH is statistically significant on camel**
231 **β -CN ($p \leq 0.01$). Therefore, there is no significant effect of temperature on both types of**
232 **β -CN where the EAI is almost constant vs. temperature.** However, EAI is higher for the
233 β -CNC than the β -CNB whatever the temperature (maximum values of 122 vs. 98.5 $\text{m}^2.\text{g}^{-1}$,
234 respectively) at pH 3 and pH 9, whereas the opposite behaviour usually emerges at pH 6 which
235 is close to the isoelectric point of the proteins.

236 These results are coherent with those of Lee et al. (2004) and Pérez-Fuentes et al. (2017)
237 which described the adsorption of the β -CNB on hydrophobic surfaces and its dependence on
238 the effect of ionic force, including pH. Indeed, the bovine (β -CN, of amphiphilic nature,
239 changes its conformation with high ionic forces, which alters its adsorption kinetics at the
240 interface.

241 Regarding ESI (Fig. 2B), the average value of emulsions stability is about 15 ± 2 min, except
242 for two β -CNC stabilized emulsions for which ESI is between 30 and 50 min when treated at
243 pH 6.0-65°C and 95°C, respectively, and except for a third emulsion stabilized by β -CNB (ESI
244 about 31 min when treated at pH 9.0-25°C). However, these trends seem to correlate with low
245 EAI values, i.e., emulsions where the oil phase is poorly dispersed. **The effects of pH as well**
246 **as the temperature are significant only for the emulsions stabilized by camel β -CN ($p \leq$**
247 **0.001). At pH values of 3.0 and 9.0, it is noted that stability decreases with the increasing**

248 treatment temperature of β -CN. This decrease is more pronounced for bovine than
249 cameline β -CN for which stability is affected immediately after homogenization process.
250 While the emulsifying activity (EAI) of treated proteins (65 and 95°C) is independent of
251 the applied thermal treatment (Fig. 2), the emulsifying stability (ESI) gets lower as the
252 applied temperature is higher. This suggests changes in the β -CN conformation either by
253 intermolecular aggregations and associations, or by thermal distortions that diminish
254 their ability to stabilize oil-in-water interfaces. At pH 6.0, a reverse behaviour is observed:
255 an increase in emulsifying stability (ESI) with the increase of the applied heat treatment.
256 This behaviour is more pronounced for β -CNC (from 14.6 to 49.2 min) than for β -CNB
257 (from 14.5 to 16.3 min). This is due to the low activity previously observed for β -CNB
258 and/or to irreversible molecular interactions (denaturation), allowing emulsion stability
259 at these conditions.

260 *3.3 Interfacial properties of β -caseins*

261 In order to understand the mechanisms involved in the emulsification of camel milk β -CN
262 and the differences of molecular structure compared to bovine β -CN, the analysis of surface
263 hydrophobicity and interfacial tension was carried out.

264 *3.3.1 Surface hydrophobicity*

265 To characterise the surface hydrophobicity of the β -CN from camel and cow's milk, the
266 extrinsic fluorescence of the ANS chromophore fixed on its surface hydrophobic amino acid
267 residues after treatment at different pH and temperature was measured. The results are displayed
268 in Fig. 3. **Thus, the surface hydrophobicity of both proteins is greater at pH 3.0 than at
269 other pH (6.0 and 9.0), regardless of the temperature of heat treatment. Nevertheless, the
270 β -CNB are more hydrophobic under these conditions than β -CNC. At pH 6.0 and 9.0,
271 the surface hydrophobicity of β -CNC and β -CNB is almost equal.** ANOVA shows that the
272 pH effect is more significant for the β -CNC than the β -CNB. The effect of temperature is
273 significant only for β -CNC. **This result proves that both types of β -CN have flexible
274 protein conformation that depends on pH and that the β -CNB is relatively more resistant
275 to heat treatment.**

276 In the literature, it has been reported that the intrinsic hydrophobicity of the β -CNB is
277 usually lower than the β -CNC (Atamer et al., 2017; Barzegar et al., 2008; Esmaili et al., 2011),

278 which does, however, not contradict our results. **This should be due to the presence of five**
279 **residues of Tyrosine and eight residues of Phenylalanine, mainly located in the**
280 **hydrophobic part. However, the primary structure of β -CNC is devoid of Tryptophan**
281 **residues, while β -CNB contains a Tryptophan residue, although four Tyrosine residues**
282 **are missing.** In addition, intrinsic fluorescence due to these residues was shown to be more
283 important at a neutral pH (7) than at lower pH (5). Consequently, the 3D conformation of the
284 two proteins is different, **but the exposure of the hydrophobic parts on their surfaces is also**
285 **less important for β -CNC. This means that the β -CNC is more protective against a**
286 **change in pH (hidden hydrophobic residues), but not vs. temperature (Barzegar et al.,**
287 **2008; Li et al., 2016, 2019) for which this structure is relaxed, exposing higher surface**
288 **hydrophobicity after intensive heat treatment (95°C) and especially at acid pH (3.0). At**
289 **pH above the isoelectric point (pH 6.0 and 9.0) where electrostatic charges are negative,**
290 **the measured surface hydrophobicity is temperature-independent, and this is similar for**
291 **both types of proteins, which agrees with the data of this work.**

292 3.3.2 Surface pressure

293 The study of the interfacial tension of β -CN at the oil-water interface was carried out and
294 the results expressed in term of surface pressure are represented in Fig. 4A and 4B for camel
295 and bovine proteins respectively.

296 Surface pressure depends on protein's type as well as on pH and treatment temperature. For
297 the β -CNC solutions (Fig. 4A), surface pressure is greater than for the β -CNB (2.45 and 1.87
298 mN.m, respectively), *i.e.* the protein extract from camel milk reduces the interfacial tension
299 between water and rapeseed oil more than the cow's milk extract. This reduction is greater at
300 pH 3.0-25/95°C and at pH 9.0-95°C for β -CNC; the effects of pH and temperature are
301 statistically significant ($p < 0.001$) for β -CNC, but for β -CNB, the influence of pH and
302 temperature is reduced and more complex.

303 **The results also show that the increase in temperature affects the interfacial properties**
304 **of β -CN. This effect is more pronounced at 25°C and 65°C for the β -CNB where the**
305 **reduction in interfacial tension between the two phases (oil/water) is greater than at high**
306 **temperature (95°C). This agrees with the evolution of emulsifier activity. Although the**
307 **EAI (Fig. 2) of the β -CN is not influenced by thermal treatment, stabilization of emulsified**
308 **oil droplets (ESI) with β -CNB is higher at 25°C where proteins retain their native**

309 **structure (no thermal denaturation), which allows intramolecular hydrophobic**
310 **interactions and thus the maintenance of a stable protein film around the oil droplets. For**
311 **β -CNC, surface pressure is higher at pH 3.0 after high heat treatment (95°C). This result**
312 **is consistent with the previously measured surface hydrophobicity (Fig. 3). Hydrophobic**
313 **interactions and relaxed structure allow proteins to be more cohesive under the applied**
314 **treatments.**

315 *3.4 Characterization of the β -caseins stabilized emulsions*

316 Oil-in-water emulsions stabilized by the bovine and cameline β -CN were characterized in
317 terms of surface charge, droplet size distribution, and apparent viscosity.

318 *3.4.1 Surface charge*

319 The ζ -potential (Fig. 5A and B), which reflects the surface charges of the oil droplets
320 emulsified by β -CNC and β -CNB, does not exceed -30 mV for both types of proteins. The
321 electrostatic forces, which maintain the relative stability of the emulsion, as described above,
322 remain weak. The pH effect is significant for both types of β -CN, but not the temperature
323 effect. However, the interaction effect of both factors ($pH \times T$) is statistically significant for
324 the β -CNB emulsions.

325 For β -CNC stabilised emulsions (Fig. 5A), the ζ -potential is maximum at pH 9.0 for the
326 different temperature. Besides, at pH 6.0 the ζ -potential is lower than at pH 3.0 especially after
327 heat treatment (65°C and 95°C). This result, as well as that on the emulsifying activity index
328 (Fig. 2A), reveals that surface charges are the most involved in the protein adsorption at the oil-
329 water interface. **The electrostatic interactions between the charged moieties of the β -CN**
330 **proteins at their relaxed micellar structure induce high emulsifying activity (EAI). The**
331 **micellar structure is therefore, formed at higher protein concentration (0.1% w/v) than**
332 **the critical micellar concentration (CMC 0.05% w/v); then, it is relaxed by the effect of a**
333 **high pH (9.0).** These findings were also described by Pérez-Fuentes et al., (2017) for the
334 adsorption of bovine β -CN on hydrophobic surfaces.

335 For the β -CNB stabilized emulsions (Fig. 5B), the ζ -potential decreased with increasing pH
336 from -13 to -27.5 mV for untreated proteins (25°C) but increased for treated proteins from -29
337 to -18.8 mV. These results are close to those reported by McCarthy et al. (2013) for an emulsion
338 stabilized by β -CNB at 2% (w/w) concentration. These authors showed that the ζ -potential

339 was estimated to be ~ -30 mV at pH > 6 and ~ -20 mV at pH 5 (McCarthy et al., 2013).
340 Therefore, the impact of temperature causes a reversal evolution of these results. **Thus, heat-**
341 **treated β -CNB proteins lose their surface charges and adopt a compact structure. Protein**
342 **interactions and aggregation are mainly the cause of the previously observed decrease in**
343 **emulsifying stability (ESI).** These trends are primarily due to differences in the structure and
344 amino acid composition of the two studied proteins (Lam & Nickerson, 2013).

345 *3.4.2 Emulsion granulometry and microscopic observations*

346 The emulsion characterisation was also achieved by granulometry measurements of both
347 types of β -CN stabilised emulsions. The size distributions of the oil droplets stabilised by
348 β -CNB and β -CNC are bimodal (Fig. 6A and 6B, respectively). Two major populations of
349 droplets are then present; the first one (small droplets) with maximum size of 2 μm peaks at
350 about 1% of the emulsion volume; the second (large droplets) is centred around 35 μm in size
351 and peaks up to 13% in volume. The size droplet homogeneity on microscopic observations of
352 the β -CNC (Fig. 6A) emulsions is lower than the β -CNB (Fig. 6B) in terms of the different
353 pH and temperature values. It also emerges from the micrographs that emulsions stabilized by
354 β -CNC are finer at pH 3 and pH 9, but not at pH 6, than those stabilised by β -CNB. **These**
355 **observations reveal important flexibility of the β -CNC vs. the operating conditions by**
356 **allowing them to adopt different conformations and configurations at the oil-water**
357 **interface. At acidic pH (3.0), the fraction of large droplets increases slightly with the**
358 **temperature of heat treatment. Similarly, at pH 9.0 where the droplets size is the smallest**
359 **for the unheated proteins (25°C), this fraction increases more significantly after heating.**
360 Nevertheless, at pH 6.0, the highest volume of large droplets is observed at 25°C with a peak
361 about 56 μm where the proteins are at their compact micellar shape. **Otherwise, the droplets**
362 **size decreases after heat treatment due to the dissociation of their micellar structures. For**
363 **β -CNB stabilised emulsions, droplet size also increases with temperature.**

364 In parallel, the Sauter diameter (d_{32}) of the β -CNC stabilised emulsions (Fig. 6C) at pH 6.0
365 display the highest values. While for the β -CNB (Fig. 6D) stabilised emulsions, the highest
366 values are observed at pH 3.0, which confirms microscopic observations. The effect of pH is
367 more significant for the β -CNB than the β -CNC, as well as the effect of the interaction
368 ($pH \times T$) (Table 1). Microscopic observations of both types of emulsions highlight also that at
369 high temperature, oil droplets stabilized by β -CNC (Fig. 6A) are closer in terms of size (low

370 span), particularly at pH 9.0 where the smallest size is observed after high heat treatment
371 (95°C), which confirms the temperature effect previously discussed.

372 Finally, β -CNC seem to be more efficient to prepare fine emulsions under acidic and
373 alkaline conditions, whereas β -CNB must be preferred at neutral pH.

374 3.4.3 Emulsion viscosity

375 The rheological behaviour of the different prepared emulsions was evaluated, and the
376 viscosity measurements are represented in Fig. 7. Both types of emulsions represent a non-
377 Newtonian flow, as the viscosity is higher at low shear rates and decreases at high shear rates
378 (where it remains constant). For the β -CNC (Fig. 7A), viscosity varies between 5 and 65 mPa.s
379 while those of β -CNB (Fig. 7B) varies between 3 and 330 mPa.s. This latter high viscosity is
380 due, first, to hydrophobic forces of protein's surface at acid pH (Fig. 3). Besides, it was reported
381 (Ellouze et al., 2019; Maldonado-Valderrama et al., 2008; Seta et al., 2014) **that the β -CNB**
382 **proteins adsorb rapidly to the oil-water interface compared to globular proteins due to its**
383 **flexible and random molecular conformation.** Moreover, the β -CNB emulsions viscosity is
384 closely related to the oil droplets size. In such ranges, as reported previously (Ellouze et al.,
385 2019, 2020), the larger the droplets are, the higher viscosity is, resulting from cohesion forces
386 between droplets.

387 Similarly, for β -CNC (Fig. 7A) stabilized emulsions, despite the lower viscosity in
388 comparison with the β -CNB, such a correlation is observed, where at low shear rate, viscosity
389 is relatively higher at pH 6.0 due to the low emulsifying activity earlier discussed (section 3.2),
390 which results in bigger droplets at pH 3.0 due to the high surface hydrophobicity (Fig. 3).

391 4 Conclusion

392 The investigation of the emulsifying properties of camel milk β -casein allowed to identify
393 the main factors acting on the formation and the stabilization of oil/water interfaces. The
394 comparison with bovine protein showed important discrepancies explained mainly by
395 differences in molecular composition and structure. The presence of phospho-serine residues in
396 camel β -casein provides the thickness and steric stabilizing properties of the absorbed layer
397 surrounding the oil droplets. This protein shares some main characteristics with bovine
398 β -casein in terms of number of hydrophobic residues, surface hydrophobicity plots and number
399 of serine and threonine. Nevertheless, the resulting emulsifying properties has shown its ability

400 to adopt a micellar structure in such concentration ranges. The consequences are that camel
401 milk β -caseins present an enhanced ability to form softer emulsions and to stabilize small
402 droplets under acidic conditions, regardless of heat treatment as well as at pH 9 after heat
403 treatment compared to bovine β -casein.

404 The stabilization mechanisms highlighted in this study will allow a wider exploitation of
405 camel β -casein according to the treatment conditions in various pharmaceutical, food or
406 cosmetic applications.

407

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