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1 **Kinetics of heat-induced denaturation of proteins in model infant milk formulas as a function of**
2 **whey protein composition**

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9

10 Keywords: Infant formula; whey proteins; heat denaturation

11

12 Highlights:

- 13 • Whey protein composition of infant milk formulas governed the heat protein denaturation
14 yield
- 15 • Heat denaturation of α -lactalbumin is reduced in the absence of β -lactoglobulin
- 16 • Heat denaturation of β -lactoglobulin is enhanced in the presence of lactoferrin
- 17 • Heat denaturation of lactoferrin is not modified by the presence of the other whey proteins

18

19 Number of words: 6028

20 **Abstract**

21 The process of manufacturing infant milk formulas (IMFs) involves heat treatments that can lead to
22 whey protein denaturation. The objective of the study was to determine how protein composition
23 affects the denaturation kinetics of the whey proteins within IMFs. Three model IMFs (1.3% of cow's
24 milk protein) were produced with a caseins:whey proteins ratio of 40:60, differing only by the whey
25 protein composition. The kinetics of heat-induced denaturation of α -lactalbumin, β -lactoglobulin and
26 lactoferrin were investigated between 67.5°C and 80°C by chromatographic quantification of the
27 residual native proteins. Results showed that the heat-denaturation of α -lactalbumin was reduced
28 when β -lactoglobulin was absent. The heat-denaturation of lactoferrin was not affected by the
29 composition of the IMFs but its presence enhanced the heat-denaturation of β -lactoglobulin. This
30 study revealed that, for higher heat treatments (90°C/15 s, 75°C/15 min), IMF containing α -
31 lactalbumin and lactoferrin preserved a higher proportion of native whey proteins than IMFs
32 containing β -lactoglobulin.

33 1. Introduction

34 Infant milk formulas (IMFs) constitute an adequate substitute of human milk when breastfeeding is
35 not a possibility or a wish, although human milk is considered as the ideal infant food for the first five
36 months of life (World Health Organization, 2001). The majority of standards IMFs is produced using
37 defatted bovine milk and demineralized bovine whey proteins. However, human and bovine milks
38 vary in terms of nutrient composition, particularly regarding the protein fraction. The protein content
39 is approximately 9-12 g.L⁻¹ for mature human milk with an average caseins:whey proteins weight
40 ratio of 40:60, *versus* about 34 g.L⁻¹ for bovine milk with a caseins:whey proteins weight ratio of
41 80:20 (Chatterton et al., 2013). In addition, the whey protein composition varies between human and
42 bovine milks. In human milk, α -lactalbumin (α -LA) is the predominant whey protein (2.5 g.L⁻¹)
43 followed by lactoferrin (LF, 1.5 g.L⁻¹), whereas β -lactoglobulin (β -LG) is absent. In contrast, β -LG is the
44 predominant whey protein of bovine milk (3.5 g.L⁻¹), followed by α -LA (1.3 g.L⁻¹) while LF is at a lower
45 content (5.0 x 10⁻² g.L⁻¹) (Chatterton et al., 2013). To mimic the human milk composition, the
46 caseins:whey proteins ratio of bovine milk-based IMFs has to be equilibrated to match the human
47 milk ratio, while the protein nature remains usually different. However, previous studies (King et al.,
48 2007; Sandström, Lönnerdal, Graverholt, & Hernell, 2008) have demonstrated that infant feeding
49 with IMFs supplemented with specific whey proteins (LF, α -LA) had the same physiological benefits
50 (e.g. infection resistance, physical development, etc.) than breastfed infants, in contrary to infants
51 fed IMFs without specific whey protein supplementation. Moreover, thanks to the high proportion of
52 essential amino acids in α -LA and LF, the α -LA and LF supplemented IMFs allow reducing the IMF
53 total protein content while respecting the regulatory amino acid profile. This may have some health
54 benefits as lower protein content in 1st age IMFs has been shown to be associated with a lower risk
55 of obesity in 6-year-old children (Totzauer et al., 2018).

56 IMFs are submitted to several heat treatments during the manufacturing process to ensure the
57 microbial safety and extend the shelf life. Depending on the heating conditions, changes of milk
58 protein structure occur, varying potentially the properties and nutritional value of IMFs. It is

59 established that heat treatments above 60°C have undesirable consequences on the structure of
60 whey proteins, mainly α -LA and β -LG, leading to their polymerization and aggregation (Nguyen,
61 Streicher, & Anema, 2018). However, the behavior of whey proteins under heating is governed by
62 medium conditions, like the content and composition of proteins (Oldfield, Singh, & Taylor, 2005),
63 minerals (Petit et al., 2011) and sugars (Anema, Lee, & Klostermeyer, 2006) of milk protein-based
64 products. The heat denaturation of LF in bovine milk received very little attention compared to α -LA
65 and β -LG, probably because of the very low LF quantity in bovine milk. Nevertheless, previous studies
66 showed the heat-labile property of LF, depending on the presence of an iron ion in each of the two
67 homologous globular N- and C-lobes of the protein. At low ionic strength and neutral pH, LF in the
68 apo-form (iron free), native form (10-20% of iron saturation) and holo-form (iron saturated) began to
69 aggregate at 60°C, 65°C and 70°C, respectively (Brisson, Britten, & Pouliot, 2007). Overall, there is
70 very limited information in the literature on the denaturation of whey proteins in IMFs, and even less
71 in those that are mimicking the protein composition of human milk.

72 To fill the gap, the kinetics of heat-induced denaturation of individual whey proteins (α -LA, β -LG and
73 LF) were investigated in IMFs at temperatures from 67.5°C to 80°C. Three model IMFs were produced
74 at 1.3% total protein content (i.e. the protein content at which is applied the heat treatment during
75 the manufacture of liquid IMFs) with a caseins:whey proteins ratio of 40:60. The IMFs differed by the
76 whey protein composition: the “control IMF” had the same whey protein composition as bovine
77 whey whereas the IMFs denoted “LF⁺ IMF” and “LF⁺ α -LA⁺ IMF” respected the quantity of LF or of
78 both LF and α -LA found in human milk, respectively. We assumed that the level of whey protein
79 heat-denaturation was dependent on the whey protein composition of IMFs, since β -LG promotes
80 whey protein denaturation (Elfagm & Wheelock, 1978) and LF is one of the most heat-sensitive
81 protein in milk (Law, 1995).

82 **2. Material and methods**

83 2.1. Chemicals

84 The low-heat skimmed milk powder (SMP) was obtained by drying raw milk supplied by Fromagerie
85 Gillot (Saint-Hilaire-de-Briouze, France) at the semi-industrial drying unit Bionov (Rennes, France).
86 Briefly, skimmed milk obtained by centrifugation of raw milk was microfiltered on 1.4 µm membrane
87 at 50°C, concentrated by evaporation under vacuum at 64°C and spray dried. The characteristics of
88 SMP are described in Table 1. The whey protein nitrogen index (WPNI) was 9 mg of nitrogen/g of
89 SMP, which is a value for ultra-low heat SMP. Whey protein isolate (WPI; Prolacta®95), with a total
90 protein content of 84% (w/w; N x 6.38) as determined by the Kjeldahl method, was purchased from
91 Lactalis Ingredients (Bourgarré, France). Bovine lactoferrin (LF, Prodiect Lactoferrin®) and α-
92 lactalbumin (α-LA) were kindly supplied by Ingredia Dairy Experts (Arras, France) and Agropur Inc
93 (Appleton, USA), respectively. The protein content of the powder was 90% (w/w; N x 6.38) for LF and
94 84% (w/w; N x 6.38) for α-LA, as determined by the Kjeldahl method. The iron saturation level of LF,
95 determined by absorbance at 465 nm using an UV-Visible spectrophotometry, was found to be of
96 16%. The purified proteins α-LA, β-LG and LF, used as chromatographic standards, were purchased
97 from Davisco (MN, USA) for α-LA and β-LG, and from Ingredia Dairy Experts (Arras, France) for LF. All
98 protein powders were stored at -20°C until use.

99 Lactose was supplied by Armor Proteines (Saint-Brice-en-Coglès, France). CaCl₂·2H₂O was from
100 AnalaR (Leuven, Belgium), FeSO₄·7H₂O from Sigma-Aldrich (St-Louis, USA), KCl from Panreac
101 (Barcelona, Spain), Na₃PO₄·12H₂O from Merck (Darmstadt, Germany), and Na₃C₆H₅O₇·2H₂O from
102 Carlo Erba Reagents (Val-de-Reuil, France). Trifluoroacetic acid was from Fisher Scientific
103 (Loughborough, UK) and acetonitrile (Chromasolv™ for HPLC) from Fisher Scientific (Muskegon, USA).
104 All other materials were standard analytical grade. Water was Milli-Q water.

105

106 2.2. Preparation of model IMFs

107 Three IMFs were formulated at 5.5% (w/w) total protein and then diluted at 1.3% (w/w) with Milli-Q
108 water. A 40:60 caseins:whey proteins ratio was targeted for the formulation of IMFs, with either no
109 modification of the bovine whey protein proportion ("Control IMF", mixture of SMP and WPI) or the
110 replacement of a fraction of the whey proteins by LF ("LF⁺ IMF", mixture of SMP, WPI and LF) or a
111 mixture of LF and α -LA ("LF⁺ α -LA⁺ IMF", mixture of SMP, α -LA and LF) to reach the α -LA and LF
112 concentrations similar to those found in human milk. Briefly, IMFs were prepared at room
113 temperature by dispersing firstly the protein powders (WPI, LF, α -LA) and secondly the SMP, in a
114 concentrated solution of lactose at 24%. Then, the protein solutions were supplemented by minerals
115 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, KCl, $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ and $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ to ensure a similar mineral
116 composition for the three IMFs. The pH of the IMFs was adjusted at pH 6.8 with 1 M KOH, and then
117 the IMFs were stored at 4°C for at least 18h. The IMFs were prepared in triplicate for biochemical
118 characterizations and denaturation kinetic study at 75°C. The scheme of formulation is shown in
119 Supplementary Figure 1. In parallel, a solution of α -LA and a solution of LF (proteins used to replace
120 part of the WPI in IMFs) at identical α -LA and LF concentration as found in the LF⁺ α -LA⁺ IMF and
121 respecting the mineral and lactose contents of IMFs were prepared and named "Pure protein
122 solution".

123

124 2.3. Compositional analysis

125 Total nitrogen, non-protein nitrogen and non-casein nitrogen were determined by the Kjeldahl
126 method, with a nitrogen-protein conversion factor of 6.38 (International Dairy Federation, 1993).
127 Lactose content was quantified by refractometry after separation by high-performance liquid
128 chromatography (HPLC) using a Aminex A-6 ion exchange column (Bio-Rad, Hercules, CA, USA) as
129 described by Aburjaile et al. (2016). Calcium, iron, potassium and sodium contents were quantified
130 by atomic absorption spectrometry as described by Brulé, Maubois, & Fauquant (1974). Inorganic
131 phosphorus content was quantified by using the molybdenum blue method according to Jean (1969).

132 Chloride was quantified with the Sherwood Scientific chloride analyzer (Modele 926, Sherwood
133 Scientific Ltd, Cambridge, UK) as described by Berdagué, Grappin, & Duboz (1987). Ash content was
134 determined by weighing the dry mineral residue after water and volatile vaporization, and organic
135 matter oxidative burning at 550°C for 5h in a muffle furnace.

136 Each of these compositional analysis was performed in triplicate on each of the three IMFs.

137

138 2.4. Heat treatments

139 Glass capillary tubes (8 mm inner diameter and 40 mm length, Waters, USA), containing 800 µL of
140 IMFs pre-heated at 30°C during 1h, were placed in a thermally controlled water bath
141 (Fisherbrand™ Isotemp™ water bath, Thermo Fisher Scientific, Newington, USA) set at different
142 temperatures ranging from 67.5°C to 80°C (by step intervals of 2.5°C). For each condition, the heating
143 ramp (i.e. the time to reach the target temperature) was controlled with a YC-747UD Data Logger
144 Thermometer inserted in a reference tube. The heating ramp was converted into a heating time at
145 target temperature (as explained in section 2.6.1).

146 At various time intervals, tubes were removed and instantaneously chilled on ice bath to stop whey
147 protein denaturation/aggregation reactions. Unheated samples served as reference for determining
148 the total amount of native proteins before heating. Holding times for each heating temperature were
149 chosen to scan the denaturation/aggregation of whey proteins up to a yield of 80%. Three
150 independent experiments were performed for the heat treatment at 75°C, considering the standard
151 deviation equivalents at all heating temperatures investigated.

152 After cooling, 20 or 10 µL of 1.4 M acetic acid/acetate buffer was added to 150 or 200 µL of heated
153 and unheated IMFs or pure protein solutions, respectively, and the mixture was placed at 40°C for 2
154 min to precipitate the caseins and denatured/aggregated whey proteins. The proteins in the pH 4.6-
155 soluble fraction were separated from the precipitate by centrifugation at 14,000g for 20 min at 25°C,

156 using an Eppendorf Microcentrifuge MicroStar 17R (VWR, Leuven, Belgium). The supernatant was
157 diluted in Milli-Q water containing 0.106% (v/v) trifluoroacetic acid at 0.5 g.L⁻¹ of proteins for analysis
158 by reversed-phase high-performance liquid chromatography (RP-HPLC).

159

160 2.5. Quantification of native whey proteins

161 Native whey proteins were separated on a PLRP-S 300 Å, 8 µm, 150 x 3 mm separation column
162 combined with a guard column (Agilent Technologies, UK) connected to a Dionex UltiMate 3000
163 HPLC System (Thermo Fisher, Dreieich, Germany), equipped with a detector VWD3400RS (Thermo
164 Fisher, Dreieich, Germany) operating at 280 nm for the quantification of eluted proteins. Proteins
165 were eluted at a flow rate of 0.2 mL.min⁻¹ and at 40°C, using a gradient of acetonitrile obtained by
166 mixing the mobile phase A (0.106% (v/v) of trifluoroacetic acid in Milli-Q water) and the mobile phase
167 B (1% (v/v) of trifluoroacetic acid in acetonitrile). The acetonitrile gradient started with a plateau of
168 35.2% of mobile phase B during 14 min, and then continued with successive increases of mobile
169 phase B to 38.4% in 6 min, 40% in 7 min, 42.4% in 14 min, 49.6% in 1 min, and finally 80% in 4 min.

170 Native whey proteins in unheated and heated samples were quantified using a calibration curve
171 established by direct injection of α-LA, β-LG and LF at 0.5 g.L⁻¹ of proteins. Chromatographs were
172 analyzed by Chromeleon® software (Version 7.2 SR4, Thermo Fisher, Waltham, USA). The term
173 “native whey proteins” referred in this study to the proteins in the pH 4.6-soluble fraction that have
174 the same retention time in RP-HPLC than the whey protein standards. Consequently, the whey
175 proteins still soluble at pH 4.6 but that underwent structural modifications during heat treatment in
176 such a way their RP-HPLC retention time is modified are not quantified as native proteins.

177

178 2.6. Kinetic parameters of individual whey protein denaturation

179 2.6.1. Calculation of D-values, Z-values, and the equivalent heating time

180 For each IMF and heating treatment, the equivalent time t_{θ} (min) was calculated, corresponding to
 181 the time required at the temperature θ ($^{\circ}\text{C}$) to obtain the same rate of protein denaturation than the
 182 reference treatment (θ_{ref} ($^{\circ}\text{C}$) at $t_{\theta_{\text{ref}}}$ (min)). t_{θ} was determined using the following equation:

$$183 \quad t_{\theta} = t_{\theta_{\text{ref}}} \cdot 10^{\frac{\theta_{\text{ref}} - \theta}{z}} \quad (1)$$

184 with z ($^{\circ}\text{C}$) corresponding to the mean of Z-values (i.e. temperature increase required to obtain a
 185 tenfold reduction of the whey protein denaturation time) estimated for each protein (Jeantet,
 186 Croguennec, Schuck, Brulé, 2016). Z-values were calculated from the equation:

$$187 \quad Z = \frac{T_2 - T_1}{\log(D_1) - \log(D_2)} \quad (2)$$

188 with D_1 and D_2 (min) the whey protein denaturation times for having 90% whey protein denaturation
 189 at heating temperature T_1 ($^{\circ}\text{C}$) and T_2 ($^{\circ}\text{C}$), respectively. The D-values were determined as the inverse
 190 of the slope of the linear regression of the logarithm of individual whey protein concentrations *versus*
 191 heating time. **The D- and Z-values are presented in the Table 3.**

192 *2.6.2. Determination of the kinetic parameters for the heat denaturation/aggregation of whey* 193 *proteins*

194 The reaction orders n and the rate constants k_n were determined by the integration of the general
 195 rate equation, according to Croguennec et al. (2014):

$$196 \quad -\frac{dC_t}{dt} = k_n \cdot C_t^n \quad (3)$$

197 which gives, for $n \neq 1$:

$$198 \quad \left(\frac{C_t}{C_0}\right)^{1-n} = 1 + (n-1) \cdot k_n \cdot C_0^{n-1} \cdot t \quad (4)$$

199 C_t ($\text{g}\cdot\text{L}^{-1}$) is the native whey protein concentration at holding time t (min), C_0 ($\text{g}\cdot\text{L}^{-1}$) the native whey
 200 protein concentration of the unheated sample and k_n ($\text{g}^{1-n}\cdot\text{L}^{n-1}\cdot\text{min}^{-1}$) the rate constant.

201 The reaction order n was determined as the best fitting of experimental data by the equation (4)
202 considering different values of n . The rate constants k_n were determined from the linear regression
203 slope of $\left(\frac{C_t}{C_0}\right)^{1-n}$ versus holding time, as described by Dannenberg & Kessler (1988).

204 The Arrhenius representation, corresponding to the relationship between the logarithm of the rate
205 constant k_n versus the inverse of heating temperature, was plotted according to the equation:

$$206 \quad \ln(k_n) = \ln(k_{n,0}) - \frac{E_a}{R.T} \quad (5)$$

207 The activation energy E_a ($\text{kJ}\cdot\text{mol}^{-1}$) was obtained from the slope of the linear regression of the
208 Arrhenius plot and the denaturation/aggregation frequency factor logarithm, $\ln(k_{n,0})$, from the y -
209 intercept. R is the universal gas constant ($8.314 \text{ J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$) and T the heating temperature (K).

210 3. Results

211 3.1. Composition of the three IMFs

212 IMFs were formulated at 1.3% of total proteins, with a caseins:whey proteins ratio of 40:60, and
213 differing exclusively on the individual whey protein quantity, as shown in Table 2.

214 According to the RP-HPLC quantification of α -LA, β -LG and LF in unheated IMFs, the control IMF
215 contained 0.14 g of α -LA and 0.51 g of β -LG/100 g of liquid IMF. Traces of LF were observed but the
216 amount was too low to be quantified. In the LF⁺ IMF, the quantities of α -LA, β -LG and LF were 0.10 g,
217 0.33 g and 0.17 g/100 g of liquid IMF, respectively. The LF⁺ α -LA⁺ IMF was constituted by 0.39 g of α -
218 LA and 0.15 g of LF/100 g of liquid IMF. In the LF⁺ α -LA⁺ IMF, 0.06 g of β -LG/100 g of IMF came from
219 the SMP. This amount was calculated from the amount of β -LG in the SMP and the proportion of SMP
220 in the IMF. Thus, the whey protein composition differed among the three IMFs. The major whey
221 proteins were α -LA and β -LG for the control IMF, α -LA and LF for the LF⁺ α -LA⁺ IMF, and the LF⁺ IMF
222 was a mixture of α -LA, β -LG and LF. Moreover, the LF contents of the LF⁺ and LF⁺ α -LA⁺ IMFs were
223 similar.

224

225 3.2. Kinetics of thermal denaturation of whey proteins

226 The denaturation kinetics of the individual whey proteins α -LA, β -LG and LF in the temperature range
227 from 67.5 to 80°C were followed by RP-HPLC quantification of the soluble protein fraction at pH 4.6.
228 This temperature range was selected from preliminary experiments. Heat treatments of the control
229 IMF and LF⁺ IMF at temperatures higher than 80°C induced a very fast denaturation of the whey
230 proteins preventing the precise determination of the whey protein denaturation kinetic parameters.
231 In contrast, the very slow whey protein denaturation during the heating of LF⁺ α -LA⁺ IMF combined
232 with the extensive lactosylation of the proteins for the longer holding times at temperatures lower
233 than 67.5°C prevented the exact quantification of the proteins due to the elution of each protein as
234 multi-peaks by RP-HPLC.

235 As expected, the increase of the heating temperature from 67.5 to 80°C or of the holding time at
236 fixed temperature induced a higher decrease of the content of native whey proteins. To gain in
237 clarity, only the whey protein denaturation kinetics at 70 and 75°C are presented on Figure 1 for the
238 IMFs and on Supplementary Figure 2 for the pure protein solutions. Figure 1 compares the heat-
239 denaturation of individual whey proteins among IMFs but similar trends were observed at other
240 studied temperatures. The reaction orders n and the rate constant k_n of the kinetics of denaturation
241 of α -LA, β -LG and LF were calculated by the best fitting of the experimental data using the equation
242 (4) (Table 3). The Arrhenius plot of α -LA, β -LG and LF (Fig 2.1) was drawn using the values of rate
243 constants of denaturation k_n of each individual whey protein at different temperatures. Each
244 Arrhenius plot was best described by a linear regression, the slope of which gave the activation
245 energies of the denaturation of the whey proteins E_a and the y -intercept the frequency factor
246 logarithms $\ln(k_{n,0})$ (Fig 2.2).

247

248 3.2.1. Thermal denaturation of α -LA

249 The kinetics of α -LA heat-denaturation were in the same range in the control and LF⁺ IMFs but they
250 were systematically slower in the LF⁺ α -LA⁺ IMF (Fig 1.A). The reaction orders of the kinetics of
251 denaturation of α -LA in both control and LF⁺ IMFs ($n=1.2 \pm 0.2$) and in the LF⁺ α -LA⁺ IMF (1.5 ± 0.5)
252 were higher than the one determined in the pure α -LA solution ($n=0.5 \pm 0.3$). At all heating
253 temperatures, the rate constants k_n of α -LA were in the same range for the control and LF⁺ IMFs in
254 one hand and for the LF⁺ α -LA⁺ IMF and the pure α -LA solution in the other hand (Table 3). The k_n
255 values obtained for the LF⁺ α -LA⁺ IMF and the pure α -LA solution were between 3-fold and 10-fold
256 lower than those determined for the control and LF⁺ IMFs in the temperature range from 67.5°C to
257 80°C.

258 The thermodynamic parameters of the denaturation of α -LA (E_a and $\ln(k_{n,0})$) were highly dependent
259 on the amount of β -LG in the IMFs. Indeed, E_a and $\ln(k_{n,0})$ were similar between the control IMF (227

260 kJ.mol⁻¹ and 75, respectively) and the LF⁺ IMF (220 kJ.mol⁻¹ and 73, respectively), which had higher
261 content of β-LG (0.51% and 0.33% respectively). They were also similar between the LF⁺ α-LA⁺ IMF
262 (110 kJ.mol⁻¹ and 33 respectively) and the pure α-LA solution (100 kJ.mol⁻¹ and 29) (Fig 2.2), which
263 contained traces or no β-LG.

264

265 3.2.2. Thermal denaturation of β-LG

266 The kinetics of denaturation of β-LG in the control and LF⁺ IMFs at 70 and 75°C are presented in the
267 Figure 1.B. No data was recorded for the LF⁺ α-LA⁺ IMF because of the low amount of β-LG (0.06 %).
268 At all heating temperatures, the heat-denaturation of β-LG was faster in the LF⁺ IMF than in the
269 control IMF. The reaction orders of the heat-denaturation of β-LG were similar for both IMFs (n=1.5 -
270 1.6) (Table 3).

271 The rate constants of β-LG denaturation in the control and LF⁺ IMFs are presented in the Table 3. The
272 k_n-values were between 2-fold and 7-fold lower for the control IMF than for the LF⁺ IMF in the
273 studied temperature range. The k_n-values at 67.5°C were 0.3 x 10⁻² g^{-0.6}.L^{0.6}.min⁻¹ and 0.8 x 10⁻² g⁻
274 ^{0.5}.L^{0.5}.min⁻¹ for the control and LF⁺ IMFs respectively, whereas they were 10.8 x 10⁻³ g^{-0.6}.L^{0.6}.min⁻¹ and
275 70.9 x 10⁻² g^{-0.5}.L^{0.5}.min⁻¹ at 80°C, respectively. The activation energy of β-LG heat-denaturation
276 determined from the Arrhenius plot was slightly higher in the control IMF (305 kJ.mol⁻¹) compared to
277 the LF⁺ IMF (273 kJ.mol⁻¹) (Fig 2.2). In contrast, the ln (k_{n,0}) value was slightly lower for the control IMF
278 (102) than for the LF⁺ IMF (126).

279

280 3.2.3. Thermal denaturation of LF

281 The kinetics of LF heat-denaturation in the LF⁺ and LF⁺ α-LA⁺ IMFs were compared (Fig 1.C). In the
282 control IMF, the amount of LF was too low to quantify the heat-induced denaturation. Interestingly,
283 the kinetics of heat-denaturation of LF were similar between the two IMFs at all studied

284 temperatures. The reaction orders of LF heat denaturation was 1.6 ± 0.2 and 1.8 ± 0.3 for the LF⁺ and
285 LF⁺ α-LA⁺ IMFs, respectively. A slightly lower reaction order ($n=1.4 \pm 0.4$) was obtained for the heat-
286 denaturation of LF in the pure protein solution. The rate constants of LF denaturation were in the
287 same range between the LF⁺ and LF⁺ α-LA⁺ IMFs (Table 3). In fact, in the temperature range between
288 67.5°C and 80°C, the rate constants of LF heat-denaturation varied between 3.6×10^{-2} to $42.8 \times 10^{-2} \text{ g}^{-0.6} \cdot \text{L}^{0.6} \cdot \text{min}^{-1}$
289 for the LF⁺ IMF and between 2.6×10^{-2} to $42.3 \times 10^{-2} \text{ g}^{-0.8} \cdot \text{L}^{0.8} \cdot \text{min}^{-1}$ for the LF⁺ α-LA⁺ IMF.
290 These values were higher than the rate constants determined for LF heat-denaturation in the pure LF
291 solution at the same heating temperature (0.4×10^{-2} to $17.6 \times 10^{-2} \text{ g}^{-0.4} \cdot \text{L}^{0.4} \cdot \text{min}^{-1}$). As expected, the
292 activation energy of LF was similar in the LF⁺ (202 kJ.mol⁻¹) and LF⁺ α-LA⁺ (215 kJ.mol⁻¹) IMFs. Likewise,
293 the frequency factor logarithm of LF denaturation determined in the LF⁺ IMF (68) is akin to the value
294 determined for the LF⁺ α-LA⁺ IMF (73). Higher E_a and $\ln(k_{n,0})$ values were determined for the pure LF
295 solution compared to the LF⁺ and LF⁺ α-LA⁺ IMFs (286 versus 215 kJ.mol⁻¹ and 96 versus 70 kJ.mol⁻¹,
296 respectively) (Fig 2.2).

297

298 3.3. Prediction of the amount of native whey proteins in IMFs after pasteurization 299 treatments

300 Based on equation (4) and the thermodynamic parameters calculated in this study (Fig 2.2), the
301 proportion of native α-LA, β-LG and LF recovered in IMFs after the temperature/time conditions
302 usually used to pasteurize milk can be predicted (Fig 3). A summary of the calculations is given in
303 Supplementary Table 1. The whey protein denaturation yield simulated for the heat treatment 75°C/
304 10 min was confirmed by RP-HPLC quantification.

305 The simulation of the holder pasteurization (i.e. 62.5°C during 30 min) induced the denaturation of
306 4% of native whey proteins in the control IMF and of 13% and 15% in the LF⁺ and LF⁺ α-LA⁺ IMFs,
307 respectively.

308 A standard HTST pasteurization treatment (i.e. 75°C during 15 s) led to about the same total protein
309 denaturation yield (between 1% and 4% of denatured proteins) in the three IMFs. Rising up the
310 temperature of the pasteurization treatment from 75°C to 90°C but keeping the holding time
311 constant (15 s) resulted in a decrease of the fraction of native whey proteins in all IMFs, with 48%,
312 27% and 81% of residual native whey proteins in the control, LF⁺ and LF⁺ α-LA⁺ IMFs, respectively. The
313 native protein loss was mainly due to the heat-denaturation of β-LG and LF during the treatment at
314 90°C/15 s. A similar trend was observed when the holding time at 75°C was prolonged to 10 min but
315 the proportion of the residual native proteins was slightly different: 61%, 32% and 71% of residual
316 native whey proteins were calculated in the control, LF⁺ and LF⁺ α-LA⁺ IMFs, respectively. The relative
317 denaturation yield of α-LA and LF was higher after a heat treatment at 75°C/10 min compared to
318 90°C/15 s.

319 **4. Discussion**

320 The present paper evidences that the heat denaturation of whey proteins within IMFs depends on
321 both the whey protein composition and the temperature/time combination treatment.

322

323 4.1. The heat-denaturation of LF is independent of the amount of α -LA and β -LG in the
324 IMFs

325 The reaction orders of LF determined for the LF⁺ and LF⁺ α -LA⁺ IMFs and in the pure LF solution are in
326 accordance with the one previously determined for the thermal denaturation of the recombinant
327 human LF (Castillo, Pérez, Franco, Calvo, & Sánchez, 2012). These authors determined a reaction
328 order of 1.5, independently of the iron saturation level of LF.

329 The present results show that LF heat-denaturation kinetics are independent of the change of α -LA
330 and β -LG amounts in the IMFs in the whole range of the studied heating temperatures. Since LF is
331 one of the most heat-sensitive protein in milk with a reported denaturation temperature around
332 60°C (Bengoechea, Peinado, & McClements, 2011), it is assumed that its denaturation occurs before
333 the heat-denaturation of α -LA and β -LG with denaturation temperatures of about 68°C (Nielsen et al,
334 2018) and 78°C (Brodkorb, Croguennec, Bouhallab, & Kehoe, 2016), respectively. Consequently,
335 these latter have no or very little influence on the heat-denaturation of LF explaining that the rate
336 constants and the activation energy of the heat-denaturation of LF are similar in the LF⁺ and LF⁺ α -LA⁺
337 IMFs, in spite of different contents in α -LA and β -LG.

338 Interestingly, the LF heat-denaturation rate constants were lower and the activation energy was
339 higher in the pure LF solution compared to those obtained in the LF⁺ and LF⁺ α -LA⁺ IMFs, indicating
340 that the physicochemical environment in the IMFs favors the denaturation of LF during heating. LF is
341 able to bind to casein micelles mainly by electrostatic interactions (Anema & de Kruif, 2012) and this
342 was confirm in this study since 43% of LF was recovered with the casein micelle fraction in the
343 ultracentrifugation pellet (100,000g - 1h - 25°C) of the LF⁺ and LF⁺ α -LA⁺ IMFs (data not shown). The

344 binding of LF to caseins is facilitated by heating and results in the formation of LF-casein complexes
345 (Li & Zhao, 2017). This binding is susceptible to modify the heat-stability of LF and can be an
346 explanation for the faster heat-denaturation of LF in the IMFs compared to the pure protein solution.
347 Casein micelles also have the ability to bind various types of cations such as iron ions (Fe^{2+} and Fe^{3+}).
348 This binding was observed in a suspension of casein micelles (Philippe, Legraet, & Gaucheron, 2005)
349 and in bovine milk (Broyard & Gaucheron, 2015). The binding of iron to casein micelles could reduce
350 the amount of iron bound to LF, and therefore accelerate its heat-denaturation kinetics because LF
351 heat-stability is dependent on the iron saturation level (Brisson et al., 2007; Sánchez, Peiró, Castillo,
352 Pérez, Ena & Calvo, 1992; Castillo et al., 2012). However, the quantification of LF and iron in the
353 supernatant after ultracentrifugation of unheated LF⁺ and LF⁺ α -LA⁺ IMFs indicated that LF was fully
354 saturated with iron (iron/LF molar ratio of 3.7 ± 0.0 , data not shown) considering a binding constant
355 of about $10^{22} - 10^{24} \text{ M}^{-1}$ (Majka et al., 2013). Therefore, the faster heat-denaturation of LF in IMFs
356 compared to pure LF solution was rather a consequence of the binding of LF to casein micelles than a
357 reduction of the iron saturation level of LF.

358

359 4.2. The heat-denaturation of α -LA is enhanced in the presence of β -LG

360 The reaction orders of α -LA determined for the three IMFs (between 1.2 and 1.5) were higher than
361 the one reported in the literature for the heat-denaturation of α -LA in bovine milk ($n=1.0$, Anema et
362 al., 2006; Dannenberg & Kessler, 1988) and the value determined in the pure α -LA solution ($n=0.5$).
363 This could be a consequence of the different protein content (Wehbi et al., 2005) and/or distinct
364 medium conditions between bovine milk, the pure α -LA solution and the IMFs.

365 The denaturation kinetics of α -LA in the different IMFs revealed that α -LA was more rapidly
366 denatured in the control and LF⁺ IMFs that contain 0.51 g and 0.33 g of β -LG/100 g of liquid IMF,
367 respectively. When the β -LG content is as low as in the LF⁺ α -LA⁺ IMF, the denaturation rate constant
368 of α -LA is comparable to that of α -LA in the pure solution. These results are in agreement with

369 published data showing that the denaturation of α -LA is promoted by the presence of β -LG (Wehbi et
370 al., 2005). In the absence of β -LG, α -LA reversibly unfolds on heating but does not form irreversible
371 aggregates (Nielsen et al, 2018). The aggregation of α -LA is enhanced in the presence of unfolded β -
372 LG molecule that exposes its free thiol group to the solvent, making it available to react with
373 unfolded α -LA *via* thiol-disulfide interchanges (Dalglish et al, 1997). This covalent cross-link between
374 β -LG and α -LA traps α -LA in an irreversibly unfolded structure in the formed aggregates. The
375 irreversible denaturation of α -LA induced by addition of thiol reagents (Nguyen et al., 2018) confirms
376 that the presence of available thiol groups cleaving intramolecular α -LA disulfide bonds is necessary
377 to promote α -LA denaturation/aggregation .

378 To the best of our knowledge, the effect of LF on the heat-denaturation of α -LA was less studied than
379 that of β -LG. The similarities of the activation energies of α -LA in the LF⁺ α -LA⁺ IMF and in the pure α -
380 LA solution, both containing the same quantities of α -LA and the same lactose and mineral contents,
381 demonstrate that LF does not interfere with the mechanism of heat-denaturation of α -LA. LF has 34
382 cysteine residues in its structure, all involved in intramolecular disulfide bonds, but no free thiol
383 group (Pierce et al., 1991). The absence of a free thiol group in the structure of LF is a plausible
384 explanation for the absence of LF effect on α -LA heat-denaturation that is promoted by thiol
385 containing molecules. However, the profile of the α -LA denaturation kinetics in the LF⁺ α -LA⁺ IMF and
386 the pure α -LA solution differs due to the variation of the reaction order (1.5 ± 0.5 and 0.5 ± 0.3 ,
387 respectively). The higher reaction order in the LF⁺ α -LA⁺ IMF could be a consequence of the presence
388 of other proteins (LF, caseins, β -LG traces) facilitating the aggregation of denatured α -LA.

389

390 4.3. The heat-denaturation of β -LG is enhanced in the presence of LF

391 The reaction orders of the heat-denaturation of β -LG determined for the control and LF⁺ IMFs (n=1.5-
392 1.6) are in adequacy with the reaction order widely reported in the literature for the heat-
393 denaturation of the protein in bovine milk (Anema & McKenna, 1996).

394 The heat-denaturation of β -LG was faster in the LF⁺ IMF (containing LF protein) than in the control
395 IMF (lacking of LF protein), with the rate constants of denaturation of β -LG being 2 to 6-times higher
396 in the LF⁺ IMF than in the control IMF, and the denaturation activation energy being higher in the
397 control IMF than in the LF⁺ IMF (305 *versus* 273 kJ.mol⁻¹, respectively).

398 The mechanism of heat-denaturation of β -LG has been well-described (de Wit, 2009). Upon heating,
399 the dimer/monomer equilibrium of native β -LG is shifted to the monomeric form that unfolds and
400 exposes its free thiol group and hydrophobic patches to the solvent. This reactive entity aggregates
401 with proteins in its close surrounding mainly *via* disulfide interchange reactions, but also by
402 hydrophobic driven association reactions. β -LG reacts rapidly with proteins having exposed disulfide
403 bonds such as α -LA (Dalgleish et al, 1997) or LF (Brisson et al., 2007). Among these proteins, LF is
404 particularly rich in disulfide bonds (17 disulfide bonds) and is already unfolded when β -LG starts to
405 denature. This could facilitate the formation of intermolecular disulfide bonds between LF and β -LG
406 supporting the hypothesis of Brisson et al. (2007), who showed that upon heating, LF aggregates with
407 proteins containing cysteine residues such as β -LG to form disulfide bound aggregates.

408

409 4.4. The impact of the pasteurization treatments is dependent on the whey protein
410 composition of IMFs

411 The simulation of the heat treatments at 90°C/15 s and 75°C/10 min revealed a lower content of
412 native whey proteins in the pasteurized LF⁺ IMF than in the control and LF⁺ α -LA⁺ IMFs, explained by
413 the simultaneous presence of LF that rapidly denatures on heating and β -LG that initiates covalent
414 cross-links between unfolded whey proteins. Surprisingly, the decrease of the native whey protein
415 fraction in the control and LF⁺ IMFs heat-treated at 90°C/15 s is mainly explained by the heat-
416 denaturation of β -LG, and to a lesser extent by the heat-denaturation of LF. However, it is probable
417 that the fraction of native β -LG in these heated IMFs is underestimated because the Arrhenius plot of
418 β -LG exhibits a specific profile with a change of the denaturation activation energy around 80°C. In

419 the aggregation-limited domain (temperatures above 80°C), the denaturation activation energy is
420 between 2 and 3-times lower than in the unfolding-limited domain (temperatures below 80°C) (Petit
421 et al., 2011). As the denaturation of the whey proteins in IMFs was not studied above 80°C for the
422 reasons presented above (section 3.2), the change of the denaturation activation energy at
423 temperature higher than 80°C cannot be taken into account for the estimation of the residual native
424 β -LG after heating the IMFs at 90°C. Consequently, the rate constant used to calculate the fraction of
425 residual native β -LG is likely overestimated, thus potentially explaining the lower amount of native
426 whey protein fractions in the control and LF⁺ IMFs heat-treated at 90°C/15 s than at 75°C/10 min.

427 Almost all whey proteins remained native after simulation of HTST treatment (75°C/15 s) for the
428 three IMFs. This is consistent with previous **work** that showed that HTST treatment of human
429 (Cavallarin, 2011) and bovine (Douglas, Greenberg, Farrell, & Edmondson, 1981) milks did not
430 significantly denature whey proteins.

431 Moreover, the lower native whey protein quantity for the LF⁺ and LF⁺ α -LA⁺ IMFs after the holder
432 pasteurization simulation, a classical heat treatment used in human milk bank to ensure the sanitary
433 quality before feeding newborn, is due to the denaturation of LF, which is more heat-sensitive at low
434 temperature than other whey proteins (Law, 1995). This confirms previous experiments showing that
435 the holder pasteurization of human milk mainly lead to the LF denaturation, whereas α -LA was not
436 affected (Deglaire et al., 2019).

437

438 **5. Conclusion**

439 This study demonstrates that the kinetics of heat-denaturation of α -LA, β -LG and LF were influenced
440 by the whey protein composition of the IMFs. The heat-denaturation of LF was not modified by the
441 presence of α -LA or β -LG in the IMFs. As expected, the heat-denaturation of α -LA was enhanced in
442 the presence of β -LG, but LF did not modify it. The presence of LF in the IMFs boosted the heat-
443 denaturation kinetics of β -LG, probably by exposing numerous disulfide bonds that are accessible to
444 the free thiol group of β -LG. The simulations of pasteurization treatments revealed that the yield of
445 total whey protein denaturation is function of both the whey protein composition of the IMFs and
446 the heat-treatment intensity. Standard IMF, formulated with WPI and SMP as protein sources,
447 preserved a higher amount of native whey proteins after heat treatment at 62.5°C/30 min or at
448 75°C/15 s than IMF mimicking human milk protein content (enriched in α -LA and LF), whereas a
449 reverse trend was observed for heat treatments at higher temperature (90°C/15 s) or longer holding
450 time (75°C/10 min). Finally, the formulation of IMFs containing both α -LA and LF, and so mimicking
451 the protein profile of human milk, is a way to preserve a higher proportion of native whey proteins
452 after heating, as long as the heat intensity applied is in the upper range of the intensity tested in the
453 present study, conditions usually encountered at industrial scale to ensure microbial safety and to
454 extend IMF shelf-life.

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Figure legends

Figure 1. Kinetics of thermal denaturation of α -LA (A), β -LG (B) and LF (C) in the control (green), LF⁺ (orange), LF⁺ α -LA⁺ (blue) IMFs and the pure protein solutions (grey) at 70°C (square) and 75°C (circle). Residual native fractions were calculated as a C_t/C_0 ratio. C_t (g.L⁻¹) is the native protein concentration at t holding time (min) and C_0 is the native protein concentration of unheated IMFs or protein solutions (g.L⁻¹). The results at 75°C represent means \pm error deviations from three independent experiments.

Figure 1 1. Arrhenius plots for the thermal denaturation of α -LA (A), β -LG (B) and LF (C) for the control (green), LF⁺ (orange), LF⁺ α -LA⁺ (blue) IMFs, the pure protein solutions (grey) and **2.** Kinetic parameters associated. 1. K_n is the unfolding reaction rate constant (g¹⁻ⁿ.Lⁿ⁻¹.min⁻¹) and 1/T the inverse of the heating temperature (K⁻¹). The point at 75°C represents an average \pm error deviation of three independent experiments. 2. $\ln(k_{n,0})$ represents the frequency factor logarithm and E_a the activation energy (kJ.mol⁻¹), determined by Arrhenius plots shown in the Figure 2.1.

Figure 2. Whey protein contents and fractions of total native whey proteins for pasteurization treatments of the control (green), LF+ (orange) and LF+ α -LA+ (blue) IMFs. The patterns represent the amount of native α -LA (hatched bars), β -LG (spots) or LF (without pattern). The data are estimations from the equation $\frac{C_t}{C_0} =$

$1 - n \sqrt{\left(1 + (n - 1) \times e^{\left(-\frac{E_a}{R} \times \frac{1}{T+273}\right)} \times k_{n,0} \times C_0^{n-1} \times t\right)}$, using the thermodynamic parameters (reaction orders n, activation energies E_a and frequency factors $k_{n,0}$) referenced at the Figure 2.2. C_t (g.L⁻¹) corresponds to the protein content at holding time t (min), C_0 is the protein content in the unheated IMFs (g.L⁻¹). T corresponds to the heating temperature (°C).

Figure 1

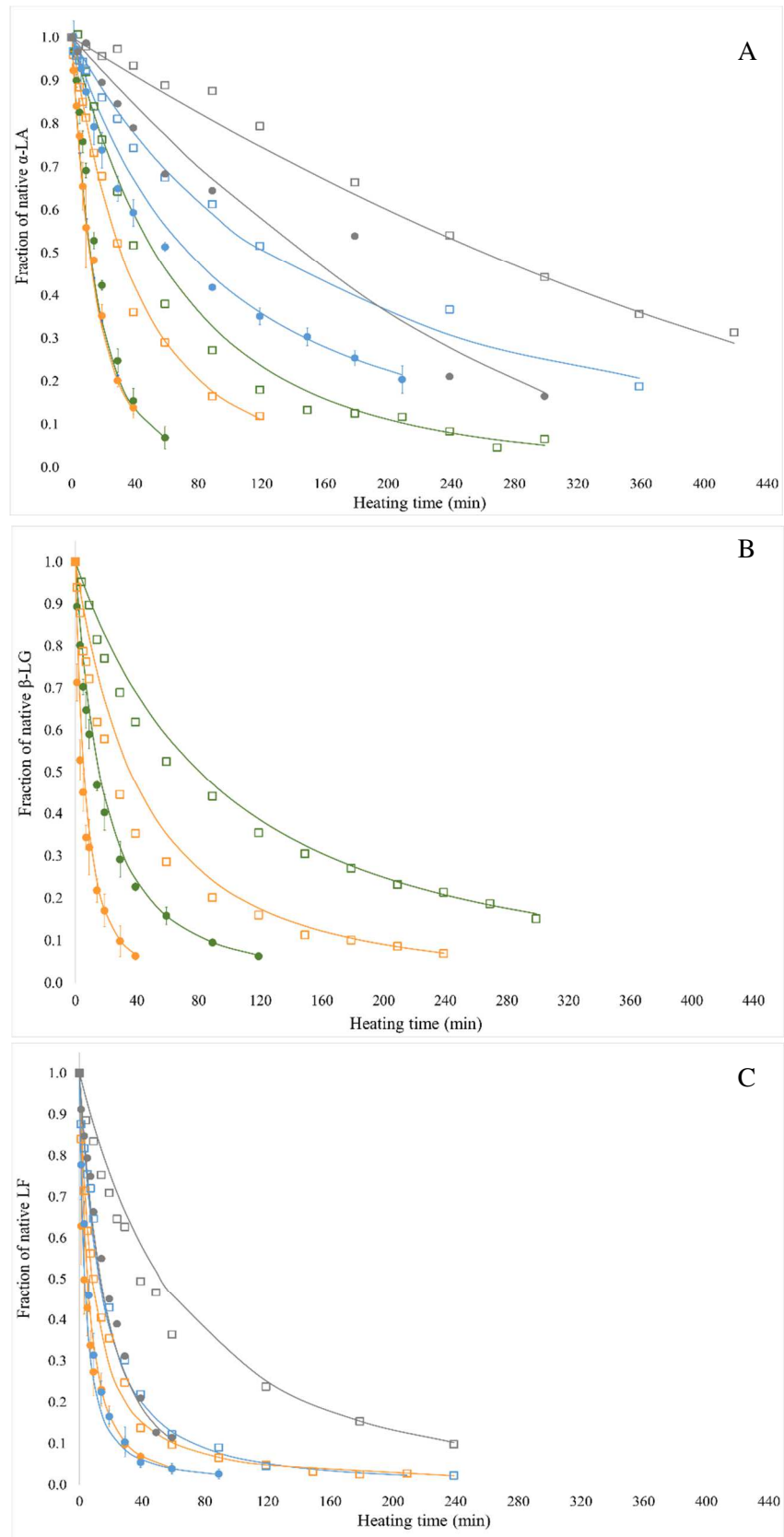
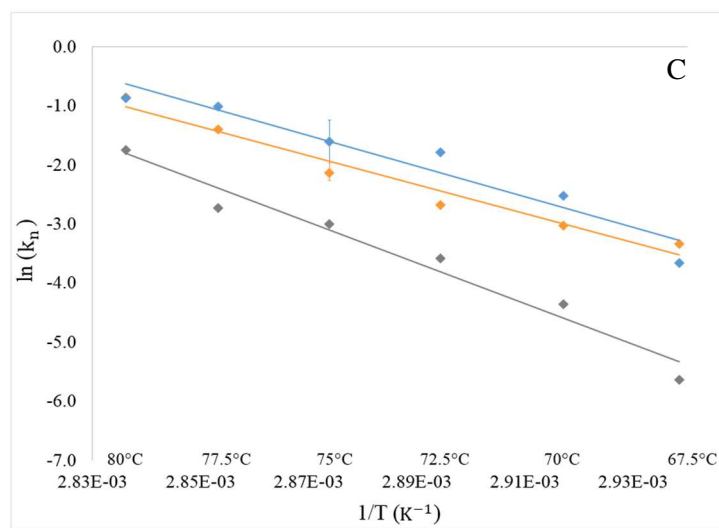
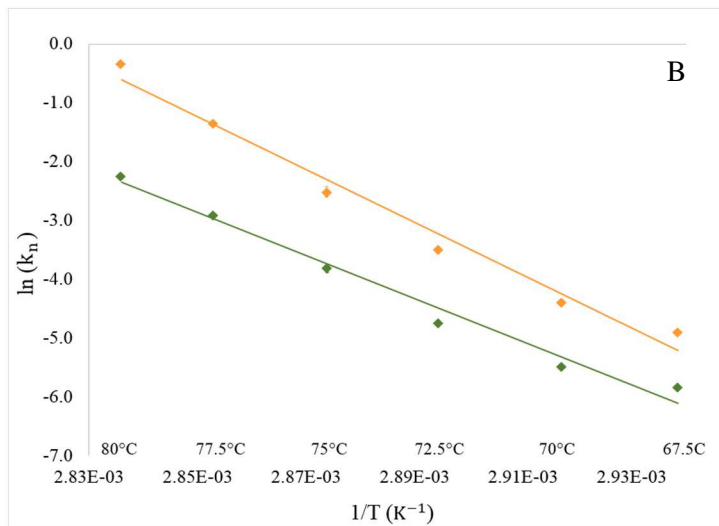
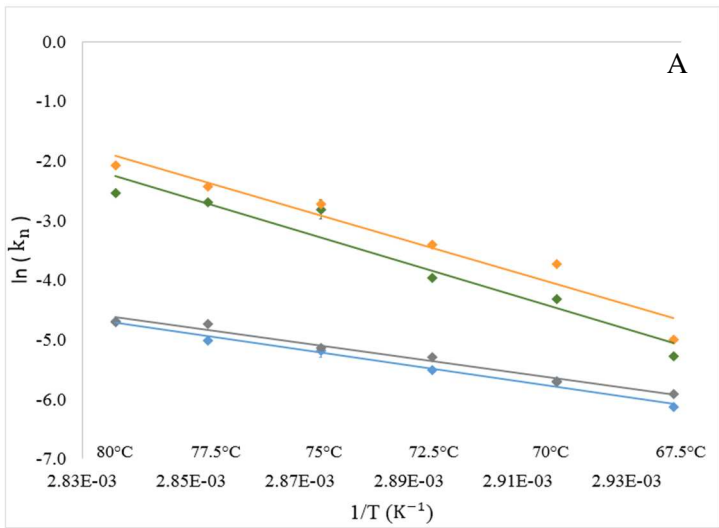


Figure 2

1.



2.

IMFs	Whey proteins	E_a ($kJ \cdot mol^{-1}$)	$\ln(k_{a,0})$
Control	α -LA	227	75
	β -LG	305	102
LF+	α -LA	220	73
	β -LG	273	126
	LF	202	68
LF+ α -LA+	α -LA	110	33
	LF	215	73
Pure protein solutions	α -LA	105	31
	LF	286	96

Figure 3

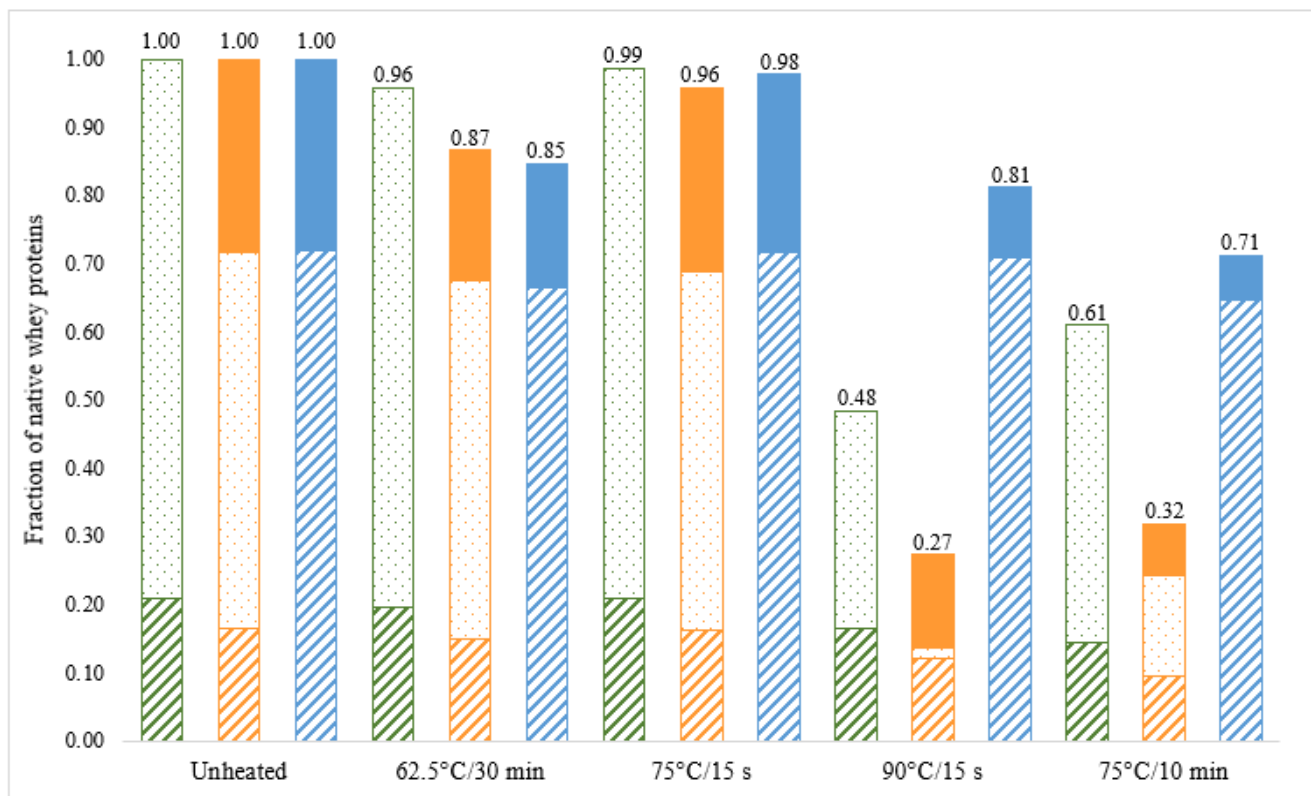


Table 1 Biochemical characteristics of the skim milk powder (SMP).

	Composition	Quantity
g/ 100 g of SMP	Total protein ^{1,4}	36
	Whey proteins ^{2,4}	7
	Caseins ^{3,4}	29
	Lactose	48
mg / 100 g of SMP	Calcium	1401
	Chloride	893
	Inorganic phosphorus	1014
	Magnesium	104
	Potassium	1616
	Sodium	382

¹ Total protein = [Total Nitrogen (TN) - Non Protein Nitrogen (NPN)] x 6.38

² Whey proteins = [Non Casein Nitrogen (NCN)- NPN] x 6.38

³ Caseins = (TN-NCN) x 6.38

⁴ TN, NCN, NPN determined by the Kjeldahl method

Table 2 Composition of the model IMFs - Comparison with the commercial IMFs and human term milk.

Elements	Model IMFs			Commercial IMF ⁵	Human term milk ⁶
	Control	LF ⁺	LF ⁺ α-LA ⁺		
Total proteins	1.33 ± 0.01	1.35 ± 0.01	1.31 ± 0.01	1.20 ± 0.07	0.90
Whey proteins	0.83 ± 0.04	0.82 ± 0.00	0.75 ± 0.00	0.72 ± 0.05	0.60
α-LA	0.14 ± 0.00	0.10 ± 0.00	0.39 ± 0.02	0.19	0.25
β-LG	0.51 ± 0.01	0.33 ± 0.00	0.06 ⁷	0.35	Trace
LF	n.d.	0.17 ± 0.00	0.15 ± 0.01	0.48.10 ⁻³	0.15
Caseins	0.50 ± 0.03	0.53 ± 0.01	0.56 ± 0.01	0.48 ± 0.05	0.40
Lactose	5.6 ± 0.4	5.8 ± 0.4	5.6 ± 0.2	6.7 ± 0.9	7.6
Calcium	41.4 ± 0.7	41.1 ± 1.4	42.2 ± 0.7	52 ± 6	27
Chloride	65.8 ± 1.7	69.6 ± 0.9	68.5 ± 0.4	44 ± 5	40
Iron	0.42 ± 0.02	0.41 ± 0.02	0.43 ± 0.01	0.70 ± 0.11	0.50
Inorganic phosphorus	21.1 ± 0.6	20.6 ± 0.6	20.8 ± 0.3	30 ± 3	15
Potassium	60.2 ± 6.3	63.4 ± 1.3	62.4 ± 0.4	67 ± 4	54
Sodium	21.5 ± 1.5	20.9 ± 1.2	25.0 ± 0.7	20 ± 2	18
Ash	0.18 ± 0.04	0.17 ± 0.04	0.16 ± 0.03	n.d.	n.d.
pH	6.82 ± 0.03	6.80 ± 0.02	6.81 ± 0.04	n.d.	n.d.

⁵ The data of commercial IMF are obtained from Chatterton et al., 2013 for the α-LA, β-LG and LF contents and mean of the five 1st age commercial IMFs for the rest of elements: Guigoz, Gallia, Milumel 1 Lactel, Novalac and Bledilait Bledina.

⁶ The data of human term milk are obtained from Lönnerdal, Forsum, & Hambraeus, 1976, Altomonte, Salari, Licitra, & Martini, 2019, Wack, Lien, Taft, & Roscelli, 1997.

⁷ Value of β-LG content in the LF⁺ α-LA⁺ IMF is determined by calculation from the amount of β-LG in SMP and the proportion of SMP in the IMF.

Table 3 Reaction orders n , **Z-values**, reaction rate constants k_n and **D-values** for the thermal denaturation of α -LA, β -LG and LF for the control, LF⁺, LF⁺ α -LA⁺ IMFs and pure protein solutions, at temperature range 67.5-80°C.

IMFs	Whey proteins	Temperatures (°C)	k_n ($10^{-2} \text{ g}^{1-n} \cdot \text{L}^{n-1} \cdot \text{min}^{-1}$)	R ²	D-value (min)	
Control	α -LA $n = 1.2 \pm 0.2$ Z-value = $(8.0 \pm 1.6)^\circ\text{C}$	67.5	0.5	0.994	421	
		70	1.3	0.968	161	
		72.5	1.9	0.953	113	
		75	6.0 ± 1.0	0.994	36 ± 6	
		77.5	6.7	0.988	32	
		80	7.9	0.997	27	
	β -LG $n = 1.6 \pm 0.1$ Z-value = $(9.8 \pm 2.7)^\circ\text{C}$	67.5	0.3	0.999	318	
		70	0.4	0.995	219	
		72.5	0.9	0.997	105	
		75	2.2 ± 0.1	0.999	42 ± 3	
		77.5	5.5	0.998	17	
		80	10.8	0.988	9	
LF ⁺	α -LA $n = 1.2 \pm 0.2$ Z-value = $(7.8 \pm 1.7)^\circ\text{C}$	67.5	0.7	0.980	338	
		70	2.4	0.993	96	
		72.5	3.3	0.972	70	
		75	6.6 ± 0.4	0.996	35 ± 2	
		77.5	8.8	0.999	26	
		80	12.5	0.965	19	
	β -LG $n = 1.5 \pm 0.2$ Z-value = $(7.9 \pm 1.7)^\circ\text{C}$	67.5	0.8	0.997	165	
		70	1.3	0.994	99	
		72.5	3.0	0.994	42	
		75	8.1 ± 0.8	0.997	16 ± 1	
		77.5	26.2	0.996	5	
		80	70.9	0.986	2	
	LF	$n = 1.6 \pm 0.2$ Z-value = $(15.1 \pm 3.0)^\circ\text{C}$	67.5	3.6	0.990	46
			70	5.0	0.980	34
			72.5	6.9	0.989	24
75			12.2 ± 1.8	0.998	14 ± 2	
77.5			25.3	0.995	7	
80			42.8	0.973	4	
LF ⁺ α -LA ⁺	α -LA $n = 1.5 \pm 0.5$ Z-value = $(18.5 \pm 2.7)^\circ\text{C}$	67.5	0.2	0.971	522	
		70	0.3	0.977	338	
		72.5	0.4	0.983	278	
		75	0.6 ± 0.1	0.993	204 ± 20	
		77.5	0.7	0.995	170	
		80	0.9	0.990	122	
	LF	$n = 1.8 \pm 0.3$ Z-value = $(8.2 \pm 2.0)^\circ\text{C}$	67.5	2.6	0.969	63
			70	8.1	0.988	20
			72.5	16.9	0.972	10
			75	21.3 ± 7.4	0.993	8 ± 3
			77.5	36.7	0.982	5
			80	42.3	0.993	4
Pure protein solutions	α -LA $n = 0.5 \pm 0.3$ Z-value = $(23.4 \pm 4.4)^\circ\text{C}$	67.5	0.3	0.975	1230	
		70	0.3	0.994	1019	
		72.5	0.5	0.997	672	
		75	0.6	0.960	574	
		77.5	0.9	0.990	380	
		80	0.9	0.978	369	
	LF	$n = 1.4 \pm 0.4$ Z-value = $(6.4 \pm 1.4)^\circ\text{C}$	67.5	0.4	0.980	550
			70	1.3	0.994	151
			72.5	2.8	0.984	71
			75	5.1	0.985	39
			77.5	6.6	0.990	30
			80	17.6	0.936	11

The reaction orders n and the reaction rate constants k_n were determined by the linear regression slope of the equation (4). The n -values are the means \pm error deviation of reaction order values at each temperature. **The Z-values correspond to means \pm error deviations from data calculated at each temperature.** The k_n -values and **D-values** at 75°C represent an average \pm error deviation of three independent experiments. The unit of k_n is $\text{g}^{1-n} \cdot \text{L}^{n-1} \cdot \text{min}^{-1}$.

The “Pure protein solutions” correspond to the solutions of pure α -LA or LF with the identical quantity within the LF⁺ α -LA⁺ IMF and respecting the mineral and lactose contents of IMFs.