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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  $\alpha$ -lactalbumin is reduced in the absence of  $\beta$ -lactoglobulin
- 16 • Heat denaturation of  $\beta$ -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  $\alpha$ -lactalbumin,  $\beta$ -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  $\alpha$ -lactalbumin was reduced  
28 when  $\beta$ -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  $\beta$ -lactoglobulin. This  
30 study revealed that, for higher heat treatments (90°C/15 s, 75°C/15 min), IMF containing  $\alpha$ -  
31 lactalbumin and lactoferrin preserved a higher proportion of native whey proteins than IMFs  
32 containing  $\beta$ -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<sup>-1</sup> for mature human milk with an average caseins:whey proteins weight  
40 ratio of 40:60, *versus* about 34 g.L<sup>-1</sup> 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,  $\alpha$ -lactalbumin ( $\alpha$ -LA) is the predominant whey protein (2.5 g.L<sup>-1</sup>)  
43 followed by lactoferrin (LF, 1.5 g.L<sup>-1</sup>), whereas  $\beta$ -lactoglobulin ( $\beta$ -LG) is absent. In contrast,  $\beta$ -LG is the  
44 predominant whey protein of bovine milk (3.5 g.L<sup>-1</sup>), followed by  $\alpha$ -LA (1.3 g.L<sup>-1</sup>) while LF is at a lower  
45 content (5.0 x 10<sup>-2</sup> g.L<sup>-1</sup>) (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,  $\alpha$ -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  $\alpha$ -LA and LF, the  $\alpha$ -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 1<sup>st</sup> 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  $\alpha$ -LA and  $\beta$ -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  $\alpha$ -LA  
65 and  $\beta$ -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 ( $\alpha$ -LA,  $\beta$ -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<sup>+</sup> IMF” and “LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> IMF” respected the quantity of LF or of  
78 both LF and  $\alpha$ -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  $\beta$ -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). CaCl<sub>2</sub>·2H<sub>2</sub>O was from  
100 AnalaR (Leuven, Belgium), FeSO<sub>4</sub>·7H<sub>2</sub>O from Sigma-Aldrich (St-Louis, USA), KCl from Panreac  
101 (Barcelona, Spain), Na<sub>3</sub>PO<sub>4</sub>·12H<sub>2</sub>O from Merck (Darmstadt, Germany), and Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>·2H<sub>2</sub>O 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<sup>+</sup> IMF", mixture of SMP, WPI and LF) or a  
111 mixture of LF and  $\alpha$ -LA ("LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> IMF", mixture of SMP,  $\alpha$ -LA and LF) to reach the  $\alpha$ -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,  $\alpha$ -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  $\alpha$ -LA and a solution of LF (proteins used to replace  
120 part of the WPI in IMFs) at identical  $\alpha$ -LA and LF concentration as found in the LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> 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<sup>-1</sup> 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<sup>-1</sup> 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<sup>-1</sup> 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  $t_{\theta}$  (min) was calculated, corresponding to  
 181 the time required at the temperature  $\theta$  ( $^{\circ}\text{C}$ ) to obtain the same rate of protein denaturation than the  
 182 reference treatment ( $\theta_{\text{ref}}$  ( $^{\circ}\text{C}$ ) at  $t_{\theta_{\text{ref}}}$  (min)).  $t_{\theta}$  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  $\alpha$ -LA,  $\beta$ -LG and LF in unheated IMFs, the control IMF  
215 contained 0.14 g of  $\alpha$ -LA and 0.51 g of  $\beta$ -LG/100 g of liquid IMF. Traces of LF were observed but the  
216 amount was too low to be quantified. In the LF<sup>+</sup> IMF, the quantities of  $\alpha$ -LA,  $\beta$ -LG and LF were 0.10 g,  
217 0.33 g and 0.17 g/100 g of liquid IMF, respectively. The LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> IMF was constituted by 0.39 g of  $\alpha$ -  
218 LA and 0.15 g of LF/100 g of liquid IMF. In the LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> IMF, 0.06 g of  $\beta$ -LG/100 g of IMF came from  
219 the SMP. This amount was calculated from the amount of  $\beta$ -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  $\alpha$ -LA and  $\beta$ -LG for the control IMF,  $\alpha$ -LA and LF for the LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> IMF, and the LF<sup>+</sup> IMF  
222 was a mixture of  $\alpha$ -LA,  $\beta$ -LG and LF. Moreover, the LF contents of the LF<sup>+</sup> and LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> IMFs were  
223 similar.

224

### 225 3.2. Kinetics of thermal denaturation of whey proteins

226 The denaturation kinetics of the individual whey proteins  $\alpha$ -LA,  $\beta$ -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<sup>+</sup> 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<sup>+</sup>  $\alpha$ -LA<sup>+</sup> 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  $\alpha$ -LA,  $\beta$ -LG and LF were calculated by the best fitting of the experimental data using the equation  
242 (4) (Table 3). The Arrhenius plot of  $\alpha$ -LA,  $\beta$ -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 $\alpha$ -LA

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

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

260 kJ.mol<sup>-1</sup> and 75, respectively) and the LF<sup>+</sup> IMF (220 kJ.mol<sup>-1</sup> and 73, respectively), which had higher  
261 content of β-LG (0.51% and 0.33% respectively). They were also similar between the LF<sup>+</sup> α-LA<sup>+</sup> IMF  
262 (110 kJ.mol<sup>-1</sup> and 33 respectively) and the pure α-LA solution (100 kJ.mol<sup>-1</sup> 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<sup>+</sup> IMFs at 70 and 75°C are presented in the  
267 Figure 1.B. No data was recorded for the LF<sup>+</sup> α-LA<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> IMFs are presented in the Table 3. The  
272 k<sub>n</sub>-values were between 2-fold and 7-fold lower for the control IMF than for the LF<sup>+</sup> IMF in the  
273 studied temperature range. The k<sub>n</sub>-values at 67.5°C were 0.3 x 10<sup>-2</sup> g<sup>-0.6</sup>.L<sup>0.6</sup>.min<sup>-1</sup> and 0.8 x 10<sup>-2</sup> g<sup>-</sup>  
274 <sup>0.5</sup>.L<sup>0.5</sup>.min<sup>-1</sup> for the control and LF<sup>+</sup> IMFs respectively, whereas they were 10.8 x 10<sup>-3</sup> g<sup>-0.6</sup>.L<sup>0.6</sup>.min<sup>-1</sup> and  
275 70.9 x 10<sup>-2</sup> g<sup>-0.5</sup>.L<sup>0.5</sup>.min<sup>-1</sup> 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<sup>-1</sup>) compared to  
277 the LF<sup>+</sup> IMF (273 kJ.mol<sup>-1</sup>) (Fig 2.2). In contrast, the ln (k<sub>n,0</sub>) value was slightly lower for the control IMF  
278 (102) than for the LF<sup>+</sup> IMF (126).

279

### 280 3.2.3. Thermal denaturation of LF

281 The kinetics of LF heat-denaturation in the LF<sup>+</sup> and LF<sup>+</sup> α-LA<sup>+</sup> 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  $1.6 \pm 0.2$  and  $1.8 \pm 0.3$  for the LF<sup>+</sup> and  
285 LF<sup>+</sup> α-LA<sup>+</sup> 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<sup>+</sup> and LF<sup>+</sup> α-LA<sup>+</sup> 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  $3.6 \times 10^{-2}$  to  $42.8 \times 10^{-2} \text{ g}^{-0.6} \cdot \text{L}^{0.6} \cdot \text{min}^{-1}$   
289 for the LF<sup>+</sup> IMF and between  $2.6 \times 10^{-2}$  to  $42.3 \times 10^{-2} \text{ g}^{-0.8} \cdot \text{L}^{0.8} \cdot \text{min}^{-1}$  for the LF<sup>+</sup> α-LA<sup>+</sup> 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 ( $0.4 \times 10^{-2}$  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<sup>+</sup> (202 kJ.mol<sup>-1</sup>) and LF<sup>+</sup> α-LA<sup>+</sup> (215 kJ.mol<sup>-1</sup>) IMFs. Likewise,  
293 the frequency factor logarithm of LF denaturation determined in the LF<sup>+</sup> IMF (68) is akin to the value  
294 determined for the LF<sup>+</sup> α-LA<sup>+</sup> IMF (73). Higher  $E_a$  and  $\ln(k_{n,0})$  values were determined for the pure LF  
295 solution compared to the LF<sup>+</sup> and LF<sup>+</sup> α-LA<sup>+</sup> IMFs (286 versus 215 kJ.mol<sup>-1</sup> and 96 versus 70 kJ.mol<sup>-1</sup>,  
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<sup>+</sup> and LF<sup>+</sup> α-LA<sup>+</sup> 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<sup>+</sup> and LF<sup>+</sup> α-LA<sup>+</sup> 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<sup>+</sup> and LF<sup>+</sup> α-LA<sup>+</sup> 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  $\alpha$ -LA and  $\beta$ -LG in the  
324 IMFs

325 The reaction orders of LF determined for the LF<sup>+</sup> and LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> 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  $\alpha$ -LA  
330 and  $\beta$ -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  $\alpha$ -LA and  $\beta$ -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<sup>+</sup> and LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup>  
337 IMFs, in spite of different contents in  $\alpha$ -LA and  $\beta$ -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<sup>+</sup> and LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> 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<sup>+</sup> and LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> 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 ( $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$ ).  
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<sup>+</sup> and LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> IMFs indicated that LF was fully  
354 saturated with iron (iron/LF molar ratio of  $3.7 \pm 0.0$ , 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 $\alpha$ -LA is enhanced in the presence of $\beta$ -LG

360 The reaction orders of  $\alpha$ -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  $\alpha$ -LA in bovine milk ( $n=1.0$ , Anema et  
362 al., 2006; Dannenberg & Kessler, 1988) and the value determined in the pure  $\alpha$ -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  $\alpha$ -LA solution and the IMFs.

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

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

378 To the best of our knowledge, the effect of LF on the heat-denaturation of  $\alpha$ -LA was less studied than  
379 that of  $\beta$ -LG. The similarities of the activation energies of  $\alpha$ -LA in the LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> IMF and in the pure  $\alpha$ -  
380 LA solution, both containing the same quantities of  $\alpha$ -LA and the same lactose and mineral contents,  
381 demonstrate that LF does not interfere with the mechanism of heat-denaturation of  $\alpha$ -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  $\alpha$ -LA heat-denaturation that is promoted by thiol  
385 containing molecules. However, the profile of the  $\alpha$ -LA denaturation kinetics in the LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> IMF and  
386 the pure  $\alpha$ -LA solution differs due to the variation of the reaction order ( $1.5 \pm 0.5$  and  $0.5 \pm 0.3$ ,  
387 respectively). The higher reaction order in the LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> IMF could be a consequence of the presence  
388 of other proteins (LF, caseins,  $\beta$ -LG traces) facilitating the aggregation of denatured  $\alpha$ -LA.

389

#### 390 4.3. The heat-denaturation of $\beta$ -LG is enhanced in the presence of LF

391 The reaction orders of the heat-denaturation of  $\beta$ -LG determined for the control and LF<sup>+</sup> 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  $\beta$ -LG was faster in the LF<sup>+</sup> IMF (containing LF protein) than in the control  
395 IMF (lacking of LF protein), with the rate constants of denaturation of  $\beta$ -LG being 2 to 6-times higher  
396 in the LF<sup>+</sup> IMF than in the control IMF, and the denaturation activation energy being higher in the  
397 control IMF than in the LF<sup>+</sup> IMF (305 *versus* 273 kJ.mol<sup>-1</sup>, respectively).

398 The mechanism of heat-denaturation of  $\beta$ -LG has been well-described (de Wit, 2009). Upon heating,  
399 the dimer/monomer equilibrium of native  $\beta$ -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.  $\beta$ -LG reacts rapidly with proteins having exposed disulfide  
403 bonds such as  $\alpha$ -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  $\beta$ -LG starts to  
405 denature. This could facilitate the formation of intermolecular disulfide bonds between LF and  $\beta$ -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  $\beta$ -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<sup>+</sup> IMF than in the control and LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> IMFs, explained by  
413 the simultaneous presence of LF that rapidly denatures on heating and  $\beta$ -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<sup>+</sup> IMFs heat-treated at 90°C/15 s is mainly explained by the heat-  
416 denaturation of  $\beta$ -LG, and to a lesser extent by the heat-denaturation of LF. However, it is probable  
417 that the fraction of native  $\beta$ -LG in these heated IMFs is underestimated because the Arrhenius plot of  
418  $\beta$ -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  $\beta$ -LG after heating the IMFs at 90°C. Consequently, the rate constant used to calculate the fraction of  
425 residual native  $\beta$ -LG is likely overestimated, thus potentially explaining the lower amount of native  
426 whey protein fractions in the control and LF<sup>+</sup> 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<sup>+</sup> and LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> 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  $\alpha$ -LA was not  
436 affected (Deglaire et al., 2019).

437

438 **5. Conclusion**

439 This study demonstrates that the kinetics of heat-denaturation of  $\alpha$ -LA,  $\beta$ -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  $\alpha$ -LA or  $\beta$ -LG in the IMFs. As expected, the heat-denaturation of  $\alpha$ -LA was enhanced in  
442 the presence of  $\beta$ -LG, but LF did not modify it. The presence of LF in the IMFs boosted the heat-  
443 denaturation kinetics of  $\beta$ -LG, probably by exposing numerous disulfide bonds that are accessible to  
444 the free thiol group of  $\beta$ -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  $\alpha$ -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  $\alpha$ -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

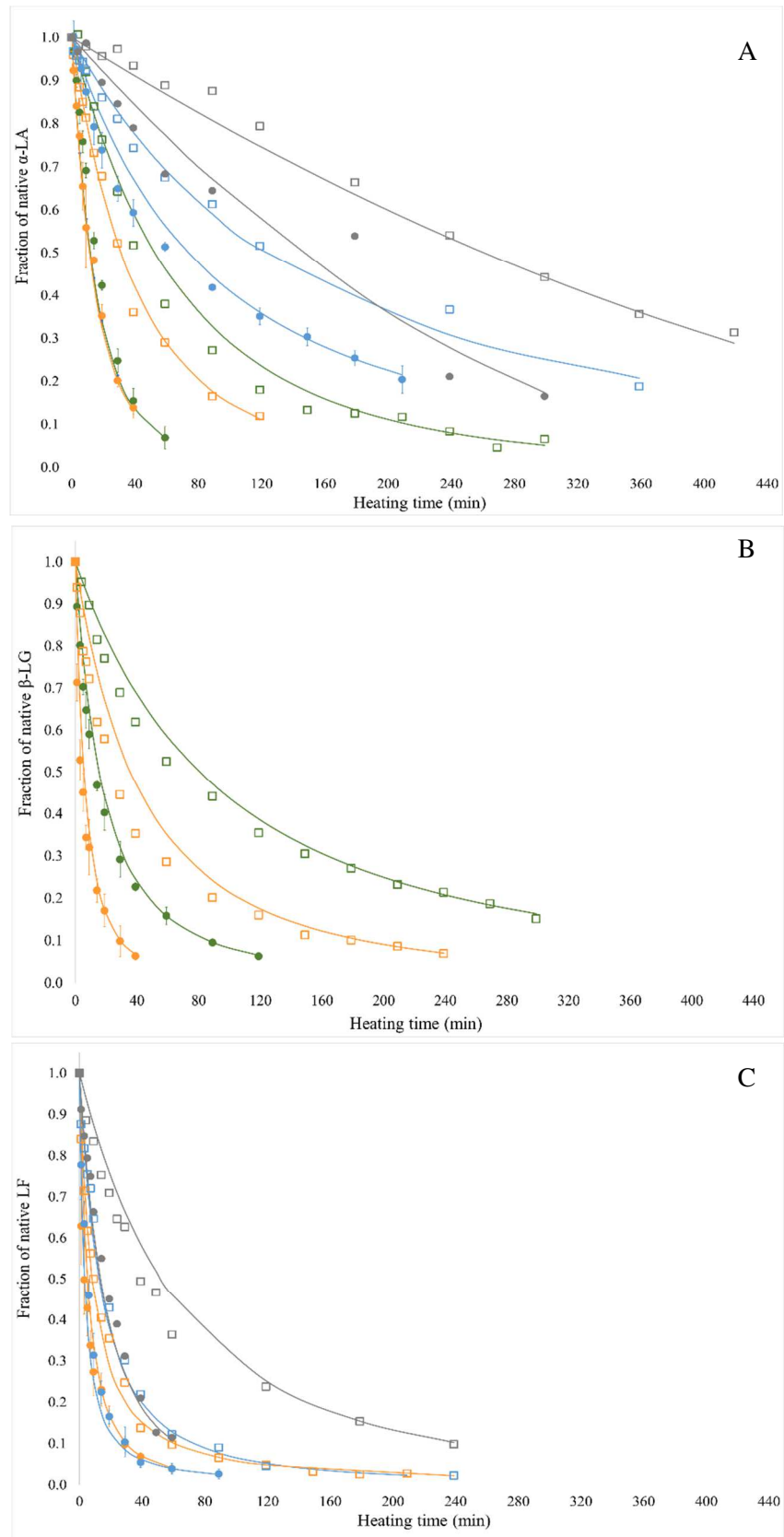
**Figure 1.** Kinetics of thermal denaturation of  $\alpha$ -LA (A),  $\beta$ -LG (B) and LF (C) in the control (green), LF<sup>+</sup> (orange), LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> (blue) IMFs and the pure protein solutions (grey) at 70°C (square) and 75°C (circle). Residual native fractions were calculated as a  $C_t/C_0$  ratio.  $C_t$  (g.L<sup>-1</sup>) 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<sup>-1</sup>). The results at 75°C represent means  $\pm$  error deviations from three independent experiments.

**Figure 1 1.** Arrhenius plots for the thermal denaturation of  $\alpha$ -LA (A),  $\beta$ -LG (B) and LF (C) for the control (green), LF<sup>+</sup> (orange), LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> (blue) IMFs, the pure protein solutions (grey) and **2.** Kinetic parameters associated. 1.  $K_n$  is the unfolding reaction rate constant (g<sup>1-n</sup>.L<sup>n-1</sup>.min<sup>-1</sup>) and 1/T the inverse of the heating temperature (K<sup>-1</sup>). 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<sup>-1</sup>), 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+  $\alpha$ -LA+ (blue) IMFs. The patterns represent the amount of native  $\alpha$ -LA (hatched bars),  $\beta$ -LG (spots) or LF (without pattern). The data are estimations from the equation  $\frac{C_t}{C_0} =$

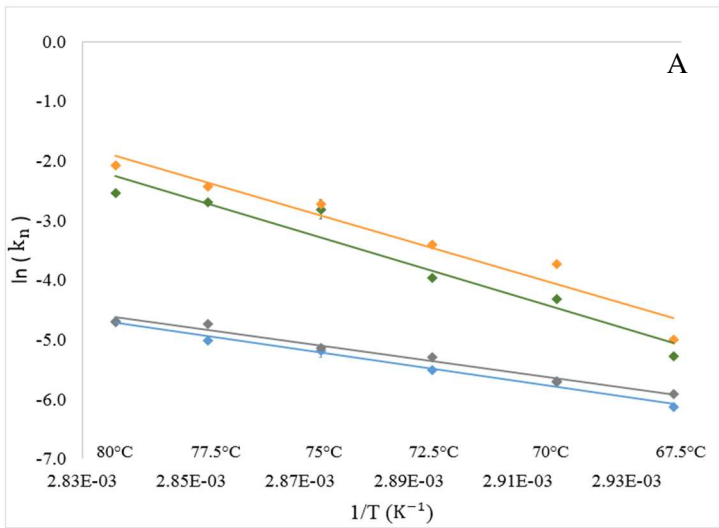
$1 - n \sqrt{\left(1 + (n - 1) \times e^{\left(-\frac{E_a}{R} \times \frac{1}{T+273}\right)} \times k_{n,0} \times C_0^{n-1} \times t\right)}$ , using the thermodynamic parameters (reaction orders n, activation energies  $E_a$  and frequency factors  $k_{n,0}$ ) referenced at the Figure 2.2.  $C_t$ 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

Figure 1



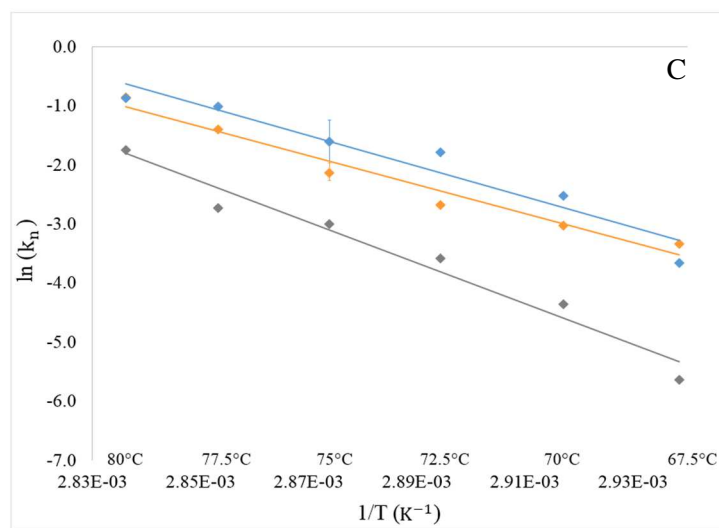
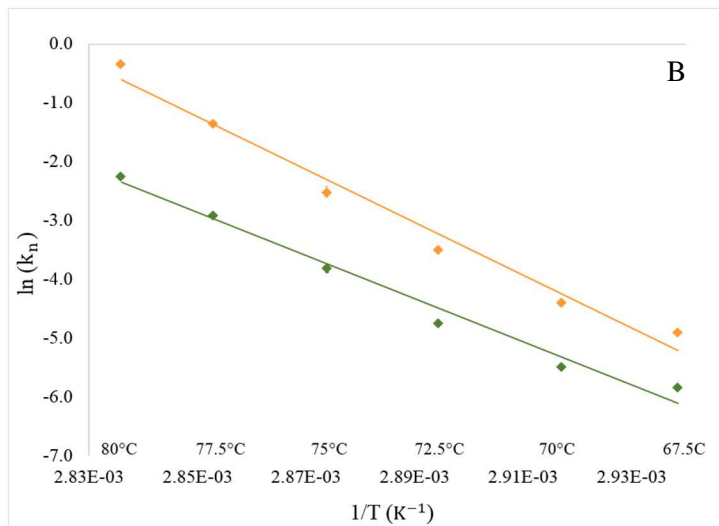
**Figure 2**

1.

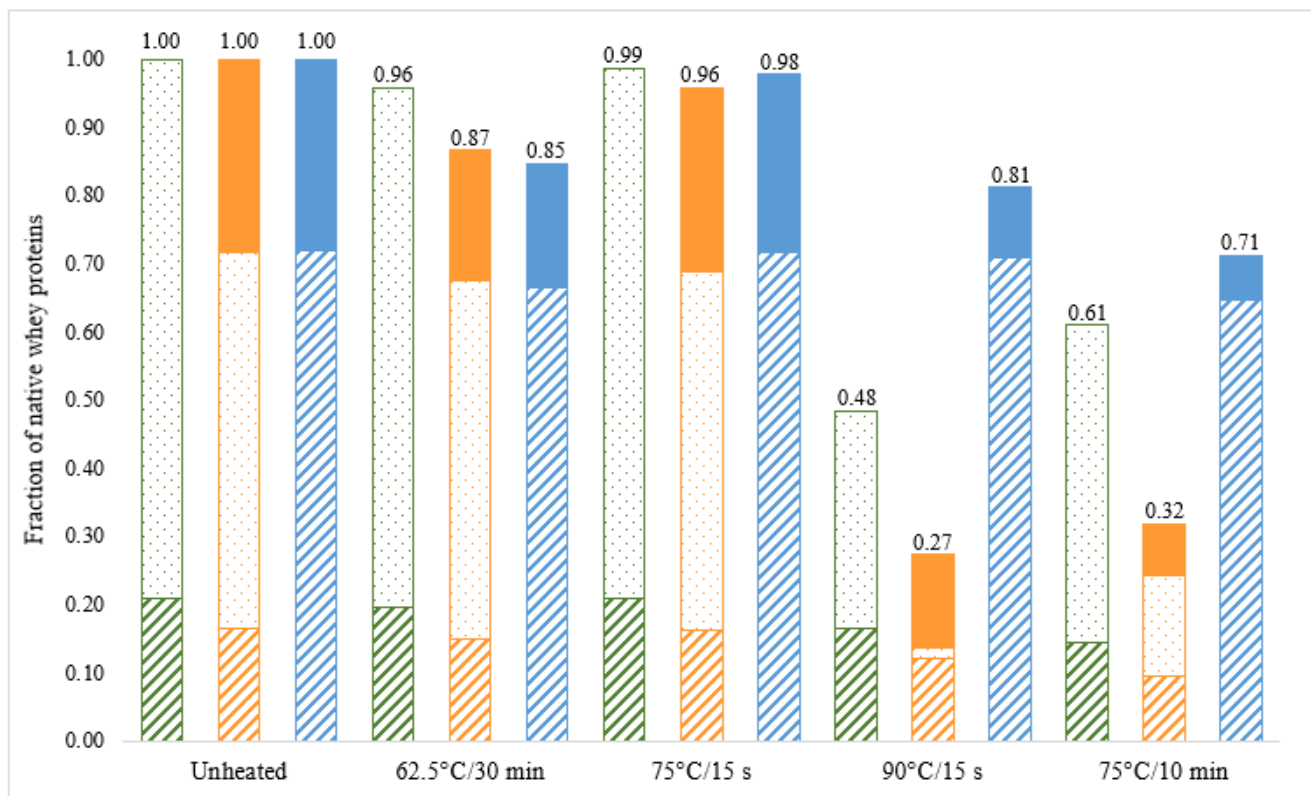


2.

IMFs	Whey proteins	$E_a$ ( $kJ.mol^{-1}$ )	$\ln(k_{a,0})$
Control	$\alpha$ -LA	227	75
	$\beta$ -LG	305	102
LF <sup>+</sup>	$\alpha$ -LA	220	73
	$\beta$ -LG	273	126
	LF	202	68
LF <sup>+</sup> $\alpha$ -LA <sup>+</sup>	$\alpha$ -LA	110	33
	LF	215	73
Pure protein solutions	$\alpha$ -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 <sup>1,4</sup>	36
	Whey proteins <sup>2,4</sup>	7
	Caseins <sup>3,4</sup>	29
	Lactose	48
mg / 100 g of SMP	Calcium	1401
	Chloride	893
	Inorganic phosphorus	1014
	Magnesium	104
	Potassium	1616
	Sodium	382

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

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

<sup>3</sup> Caseins = (TN-NCN) x 6.38

<sup>4</sup> 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 <sup>5</sup>	Human term milk <sup>6</sup>
	Control	LF <sup>+</sup>	LF <sup>+</sup> α-LA <sup>+</sup>		
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 <sup>7</sup>	0.35	Trace
LF	n.d.	0.17 ± 0.00	0.15 ± 0.01	0.48.10 <sup>-3</sup>	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.

<sup>5</sup> 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 1<sup>st</sup> age commercial IMFs for the rest of elements: Guigoz, Gallia, Milumel 1 Lactel, Novalac and Bledilait Bledina.

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

<sup>7</sup> Value of β-LG content in the LF<sup>+</sup> α-LA<sup>+</sup> 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  $\alpha$ -LA,  $\beta$ -LG and LF for the control, LF<sup>+</sup>, LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> 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 <sup>2</sup>	D-value (min)
Control	$\alpha$ -LA	67.5	0.5	0.994	421
		70	1.3	0.968	161
		72.5	1.9	0.953	113
		75	$6.0 \pm 1.0$	0.994	$36 \pm 6$
		77.5	6.7	0.988	32
		80	7.9	0.997	27
	$\beta$ -LG	67.5	0.3	0.999	318
		70	0.4	0.995	219
		72.5	0.9	0.997	105
		75	$2.2 \pm 0.1$	0.999	$42 \pm 3$
		77.5	5.5	0.998	17
		80	10.8	0.988	9
LF <sup>+</sup>	$\alpha$ -LA	67.5	0.7	0.980	338
		70	2.4	0.993	96
		72.5	3.3	0.972	70
		75	$6.6 \pm 0.4$	0.996	$35 \pm 2$
		77.5	8.8	0.999	26
		80	12.5	0.965	19
	$\beta$ -LG	67.5	0.8	0.997	165
		70	1.3	0.994	99
		72.5	3.0	0.994	42
		75	$8.1 \pm 0.8$	0.997	$16 \pm 1$
		77.5	26.2	0.996	5
		80	70.9	0.986	2
	LF	67.5	3.6	0.990	46
		70	5.0	0.980	34
		72.5	6.9	0.989	24
		75	$12.2 \pm 1.8$	0.998	$14 \pm 2$
		77.5	25.3	0.995	7
		80	42.8	0.973	4
LF <sup>+</sup> $\alpha$ -LA <sup>+</sup>	$\alpha$ -LA	67.5	0.2	0.971	522
		70	0.3	0.977	338
		72.5	0.4	0.983	278
		75	$0.6 \pm 0.1$	0.993	$204 \pm 20$
		77.5	0.7	0.995	170
		80	0.9	0.990	122
	LF	67.5	2.6	0.969	63
		70	8.1	0.988	20
		72.5	16.9	0.972	10
		75	$21.3 \pm 7.4$	0.993	$8 \pm 3$
		77.5	36.7	0.982	5
		80	42.3	0.993	4
Pure protein solutions	$\alpha$ -LA	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	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  $\alpha$ -LA or LF with the identical quantity within the LF<sup>+</sup>  $\alpha$ -LA<sup>+</sup> IMF and respecting the mineral and lactose contents of IMFs.