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Corresponding Author: Dr. Stefano Cattaneo,

Corresponding Author's Institution:

First Author: Stefano Cattaneo

Order of Authors: Stefano Cattaneo; Milda Stuknyte; Fabio Masotti; Ivano De Noni

Abstract: Protein modifications occurring during sterilisation of infant formulas can affect protein digestibility and release of bioactive peptides. The effect of glycation and cross-linking on protein breakdown and release of β-casomorphins was evaluated during in vitro gastro-intestinal digestion (GID) of six sterilised model systems of infant formula. Protein degradation during in vitro GID was evaluated by SDS-PAGE and by measuring the nitrogen content of ultrafiltration (3 kDa) permeates before and after in vitro GID of model IFs. Glycation strongly hindered protein breakdown, whereas cross-linking resulting from β-elimination reactions had a negligible effect. Only β-casomorphin 7 (β-CM7) was detected (0.187-0.858 mg L-1) at the end of the intestinal digestion in all untreated IF model systems. The level of β-CM7 in the sterilised model system prepared without addition of sugars ranged from 0.256 to 0.655 mg L-1. The release of this peptide during GID was hindered by protein glycation.

UNIVERSITÀ DEGLI STUDI DI MILANO
Department of Food, Environmental and Nutritional Sciences
Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente
DEFENS

May 17th, 2016

Dear Editor,

On behalf of all Authors, I have the pleasure to submit the manuscript "Protein breakdown and release of β-casomorphins during *in vitro* gastro-intestinal digestion of sterilised model systems of liquid infant formula" to be considered for publication in *Food Chemistry*.

In this work, we designed model systems of liquid with or without reducing sugars and including in the formulation protein ingredients with different heat damage. We explored protein digestibility and the release of five β-casomorphins, opioid-like peptides investigated due to their wide range of hypothesized physiological effects. We highlighted that the protein digestibility and the release of β-casomorphin 7 are strongly affected by protein modifications, in particular those occurring via Maillard reaction. Furthermore, we demonstrated that, interestingly, interactions between caseins and whey proteins as well as β-elimination reaction play a minor role.

For the first time, the release of β-casomorphins upon gastrointestinal digestion was linked to a specific phenomenon of heat damage (Maillard reaction). Despite referred to model systems, the data obtained in this research allow to better evaluate the effect of IF processing on their potential biological properties. The evidences emerging from this work address the manufacturers of IF towards an accurate selection of raw materials and optimization of heat treatment conditions.

I hope that the manuscript could be considered for the publication in *Food Chemistry*.

Sincerely,

dr. Stefano Cattaneo

Corresponding author
University of Milan,
Department of Food, Environmental and Nutritional Sciences (DeFENS),
Via G. Celoria 2, 20133 Milan, Italy
E-mail: stefano.cattaneo@unimi.it
Phone: +39 02 50316676
Fax: +3950316672

Highlights

- Infant formula model systems with or without sugars were designed and sterilized
- Presence of sugars affected protein digestibility of sterilized model systems
- Cross-linking via β-elimination plays a minor role in protein digestibility
- Among β-casomorphins, only β-casomorphin 7 was released upon *in vitro* digestion
- Protein glycation hindered the release of β-casomorphin 7

Protein breakdown and release of β-casomorphins during *in vitro* gastro-intestinal digestion of sterilised model systems of liquid infant formula

Stefano Cattaneo*, Milda Stuknytė, Fabio Masotti, Ivano De Noni

Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente, Università degli Studi di Milano, via G. Celoria 2, 20133 Milan, Italy

*Corresponding author. Tel.: +39 0250316676; fax: +39 0250316672.

E-mail address: stefano.cattaneo@unimi.it

Abstract

Protein modifications occurring during sterilisation of infant formulas can affect protein digestibility and release of bioactive peptides. The effect of glycation and cross-linking on protein breakdown and release of β-casomorphins was evaluated during *in vitro* gastro-intestinal digestion (GID) of six sterilised model systems of infant formula. Protein degradation during *in vitro* GID was evaluated by SDS-PAGE and by measuring the nitrogen content of ultrafiltration (3 kDa) permeates before and after *in vitro* GID of model IFs. Glycation strongly hindered protein breakdown, whereas cross-linking resulting from β-elimination reactions had a negligible effect. Only β-casomorphin 7 (β-CM7) was detected (0.187–0.858 mg L^{-1}) at the end of the intestinal digestion in all untreated IF model systems. The level of β-CM7 in the sterilised model system prepared without addition of sugars ranged from 0.256 to 0.655 mg L^{-1}. The release of this peptide during GID was hindered by protein glycation.

Keywords: infant formula model systems, heat damage, *in vitro* digestion, Maillard reaction, β-casomorphins

1. Introduction

Beta-casomorphins (β-CMs) are opioid peptides generated by the proteolysis of bovine β-casein (β-CN) both during processing and gastrointestinal digestion (GID) of dairy products. Common traits of the primary structure of these peptides are the presence of Tyr at the N-terminus and Phe and Tyr in position 3 and 4 of the amino acid sequence. These particular features result in opioid activity of β-CMs, probably acting via μ-type opioid receptors (Meisel, 1998). Among the β-CMs isolated from *in vivo* or *in vitro* hydrolysates of β-CN, the peptide β-CM7 was the most investigated due to its wide range of hypothesized physiological effects (Laugesen & Elliot, 2003; McLachlan, 2001). Despite this, a scientific report by EFSA stated that a clear relationship between dietary intake of β-CM7 and any suggested non-communicable diseases cannot be established (EFSA, 2009). On the other hand, the same report recognised β-CM7 capable to exert a regulatory effect on gastrointestinal motility and on pancreatic secretion. Moreover, Han, Zhang, Wang and Zhang (2013) demonstrated a beneficial effect of β-CM7 against oxidative stress.

Chemical and physical modifications of milk proteins induced by heat processing are of paramount importance when studying the protein breakdown and the release of certain peptides during *in vitro* GID of infant formulas (IFs). Indeed, IFs are manufactured adopting severe heating conditions to achieve both microbiological safety and prolonged shelf life (Birluez-Aragon et al., 2004). During thermal processing, proteins undergo chemical changes leading to a reduction of their nutritional value. In addition, the use of already heat-treated protein ingredients and the relevant content of both lactose and whey proteins (WP) in the initial recipe contribute to make IFs very prone to heat damage during manufacturing (Cattaneo, Masotti, & Pellegrino, 2009). The impact of processing on the nutritional quality is of particular importance for milk IFs as these products are characterised by a higher heat damage compared to regular milk products (Pischetsrieder & Henle, 2012). Chemical modifications of milk proteins occurring upon heating are mainly represented by glycation via Maillard reaction (MR) and crosslinking arising from both MR

and β-elimination phenomena. The extent of these reactions can be evaluated by determining some targeted chemical indices (Contreras-Calderon, Guerra-Hernandez, & Garcia-Villanova, 2009). For instance, furosine (FUR) and pyrraline (PYR) represent reliable markers of the early and the advanced stage of MR, respectively. Protein crosslinking via β-elimination has been widely studied by monitoring the level of lysinoalanine (LAL), a molecule the formation of which is enhanced by alkaline environment and low content of reducing sugars (Friedman, 1999). All the mentioned chemical modifications could influence protein digestibility, as they may hinder or enhance proteolysis by gastrointestinal enzymes (Hiller & Lorenzen, 2010; Kananen, Savolainen, Makinen, Perttila, Myllykowsky, & Pihlanto-Leppala, 2000; Gilani, Wu Xiao, & Cockell, 2012), and hence they may affect the release of β-CMs during *in vitro* GID of IFs.

The aim of this work was to evaluate the impact of targeted chemical modifications of milk proteins induced by MR and β-elimination on protein degradability and the release of β-CMs during *in vitro* GID of sterilised model systems of liquid IFs.

2. Materials and methods

2.1 Synthetic bovine β-CMs peptides

The synthetic bovine β-CN-derived peptides YPF (f60–62, β-CM3), YPFP (f60–63, β-CM4), YPFPG (f60–64, β-CM5), YPFPGP (f60–65, β-CM6) and YPFPGPI (f60–66, β-CM7) were purchased from GenScript (Piscataway, NJ, USA).

2.2 Laboratory-prepared ingredients

A volume of 1000 mL of commercial freshly prepared pasteurised skim milk was acidified (with 1 M HCl) to pH 4.60 and centrifuged at 5,000 *g* for 10 min at 20 °C. The pellet was suspended in 200 mL of milliQ-treated water and centrifuged adopting the same conditions. This last step was repeated three times. The final pellet was resuspended in 200 mL of milliQ-treated water, and the pH was adjusted to 7.0 (with 1 M NaOH) to obtain sodium caseinate (NaCas). The

77 supernatant (acid whey) was alkalinised to pH 6.80 with 0.1 M NaOH and subjected to
78 ultrafiltration (UF) on a 200-mL stirred UF cell (Amicon 8200, , EMD Millipore, Billerica, MA,
79 USA) using a regenerated cellulose membrane (10 kDa) (EMD Millipore) to obtain a whey protein
80 concentrate (WPC). Diafiltration against distilled water was applied in order to remove lactose and
81 other soluble compounds from WPC. Subsequently, NaCas and WPC were freeze-dried. Their
82 protein contents (ISO Standards 8968-2014) were 82 g 100 g^{-1} and 80 g 100 g^{-1}, respectively.
83
84 *2.3 Commercial ingredients*
85 Commercial samples of sodium caseinate (C-NaCas) and whey protein isolate (WPI) were
86 purchased from Fonterra (Auckland, New Zealand). Their protein contents were 85 g 100 g^{-1} and 80
87 g 100 g^{-1}, respectively. Lactose and maltodextrins were from Sigma-Aldrich (St. Louis, MO, USA).
88
89 *2.4 Model systems of IFs*
90 Six model systems of IFs consisting of different amounts and type of protein-based ingredients
91 and carbohydrates were designed and prepared in 100 mL 0.2 M sodium phosphate buffer (pH
92 7.00). The composition of IF model systems is reported in Table 1.
93 The in batch sterilisation of IF model systems was performed at 110 °C for 38 min in a Vapor
94 Matic 770 autoclave (Sacco, Cadorago, Italy). The applied heat treatment corresponded to a F_0
95 value of 3, which represents the minimal heating conditions adopted for sterilisation of drinking
96 milk (Bylund, 2015).
97
98 *2.5 Determination of targeted heat-damage indices of IF model systems*
99 Furosine was determined according to the ISO Standard 18329-2004. For the determination of
100 PYR the method proposed by Resmini and Pellegrino (1994) was adopted. The LAL content was
101 evaluated by the method reported by Pellegrino, Resmini, De Noni and Masotti (1996).
102

2.6 In vitro *static gastrointestinal digestion (GID) of IF model systems*

Digestions of IF model systems were carried out using the *in vitro* GID protocol reported by Minekus et al. (2014) with some modifications to better mimic the physiological parameters of infant digestive tract (Dupont et al. 2010a). In detail, IF model systems (5 mL) were mixed with 5 mL of simulated gastric fluid supplemented with porcine pepsin (22.75 U mg^{-1} protein). The gastric digestion was performed at 37 °C for 2 h at pH 3.0 (adjusted with 1 M HCl). Afterwards, 10 mL of simulated intestinal fluid and bile salts (2 mM, Sigma-Aldrich) were added to the gastric digestate. Enzymes for intestinal digestion were porcine trypsin (3.45 U mg^{-1} protein) and bovine chymotrypsin (0.04 U mg^{-1} protein). The intestinal phase of GID was performed at 37 °C for 2 h at pH 7.0. It was stopped by adding the protease inhibitor AESFB (Roche, Mannheim, Germany) to give a 1 mM final concentration. The gastric and intestinal digestates were immediately frozen at -40 °C and freeze-dried. All enzymes were purchased from Sigma-Aldrich. Each sample was submitted to three replicate digestions on the same day.

2.7 Evaluation of protein breakdown during in vitro *GID of IF model systems*

SDS-PAGE was performed using 12 % polyacrylamide gels. Non-digested IF model systems were diluted 10-fold with MilliQ-treated water, and the digestates were concentrated 8-fold after lyophilization and re-suspension in MilliQ-treated water. Before analysis samples were diluted 1 : 1 (v/v) with Tricine Sample Buffer (Bio-Rad Laboratories, Hercules, CA, USA) containing 2% β-mercaptoethanol and heated at 95 °C for 5 min. Gels were run in TRIS/Tricine/SDS Running Buffer (Bio-Rad) on a Mini vertical electrophoresis unit (SE250, Hoefer, Holliston, MA, USA) at a constant voltage of 60 V, and they were subsequently stained with Coomassie Blue R-250. Standard proteins of α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) (Sigma-Aldrich) and Spectra Multicolor Low Range Protein Ladder (1700–40000 Da; Thermo Fisher Scientific, Pierce Biotechnology, Rockford, IL, USA) were run together with the samples.

128 The amounts of peptides with a molecular weight not higher than 3 kDa released by protein
129 degradation during *in vitro* GID were assessed by submitting aliquots of each IF model system to
130 UF (3 kDa) before and after *in vitro* GID. The derived permeates were used for the determination of
131 nitrogen (N) content. Protein breakdown was calculated as follows:
132 Protein breakdown (%) = $(N_{GID} - N_b) - N_{SB}/ N_T \times 100$
133 with:
134 N_{GID}, N content of the UF (3 kDa) permeate of the model systems after GID.
135 N_b, N content of the UF (3 kDa) permeate of the blank sample (enzymes and simulated digestive
136 fluids) after GID.
137 N_{SB}, N content of UF (3 kDa) permeate of the model systems before GID.
138 N_T, total N content of the IF model systems.
139 The nitrogen content of each fraction was determined by Kjeldahl method according to the
140 International standard ISO 8968-2014.
141
142 *2.8 Quantification of β-CMs in digestates of IF model systems by UPLC/HR-MS*
143 The above reported permeates were submitted to the determination of the β-CM3, β-CM4, β-
144 CM5, β-CM6 and β-CM7. The UPLC/HR-MS comprised an Acquity UPLC separation module
145 (Waters, Milford, MA, USA) coupled to a Q Exactive hybrid quadrupole-Orbitrap MS through an
146 HESI-II probe for electrospray ionisation (Thermo Scientific, San Jose, CA, USA). The ion source
147 and interface conditions were: spray voltage 4 kV, capillary temperature 350 °C, sheath gas flow
148 35, auxiliary gas flow 15 and heater temperature 300 °C. Peptides in the digestates of model
149 systems were separated using an Aeris WIDEPORE XB-C18 column (150×2.1 mm, 3.6 μm)
150 (Phenomenex, Torrance, CA, USA) kept at 40 °C, using 0.1% trifluoroacetic acid (TFA) in MilliQ-
151 treated water (solvent A) and 0.1% TFA in acetonitrile (solvent B). For the UPLC separation, a
152 linear elution gradient was applied (10% to 26% of solvent B in 14 min) at a flow rate of 0.2 mL
153 min^{-1}. The LC eluate was analysed by MS using targeted selected ion monitoring (t-SIM) and data

dependent tandem MS analysis (dd-MS^2). The Xcalibur software (version 3.0, Thermo Scientific) was used for processing MS data. Peak areas were calculated from extracted t-SIM chromatograms of target peptides with a 3 ppm mass tolerance. Quantification was performed with an external standard 6-point calibration using the synthetic bovine β-CMs peptides.

2.9 Statistical analysis

Analysis of variance (ANOVA) was performed with Daniel's XL Toolbox adding for Excel, version 6.60, by Daniel Kraus, Würzburg, Germany (available at: http://xltoolbox.sourceforge.net/).

3. Results and discussion

3.1 Design of liquid IF model systems and evaluation of their heat damage

IFs are usually manufactured using pasteurised skim milk as the raw material. A CN/WP ratio similar to that of human milk is achieved by adding whey powder, WPC, WPI and caseinates to the formulation (Baxter, Dimler, & Rangavajala, 2011; Nasripour, Scher, & Desorby, 2006). In this study, the IF model systems were designed and prepared to meet the following chemical composition: protein content (1.5 g 100 $m^{-1}L$), CN/WP (1/1) ratio, lactose level (6.0 g 100 mL^{-1}), maltodextrin amount (1.0 g 100 mL^{-1}) and pH value (pH=7.0) (Table 1). These chemical features were in the ranges stated by the EFSA for the IF composition (EFSA, 2014). These parameters were also similar to those previously observed in several commercial IFs (Cattaneo et al., 2009). For the formulation of the model systems A, B, C and D, the protein ingredients (NaCas and WPC) were laboratory-prepared from pasteurised skim milk as described in Materials and Methods. To mimic the use of previously heat-treated ingredients, we utilised commercial samples of sodium caseinate (C-NaCas) and WPI as protein sources for model systems E and F. We included lactose and maltodextrins only in model systems C, D and F to distinguish the impact of protein glycation from

179 the effect of cross-linking deriving from β-elimination reactions on protein degradability and
180 release of β-CMs upon *in vitro* GID of sterilised IF model systems.

181 The levels of heat-damage indices both in protein ingredients and IF model systems were
182 measured. The samples of NaCas and WPC, prepared from pasteurised skim milk, were
183 characterised by a FUR level of 3.5 mg 100 g^{-1} protein and 12.8 mg 100 g^{-1} protein, respectively.
184 The higher FUR level of WPC was attributable to the higher reactivity of WP towards the MR
185 during heat treatment (Pischetsrieder & Henle, 2012). As expected, the untreated model system B
186 prepared with an equal amount (1:1) of NaCas and WPC showed an intermediate (8.3 mg 100 g^{-1}
187 protein) FUR level (Table 2). The FUR contents of the C-NaCas and WPI were 19.9 and 18.8 mg
188 100 g^{-1} protein, respectively. Accordingly, the model systems E and F were characterised by a
189 similar FUR value (19.5 mg 100 g^{-1} protein).

190 All IF model systems were subsequently in batch sterilised to mimic the severe heating
191 conditions adopted during manufacturing of IFs to achieve both microbiological safety and
192 prolonged shelf life (Birluez-Aragon et al., 2004). Following this treatment, heat damage indices
193 were measured in IFs. As expected, the in batch sterilisation dramatically increased the FUR level
194 of the IFs containing lactose and maltodextrins (Table 2). In particular, those prepared with WP
195 (samples D and F) were more reactive towards protein glycation than sample C containing only
196 NaCas as a protein source. As mentioned, this finding could be mainly attributed to the higher
197 content of lysine residues in WP and hence higher reactivity towards MR (Pischetsrieder & Henle,
198 2012). When sterilised in presence of lactose and maltodextrins, sample F showed the highest extent
199 of the "early stage" of MR giving rise to a FUR quantity of 637 mg 100 g^{-1} protein. This level
200 accounted also for the pre-existing heat damage of the commercial protein ingredients used in the
201 initial formulation of this IF model system. In the model IFs prepared without sugars, the FUR level
202 decreased in sample B, and this molecule was not detectable at all in samples A and E. These
203 findings could be explained by the degradation of the Amadori compound to advanced glycation
204 end-products (AGEs). The decrease of FUR content upon sterilisation in presence of reducing

sugars was previously observed by Rufian-Henares, Guerra-Hernandez and Garcia-Villanova (2002) in model systems of enteral formulas based on caseinate and whey protein. Overall, the content of FUR in IF model systems containing sugars was in the range (92.5–1688 mg 100 g^{-1} protein) reported by Rufian-Henares, Garcia-Villanova and Guerra-Hernandez (2004) for IF model systems containing calcium caseinate or whey protein and lactose or maltodextrins, heated at 120 or 140 °C for 5 to 30 min. The content of FUR also overlapped the data previously found by Cattaneo et al. (2009) in commercial liquid IFs.

The compound PYR is a protein-bound glycation molecule. It has been identified among AGEs that form during severe thermal treatment of foods (Pischesrieder & Henle, 2012). Rufian-Henares et al. (2002) reported PYR to be a suitable index for monitoring the heat damage in sterilised products containing previously heat-processed protein ingredients. In the present research, PYR was determined only in samples containing both proteins and sugars (Table 2). The PYR levels in untreated samples C, D and F were under the LOD (< 0.2 mg 100 g^{-1} protein) of the analytical method. After in batch sterilisation, the levels increased to 3.0, 6.4, and 5.5 mg 100 g^{-1} protein, respectively. Differently from our results, Rufian-Henares, Guerra-Hernandez and Garcia-Villanova (2004) did not detect PYR in liquid model systems obtained from laboratory-prepared WP supplemented with sugars and submitted to a sterilisation at 120 °C for 30 min. Despite this, we found PYR levels from 0.6 to 19.0 mg 100 g^{-1} protein in a survey on six commercial liquid IFs (unpublished data). Furthermore, Hellwig, Matthes, Peto, Lobner and Henle (2014) found PYR to be present at 0.7 and 1.2 and mg 100 g^{-1} protein in milk and whey powders, respectively.

The occurrence of protein crosslinking arising from β-elimination reactions was monitored by the determination of LAL (Table 2). The amount of this marker in the laboratory-prepared protein ingredients and in the untreated samples A and B was lower than the LOD of the analytical method. This crosslinker formed only when proteins were submitted to severe heating conditions. In contrary, the LAL level (16.8 mg 100 g^{-1} protein) of the untreated sample E was attributable to the LAL contents of commercial WPI and C-NaCas (1.5 and 27.9 mg 100 g^{-1} protein, respectively).

231 Such difference could be explained by both, different manufacturing conditions and high reactivity
232 of CN towards β-elimination reaction. Indeed, caseins contain phosphoserine, which is one of the
233 amino acid residues involved in LAL formation via β-elimination (Friedman, 1999).

234 The in batch sterilisation increased the LAL level in all sugar-free model IFs (Table 2). In
235 particular, the model systems A and E containing caseinates as protein source showed the highest
236 levels measured in this set (157.5 mg and 277.1 mg 100 g^{-1} protein, respectively). Cattaneo, Masotti
237 and Pellegrino (2012) observed that in absence of sugars the β-elimination reaction prevailed during
238 industrial manufacturing of milk protein powders. Indeed, sample F containing sugars showed a
239 LAL value (99.9 mg 100 g^{-1} protein) three times lower than the corresponding model system
240 without lactose (277.1 mg 100 g^{-1} protein, sample E). In the same way, the other IF model systems
241 with sugars (samples C and D) presented a LAL amount lower than those of the corresponding
242 sugar-free systems (samples A and B). Overall, the range of LAL levels of sterilised IF model
243 systems containing lactose resembled those observed by Cattaneo et al. (2009) in commercial liquid
244 IFs.

245

246 *3.2 Protein breakdown and release of β-CMs upon* in vitro *GID of liquid IF model systems*

247 In the present work, the *in vitro* GID of IF model systems was carried out using a static
248 protocol, which has been proposed as the standard protocol to apply for *in vitro* food digestion
249 studies (Minekus et al., 2014). This protocol was modified according Dupont et al. (2010a) to
250 mimic the conditions of the digestive tract of infant. Indeed, these conditions are far to be optimal
251 for protein degradation, and large peptides can resist gastric and intestinal proteolysis remaining
252 intact (Chatterton, Rasmussen, Heegard, Soresen, & Petersen, 2004).

253 The SDS-PAGE analysis was performed on IF model systems and on their digestates. Protein
254 fractions of the model systems were clearly identified in undigested samples (Fig. 1a). In this case,
255 the sterilisation did not cause any apparent modifications of the profiles in comparison to those of
256 the corresponding raw IF model systems. The bands with electrophoretic mobility corresponding to

257 α-LA and β-LG in sample A and in the commercial NaCas could be ascribed to incomplete removal
258 of whey proteins during casein separation from milk. *In vitro* GID (Fig. 1b) promoted in all samples
259 both the hydrolysis of α_s- and β-CN and the formation of smeared bands with molecular weight
260 between 15 and 25 kDa, likely corresponding to new peptides deriving from CN fractions. All
261 digested systems (Fig. 1b) exhibited faint bands with migration times corresponding to whey
262 proteins. Samples A and C showed electrophoretic bands with mobilities overlapping those of both
263 WP fractions (Fig. 1b). No differences in the degree of protein degradation were observed after
264 digestion between the model systems prepared with the laboratory ingredients. Only GID of
265 samples E and F submitted to sterilisation promoted a complete degradation of all milk proteins.
266 Overall, SDS-PAGE analysis did not allow achieving an exhaustive picture of the proteolysis
267 extent.

268 The protein breakdown following *in vitro* GID of IF model systems was further evaluated
269 measuring the N content of UF (3 kDa) permeates before and after digestion as described in
270 Materials and Methods (Table 3). A similar approach was recently adopted by Villemejane, Denis,
271 Marsset-Baglieri, Alric, Aymard and Michon (2016) who evaluated protein hydrolysis following *in*
272 *vitro* digestion of biscuits by measuring the TCA-soluble peptides concentration.

273 In sample A, containing NaCas but not sugars, the heat treatment did not affect the degree of
274 enzymatic degradation of the proteins. The protein breakdown level during *in vitro* GID of sample
275 A did not change after sterilisation (Table 3). Guo, Flynn and Fox (1999) reported similar
276 observations: they found that heat treatment at 120 °C for 60 min did not affect the *in vivo*
277 digestibility of sodium caseinate.

278 Similarly, the non-significant ($P > 0.05$) difference in the protein breakdown of sample B (made
279 with NaCas/WPC and not containing sugars) before and after in batch sterilisation would suggest
280 that the presence of WP during the heat treatment did not affect the protein breakdown upon *in vitro*
281 GID. In this regard, some authors reported that denaturation of WP translated in an enhanced
282 digestibility due to the easier accessibility of unfolded WP to proteinases (Kaanen et al., 2000).

283 Nonetheless, other research studies claimed that the formation of CN-WP aggregates via disulphide
284 bonds could impair CN digestion (Dupont et al., 2010b). Different levels of protein crosslinking
285 evidenced by the LAL content of heat-treated model systems A and B (Table 2) did not hinder
286 protein breakdown after *in vitro* GID. Despite several authors (Friedman, 1999; Gilani et al., 2012)
287 reported LAL to impair protein digestibility, De Vreese, Frik, Roos and Hagemeister (2000)
288 observed that LAL crosslinking played a minor role in the decrease of food protein digestibility.
289 The extensive protein glycation of samples C and D determined by sterilisation in presence of
290 lactose and maltodextrins strongly impaired the protein breakdown upon *in vitro* GID (Table 3).
291 Protein modifications occurring during MR are generally reported to decrease digestibility. Protein
292 glycation can hinder digestibility because of reduced susceptibility to proteolysis due to steric
293 hindrance of carbohydrate moiety (Sanz, Corzo-Martinez, Rastall, Olano, & Moreno, 2007). A
294 similar mechanism can be hypothesized to explain the decrease of protein breakdown of samples C
295 and D after sterilisation, considering the presence of high molecular weight carbohydrates as
296 maltodextrins in the formulations. Different authors reported lower *in vitro* digestibility of glycated
297 protein due to both poor trypsin accessibility to peptide C-terminal bonds and occurrence of
298 glycated lysyl and arginyl residues (Chevalier, Chobert, Popineau, Nicolas, & Haertl, 2001; Nacka,
299 Chobert, Burowa, Leonil, & Haertl, 1998). The reduction of protein hydrolysis during digestion can
300 also result from the formation of protein cross-linking, since the covalently cross-linked protein
301 aggregates are scarcely susceptible to enzymatic proteolysis (Gerrad et al., 2012). Several studies
302 highlighted formation of protein cross-linking via MR in food (Biemel, Buhler, Reihl, & Lederer,
303 2001; Bouhallab, Morgan, Henry, Mollé, & Léonil, 1999). The reduction of protein breakdown, we
304 observed in model systems C and D, is therefore consistent with the above-reported protein
305 modifications, the extension of which is highlighted by high levels of FUR and PYR following
306 sterilisation of these samples.
307 Among untreated IF model systems, we found the lowest protein breakdown after *in vitro* GID
308 in samples E and F, obtained by the inclusion in the initial formulation of previously heat-processed

309 protein ingredients. Differently from the IF model systems formulated with laboratory-prepared
310 ingredients, the in batch sterilisation of the samples E and F slightly enhanced the protein
311 breakdown (Table 3) during *in vitro* GID. Hiller and Lorenzen (2010) hypothesised that partial
312 unfolding of protein aggregates formed during heat treatment of previously heated WPI in presence
313 of saccharides resulted in an increased accessibility of intestinal proteinases on the peptide bonds.
314 Kim et al. (2007) reported the same phenomenon. They evaluated the digestion of heated
315 commercial WPC by pepsin and trypsin. According to these authors, the enhanced digestibility of
316 commercial WP could be explained by the unfolding of protein aggregates due to the breakdown of
317 the disulphide bonds. Beside modifications involving the whey protein fraction, the effect of severe
318 heating on susceptibility of casein to proteolysis should be considered as well. The extent of casein
319 proteolysis is largely enhanced by dephosphorylation. Dalgleish, Pouliot and Paquin (1987)
320 reported about two-thirds of the casein phosphates to be hydrolysed after heating at 130 °C for 60
321 min. Similarly, multiple heat treatments, which commercial Na caseinate in model systems E and F
322 underwent, could be hypothesised to result in a partial dephosphorylation. Therefore, the increased
323 digestibility of samples E and F could be potentially ascribed to severe heat load undergone by
324 whey protein and casein fractions during the industrial manufacturing of the commercial powders
325 and the laboratory sterilization of the IF model systems.

326 Finally, we investigated the presence of the β-CM3, β-CM4, β-CM5, β-CM6 and β-CM7 in the
327 *in vitro* digestates of liquid IF model systems. After the gastric step of GID, none of the mentioned
328 β-CMs was detected in the digestates of all IF model systems, neither before nor after the
329 sterilisation process. After the intestinal step of *in vitro* GID, only the β-CM7 was found at 0.187–
330 0.858 mg L^{-1} in the digestates of all unheated model systems (Table 4). On the contrary, after in
331 batch sterilisation the β-CM7 was revealed only in the digestates of the model IFs prepared without
332 sugars, whereas no other β-CMs were detected. In detail, 0.655, 0.414 and 0.256 mg L^{-1} of β-CM7
333 were estimated to be present in samples A, B and E, respectively (Table 4). These data are
334 comparable to those (0.028 to 0.470 mg L^{-1}) previously found by De Noni (2008) in commercial

IFs. The in batch sterilisation slightly hindered the β-CM7 release during GID of sample A, despite the degree of NaCas degradation was not affected according to the protein breakdown before and after heating (Table 3).

In the same manner, the similar β-CM7 content of sample B before and after in batch sterilisation would suggest that the release of this peptide during *in vitro* GID was not affected by either the presence of WP during heat treatment or by the different level of protein crosslinking, via LAL formation. The high content of FUR together with the presence of PYR accounted for the extensive protein glycation of samples C and D, determined by heat treatment in presence of lactose and maltodextrins. These post-translational modifications likely impaired the release of β-CM7, as well as protein breakdown, upon *in vitro* GID. Untreated samples E and F showed the lowest contents of β-CM7 after *in vitro* GID (0.191 and 0.187 mg L^{-1}, respectively). These data fit the very low protein breakdown of these model systems after *in vitro* GID. Despite the heat treatment of samples E and F slightly enhanced their protein digestibility (Table 3), the release of β-CM7 occurred only after *in vitro* GID of sample E.

The link between the release of β-CM7 upon GID and the protein breakdown of IF model systems was well highlighted by the correlation (R^2=0.884) found between the content of this peptide on casein basis and the protein breakdown of IF digestates (Figure 1).

4. Conclusions

Protein modifications occurring via MR represented the key factor affecting the release of β-CM7 during *in vitro* GID of IF model systems. At this regard, modifications induced by WP-CN interactions and β-elimination reactions seemed to play a minor role in the modification of accessibility of gastrointestinal proteases to milk proteins. Despite referred to model systems, the data obtained in this research address some indications to better evaluate the potential biological properties of IFs in relationship to processing and digestive conditions. Evidences emerging from this research can address the manufacturers of IF towards modulation of heat treatment conditions

and careful selection of the raw materials. The knowledge of these technological aspects are of basic importance to define some nutritional properties of protein fractions of IF. Finally, these data constitute an additional background for further studies that deal with the nutritional consequences of consumption of IFs, which are still largely unknown.

Conflict of interests

Authors declare no conflict of interests.

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477

Captions to figures

Figure 1. SDS-PAGE analysis of infant formula model systems: (A) undigested and (B) after *in vitro* static gastrointestinal digestion.

T, sterilised, M, molecular weight marker 1.7–40 kDa; C-NaCas, commercial sodium caseinate; WPI, whey protein isolate; α-LA, α-lactalbumin; β-LG, β-lactoglobulin. In B, C-NaCas, α-LA and β-LG, undigested.

Figure 2. Correlation between the protein breakdown (%) and the content of β-CM7 after *in vitro* GID in IF model systems.

Table 1. Composition of the IF model systems.

Model system	Composition	Caseins	Whey proteins	Lactose	Maltodextrins
		g 100 mL^{-1}			
A	NaCas	1.500	-	-	-
B	NaCas + WPC	0.750	0.750	-	-
C	NaCas + L + M	1.500	-	6.000	1.000
D	NaCas + WPC + L + M	0.750	0.750	6.000	1.000
E	C-NaCas + WPI	0.750	0.750	-	-
F	C-NaCas + WPI + L + M	0.750	0.750	6.000	1.000

NaCas: sodium caseinate, laboratory-prepared from pasteurised skim milk; WPC: whey proteins, laboratory-prepared by ultrafiltration from pasteurised skim milk; C-NaCas: commercial sodium caseinate; WPI: commercial whey protein isolate; L: lactose; M: maltodextrins.

Table(s)

Table 2. Levels of furosine, pyrraline and lysinoalanine in the liquid IF model systems before and after in batch sterilisation.

	Furosine		Pyrraline		Lysinoalanine	
Model system	Before heating	After heating	Before heating	After heating	Before heating	After heating
					mg 100 g^{-1} protein	
A	3.5^{a}±0.3	<0.2	<0.2	<0.2	<0.2	157.5^{b}±10.2
B	8.3^{b}±0.4	1.9^{d}±0.2	<0.2	<0.2	<0.2	87.5^{c}±5.8
C	3.3^{a}±0.2	321.0^{e}±12.2	<0.2	3.0^{a}±0.3	<0.2	41.9^{d}±4.1
D	8.4^{b}±0.2	349.2^{e}±11.0	<0.2	6.4^{b}±0.3	<0.2	38.5^{d}±4.3
E	19.6^{c}±0.6	<0.2	<0.2	<0.2	16.8^{a}±1.1	277.1^{e}±15.3
F	19.5^{c}±1.1	637.8^{f}±22.5	<0.2	5.5^{b}±0.2	17.0^{a}±0.8	99.9^{f}±9.5

Values are presented as means ± SD (n = 3). Data with different superscripts differ ($p<0.05$).

Table 3. Degree of protein breakdown (%) after *in vitro* GID of unheated or in batch sterilised IF model systems.

Model system	Protein breakdown (%)	
	Before heating	After heating
A	48.9^{a}±1.0	48.2^{a}±0.7
B	54.3^{b}±0.9	53.8^{b}±1.2
C	48.1^{a}±1.0	43.7^{c}±1.3
D	54.0^{b}±0.9	35.0^{d}±0.9
E	36.0^{d}±0.6	45.1^{c}±1.0
F	35.6^{d}±0.6	40.3^{e}±1.2

Values are presented as means ± SD (n = 3). Data with different superscript differ ($p<0.05$).

Table 4. Content of β-CM7 in *in vitro* digestates of untreated and in batch sterilised IF model systems.

Model system	β-CM7	
	Before heating	After heating
	mg L^{-1}	
A	0.858^{a}±0.043	0.655^{a}±0.009
B	0.411^{b}±0.022	0.414^{b}±0.040
C	0.823^{a}±0.016	n.d.
D	0.428^{b}±0.031	n.d.
E	0.191^{c}±0.015	0.256^{c}±0.010
F	0.187^{c}±0.029	n.d.

Values are presented as means ± SD (n = 3). Data with different superscripts differ ($p < 0.05$). n.d., not detected.

Figure(s)

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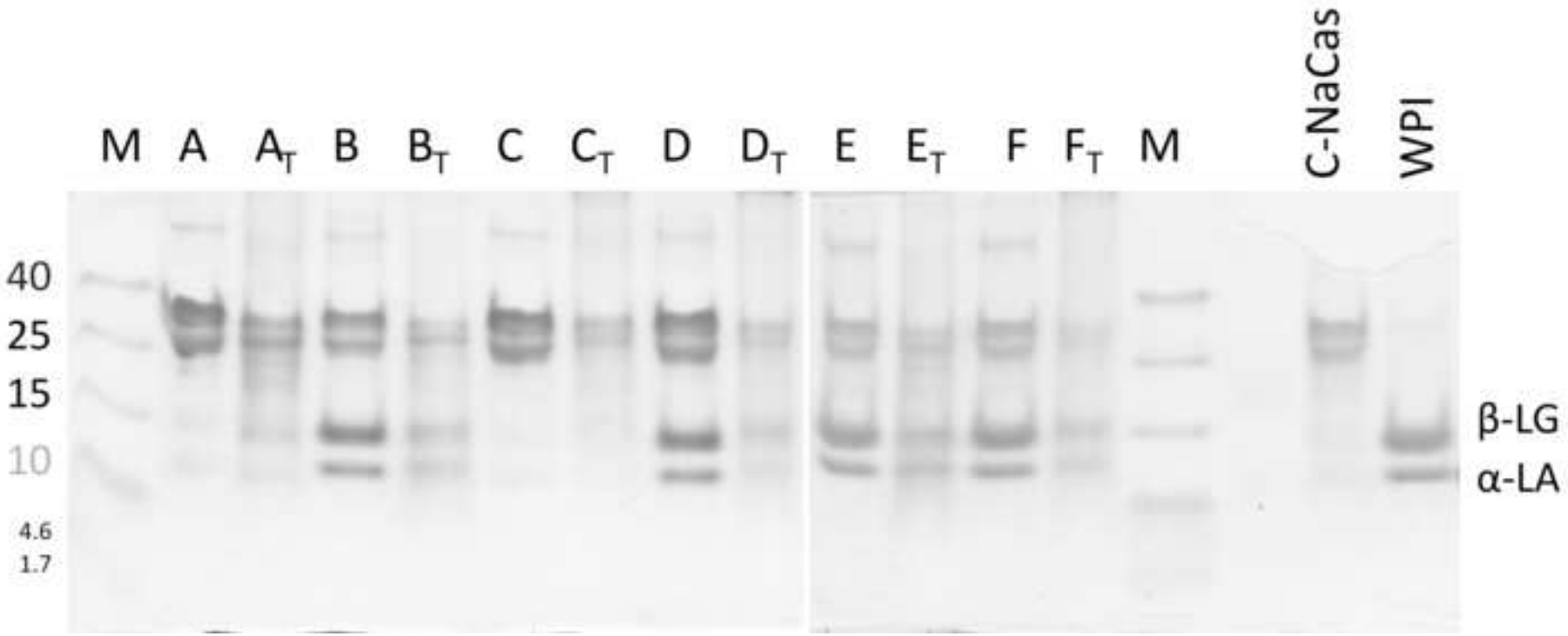

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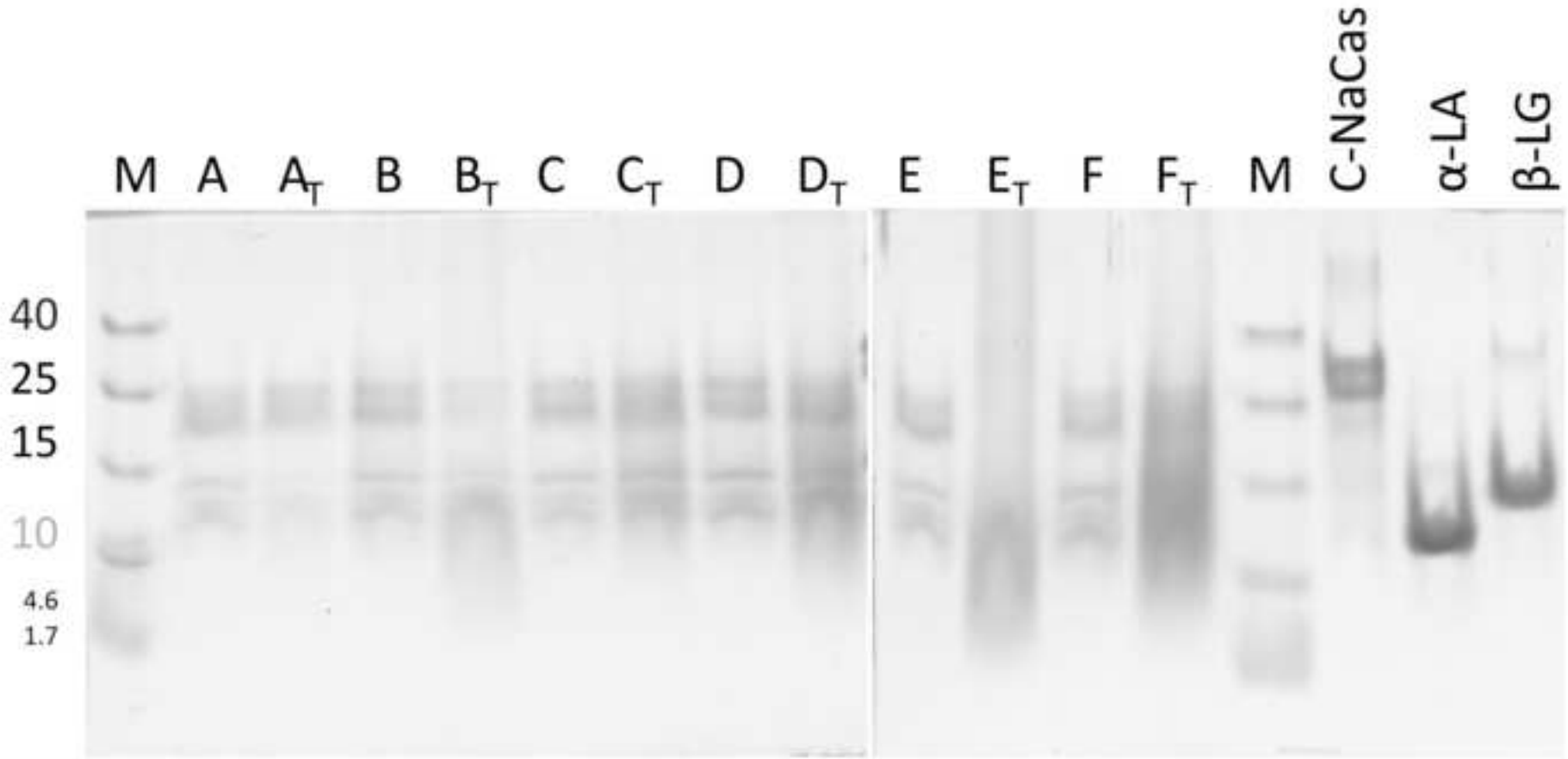

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Figure 2- Cattaneo