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

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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 systems 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.

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## 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 Pro 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 & Elliott, 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 in rats.

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 (Birlouez-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-Calderón, Guerra-Hernández, & García-Villanova, 2009). For instance, furosine (FUR) and pyrraline (PYR) represent reliable markers of the early and the advanced stage of MR, respectively. In detail, PYR is a protein-bound glycation molecule forming during severe thermal treatment of foods (Pischetsrieder & Henle, 2012). Rufian-Henares, Guerra-Hernandez, and Garcia-Villanova (2002) reported PYR to be a suitable index for monitoring the heat damage in sterilised products containing previously heat-processed protein ingredients. Protein crosslinking via β-elimination has been widely

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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 et al., 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 5000*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 supernatant (acid whey) was alkalinised to pH 6.80 with 0.1 M NaOH and subjected to ultrafiltration (UF) on a 200-mL stirred UF cell (Amicon 8200, EMD Millipore, Billerica, MA, USA) using a regenerated cellulose membrane (10 kDa) (EMD Millipore) to obtain a whey protein concentrate (WPC). Diafiltration against distilled water was applied in order to remove lactose and other soluble compounds from WPC. Subsequently, NaCas and WPC were freeze-dried. Their protein contents (ISO Standards 8968-2014) were 82 g 100 $g^{-1}$ and 80 g 100 $g^{-1}$, respectively.

### 2.3. Commercial ingredients

Commercial samples of sodium caseinate (C-NaCas) and whey protein isolate (WPI) were purchased from Fonterra (Auckland, New Zealand). Their protein contents were 85 g 100 $g^{-1}$ and 80 g 100 $g^{-1}$, respectively. Lactose and maltodextrins were from Sigma-Aldrich (St. Louis, MO, USA).

### 2.4. Model systems of IFs

Six model systems of IFs consisting of different amounts and type of protein-based ingredients and carbohydrates were designed and prepared in 100 mL 0.2 M sodium phosphate buffer (pH 7.00). The composition of IF model systems is reported in Table 1.

The in batch sterilisation of IF model systems was performed at 110 °C for 38 min in a Vapor Matic 770 autoclave (Sacco, Cadorago, Italy). The applied heat treatment corresponded to a $F_0$ value of 3, which represents the minimal heating conditions adopted for sterilisation of drinking milk (Bylund, 2015).

### 2.5. Determination of targeted heat-damage indices of IF model systems

Furosine was determined according to the ISO Standard 18329-2004. For the determination of PYR the method proposed by Resmini and Pellegrino (1994) was adopted. The LAL content was evaluated by the method reported by Pellegrino, Resmini, De Noni, and Masotti (1996).

### 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

**Table 1**
Composition of the IF model systems.

| Model system | Composition | Caseins<br>g 100 $mL^{-1}$ | Whey proteins | Lactose | Maltodextrins |
|---|---|---|---|---|---|
| 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.

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