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Abstract: Pasteurized donor human milk (PDHM) for preterm infant nutrition is fortified with hydrolyzates of cow's milk proteins, which have been poorly investigated in relation to heat damage and occurrence of the bioactive peptides β-casomorphins (BCMs). Therefore, the heat load of three commercial fortifiers was assessed by measuring well-recognized indexes of thermal protein modifications. The fortifiers did not contain pyrraline, whereas furosine and lysinoalanine levels overlapped the lowest values reported for liquid formulas addressed to term infant nutrition. Bovine BCMs 3 to 7 and human BCMs 3 to 9 were searched. Bovine BCMs 3, 4, 6 and 7 were found in the undigested fortifiers. Following in vitro digestion simulating the digestive conditions of premature infant, bovine BCMs still occurred in fortified PDHM; the human BCMs 3, 7, 8 and 9 formed. Overall, these results better address the nutritional features of protein fortifiers and fortified PDHM intended for nutrition of preterm infants.

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Department of Food, Environmental and Nutritional Sciences
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July 25th, 2019

Dear Editor,

we have the pleasure to submit the manuscript "Effect of protein fortification on heat damage and occurrence of β-casomorphins in (un)digested donor human milk intended for nutrition of preterm infants" by Stefano Cattaneo, Valentina Pica, Milda Stuknytė, Fabio Masotti, Domenica Mallardi, Chiara Tabasso, Paola Roggero, and myself to be considered for publication in the "Food Chemistry".

In this work, we investigated the heat damage of three protein fortifiers of pasteurized donor human milk (PDHM) intended for nutrition of preterm infants. In the same samples and in the PDHM, we studied the occurrence of bioactive peptides, bovine and human β-casomorphins (BCMs), prior to and after *in vitro* gastrointestinal digestion. To this aim, we adopted an *in vitro* static digestion protocol tailored to mimic the digestive physiological conditions of preterm infant.

In the present study, we demonstrated that fortifiers accounted for the overall heat damage and for the occurrence of bovine BCMs in fortified (digested) PDHM. We found that human BCMs were released during gastrointestinal digestion of fortified PDHM.

To the best of our knowledge, this is *the first* investigation dealing with the chemical characterization of commercial protein fortifiers used to supplement PDHM for nutrition of preterm infants. Moreover, it is *the first* study providing quantitative data regarding the occurrence of both human and bovine BCMs in (digested) fortifiers and fortified PDHM. Overall, the obtained results bring additional knowledge into the field of premature baby nutrition and provide supplementary evidence for the development of more tailored PDHM fortifiers.

Sincerely,

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Highlights

- Pasteurized donor human milk (PDHM) is fortified for preterm infant nutrition
- Hydrolyzates of bovine milk proteins are used for fortification of PDHM
- Hydrolyzates contain casomorphins (BCMs) and present protein chemical artifacts
- Hydrolyzates account for the protein artifacts of PDHM
- Digestion of fortified PDHM releases bovine and human BCMs

Effect of protein fortification on heat damage and occurrence of β-casomorphins in (un)digested donor human milk intended for nutrition of preterm infants

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ABSTRACT

Pasteurized donor human milk (PDHM) for preterm infant nutrition is fortified with hydrolyzates of cow's milk proteins, which have been poorly investigated in relation to heat damage and occurrence of the bioactive peptides β-casomorphins (BCMs). Therefore, the heat load of three commercial fortifiers was assessed by measuring well-recognized indexes of thermal protein modifications. The fortifiers did not contain pyrraline, whereas furosine and lysinoalanine levels overlapped the lowest values reported for liquid formulas addressed to

term infant nutrition. Bovine BCMs 3 to 7 and human BCMs 3 to 9 were searched. Bovine BCMs 3, 4, 6 and 7 were found in the undigested fortifiers. Following *in vitro* digestion simulating the digestive conditions of premature infant, bovine BCMs still occurred in fortified PDHM; the human BCMs 3, 7, 8 and 9 formed. Overall, these results better address the nutritional features of protein fortifiers and fortified PDHM intended for nutrition of preterm infants.

Keywords: preterm infant; milk protein hydrolyzates; heat damage; casomorphins; digestion

1. Introduction

The term “preterm” (or “premature”) infants refers to newborns whose parturition occurs before the 37th week of pregnancy (WHO, 2006). Birth weight is an important parameter for addressing treatments to guarantee survival of preterm infants. This especially accounts for Very Low Birth Weight (VLBW) premature infants, who weigh less than 1500 g at birth (WHO, 2006). Increasing their weight by intensive medical care and personalized nutritional aid avoids complications and increases their survival probabilities. When mother’s own milk is not available, pasteurized donor human milk (PDHM), coming from mothers delivering at term, is used. It represents the best form of nutrition for preterm infants providing strong health advantages both in the short and long-term (Arslanoglu et al., 2019). Although HM is biologically tailored to meet the nutritional demands of the baby born at term, it is inadequate for the peculiar nutritional needs of VLBW neonate when fed at the usual feeding volumes (135–200 mL kg^{-1} d^{-1}) (Arslanoglu et al., 2019; O’Connor et al., 2018; WHO, 2006). In this regard, the adequate protein supplementation is the main aid to support nutritional needs of preterm infants and to ensure fast growth and optimal neurocognitive outcomes (Arslanoglu et al., 2019). “Standard Fortification” is currently the most utilized regimen in neonatal intensive care

units, and it is based on the addition of a fixed amount of (multicomponent) fortifier to a certain volume of PDHM (Arslanoglu et al., 2019). Commonly, powdered proteins extracted from bovine milk are used for "Standard protein fortification" of PDHM (WHO, 2006). The most commonly used protein fortifiers are hydrolyzates of casein(ate) and protein concentrate/isolate from bovine whey or milk. They also contain variable quantities of energy, minerals, trace elements, vitamins and electrolytes (Arslanoglu et al., 2019). These protein hydrolyzates are less expensive and more available compared to HM-based fortifiers, which have been recently adopted in neonatal care, even if their efficacy has not yet fully proved (Arslanoglu et al., 2019; Ziegler, 2014).

To date, commercial cow's milk fortifiers have been poorly investigated in relation to protein artifacts, which could affect their biological and nutritional properties. For instance, heat treatment applied during manufacturing of fortifiers can lead to protein modifications, which can be evaluated through quantitation of the markers furosine (ε-N-2-furoylmethyl-L-lysine, FUR), pyrraline (2-formyl-5-(hydroxymethyl)pyrrole-1-norleucine, PYR) and lysinoalanine (N6-(DL-2-amino-2-carboxyethyl)-L-lysine, LAL). FUR is a widely used index for the evaluation of the extent of protein glycation via Maillard Reaction (MR) in milk products (Pischetsrieder & Henle, 2012). PYR is a protein-bound marker of the advanced stage of MR in dairy products submitted to severe heat treatment, and its presence has been related to decreased protein digestibility (Pischetsrieder & Henle, 2012). Finally, LAL is a protein crosslinker resulting from the thermal degradation of milk proteins, especially casein (CN) (Friedman, 1999). The enzymatic process the fortifiers undergo can accelerate some of these phenomena. Indeed, because of protein breakdown, fortifiers contain huge quantities of peptides, the amino groups of which can promptly interact via the MR during sterilization and spray-drying manufacturing steps (Pischetsrieder & Henle, 2012).

In addition, the enzymatic hydrolysis of bovine milk proteins can potentially release bioactive peptides (BAPs) in fortifiers or favor their release from precursor peptides during gastrointestinal digestion of fortifiers. The most known BAPs of bovine milk proteins are the opioid-acting bovine β-casomorphins (bBCMs) and, in particular, the bBCM7 and bBCM5 that are fragments f60–66 and f60–64 of the precursor bovine β-CN (UniProtKB, CASB_Bovin, P02666), respectively. Other bBCMs are bBCM3 (f60–62), bBCM4 (f60–63) and bBCM6 (f60–65). Many studies reported the potential biological effect of bBCMs in adults and infants as mainly caused by interaction of bBCM7 with the μ opioid receptor (MOR) (Banks, 2015; Enjapoori, Kukuljan, Dwyer, & Sharp, 2019; Fiedorowicz et al., 2016; Kost et al., 2009). In fact, bBCMs are suspected to be involved in the onset or worsening of several non-communicable diseases (EFSA, 2009). Whether the same or even amplified effects can be predicted in preterm newborns, is a topic that has not been addressed to date.

Human β-casomorphins (hBCMs) can be potentially released from the region 51–62 of mature human β-CN (UniProtKB, CASB_Human, P05814) upon endogenous and/or exogenous proteolytic phenomena. Accordingly, hBCMs 3 to 11 are fragments resulting from cleavage of 50–51 bond and a sequential cleavage of the C-terminal residue from the 51–62 region. Some hBCMs have been demonstrated to be present in human biological systems (Banks, 2015), and hBCMs 3 to 7, released from the sequence 51–57, have been shown to elicit opioid activity by binding to MOR (Koch, Wiedemann, & Teschemacher, 1985).

Human and bovine BCMs can be potentially released during gastrointestinal digestion of (fortified) HM. The digestive conditions are peculiar to preterm infants. Indeed, relevant differences in functionality between term and preterm infants are observed and, generally, the former present a number of physiological conditions that make the digestion more difficult and less efficient (Dallas, Underwood, Zivkovic, & German, 2012). For instance, the more neutral pH values in the stomach impair the pepsin activity and the intestinal digestive function is lower

than in infants born at term (Dallas et al., 2012). These digestive conditions could affect the enzymatic degradation of CN and, therefore, the potential release of bBCMs and hBCMs during *in vivo* gastrointestinal digestion of (fortified) PDHM.

To the best of our knowledge, so far, no studies assessed the chemical artifacts related to heat damage of hydrolyzed cow's milk fortifiers intended for supplementation of PDHM. Moreover, no studies attempted to ascertain the occurrence of bBCMs in fortifiers, as well as bBCMs and hBCMs during *in vitro* digestion of PDHM fortified with hydrolyzed cow's milk proteins. On these bases, this work aimed to: investigate the thermal damage of three commercial hydrolyzed cow's milk fortifiers; determine the presence and quantity of bBCMs in the same undigested samples; and, finally, evaluate the occurrence and fate of bBCMs and hBCMs during *in vitro* digestion of PDHM supplemented using the three fortifiers. To this end, a static *in vitro* gastrointestinal digestion (SGID) model simulating the physiological parameters of the digestive tract of premature infants was adopted. Human and bovine casomorphins were searched and quantified by UPLC/HR-MS.

2. Materials and methods

2.1. Pasteurized donor human milk (PDHM)

Donor mature raw HM (20 L) was collected by Donor Human Milk Bank of "Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico" (Milan, Italy). The study was approved by the Ethics Committee of the same institution [Approval No. 289-2017]. Informed written consent was obtained from lactating women enrolled for the study. Mature HM samples were collected using an electrical breast pump by healthy donors who delivered at term. Prior to fortification the pool of previous samples was constituted, and then it was Holder pasteurized (63 °C for 30

125 min). Samples were stored at -24 °C for the entire duration of the trial. The composition (g 100
126 mL^{-1}) of the pooled PDHM was the following: proteins 1.3, carbohydrates 7.0, lipids 2.9.
127
128 *2.2. Fortifiers of PDHM*
129
130 In the present work, three commercial powdered fortifiers (NF, BF, PF) intended for
131 protein fortification of PDHM meant for nutrition of preterm infant were considered. The
132 samples were provided by the Donor Human Milk Bank and commonly used for fortifying
133 PDHM. The composition (g 100 g^{-1}) of NF, BF and PF, as declared on their labels, is reported in
134 Table 1.
135
136 **Table 1**
137 Composition (g 100 g^{-1}) of the commercial fortifiers intended for protein fortification of PDHM
138 for nutrition of preterm infants.

Fortifier	Composition
NF	Hydrolyzed whey proteins 20, maltodextrin 60, fat 0.4, mineral salts, vitamins, emulsifier soya lecithin, inositol, L-carnitine, taurine. Carbohydrates: 66 (1.8 sugars and 4.2 other carbohydrates).
BF	Maltodextrins, hydrolyzed caseins 12.5, hydrolyzed whey proteins 12.5, fat 0.0, calcium salts, sodium chloride, vitamins and minerals. Carbohydrates: 62.2 (5.5 sugars).
PF	Casein 41, hydrolyzed whey proteins 41, vitamins, sodium 0.776, potassium 1.226, chloride 0.066, calcium 0.524, phosphorus 0.516, magnesium 0.046, manganese 0.0002 selenium 0.00003. Carbohydrates: 2.2 (sugars 1.3).

139
140 *2.3. Fortification of PDHM*
141
142 The PDHM fortification was addressed to achieve a protein content of 4.1 g 100 $kcal^{-1}$,
143 according to the nutritional program for preterm infants adopted at the “Fondazione IRCCS Ca’

144 Granda Ospedale Maggiore Policlinico". In detail, two fortification modes were adopted by
145 adding 5.0 g NF and 0.36 g PF, or 4.4 g BF and 0.24 g PF to 100 mL of PDHM. The daily
146 nutritional requirement was assessed based on the minimum acceptable weight growth rate for
147 correct postnatal development of premature infants (1000–1500 g body weight) corresponding
148 to 17.4 g per kg of body weight per day (Ziegler, 2014).

149 Other three samples were prepared by dissolving 5.47 g of BF, 6.83 g NF or 1.67 g of PF,
150 respectively, in 100 mL of permeate deriving from ultrafiltration (UF) of PDHM (Figure 1). To
151 this purpose, the permeate was prepared using an Amicon stirred cell 8440 (Merck, Darmstadt,
152 Germany) equipped with an Ultracel (Merck) membrane (cut-off 1 kDa). The used amount of
153 each fortifier corresponded to the same bovine protein content of the two fortified PHDM
154 samples. The different samples arising from the experimental plan adopted in the present work
155 and further submitted to *in vitro* SGID are depicted in Figure 1.

156

157 *2.4. Heat damage assessment*

158

159 Furosine was determined according to ISO Standard 18329-2004. The method
160 developed by (Pellegrino, Resmini, De Noni, & Masotti, 1996), consisting of an HPLC
161 chromatographic analysis carried out after acid hydrolysis and derivatization with 9-
162 fluorenilmethyl-chloroformate (FMOC), was used to quantify LAL. PYR was determined by
163 adopting the procedure described by (Pellegrino, De Noni, & Cattaneo, 2000). Analyses were
164 performed in duplicate and mean values are reported.

165

166 *2.5.* In vitro *SGID of fortifiers, PDHM and fortified PDHM samples*

167

Digestion *in vitro* was performed simulating the gastrointestinal digestive conditions of preterm infants according protocols available so far (de Oliveira et al., 2016; Poquet & Wooster, 2016) adapted for static conditions. In detail, the samples, consisting of 10 mL of fortifiers dissolved in UF permeate, PDHM or fortified PDHM, were supplemented with 3 mL of simulated gastric fluid, pepsin (120 U mL^{-1}) and gastric lipase (8.6 U mL^{-1}). They were stirred at 37 °C for 3 h, varying the pH value by HCl addition as follows: 1^{st} h pH 6.0, 2^{nd} h pH 5.0, 3^{rd} h pH 4.0. Subsequently, the intestinal phase of the digestion was performed by adding 4 mL of simulated intestinal fluid, bile salts (1.6 mM), trypsin (395 BAEEU mL^{-1}) and pancreatic lipase (59 U mL^{-1}) to the gastric digest. The samples were then kept under stirring at 37 °C for 3 h at pH 7.0. After the intestinal phase, the digestates were immersed in ice and their pH was set to 5.5 by adding 6 N HCl. Finally, the volume of each sample was increased to 25 mL by adding MilliQ-treated water, and the samples were frozen at -24 °C. The enzymes were from Sigma-Aldrich (St. Louis, MO, USA).

2.6. Synthetic human and bovine BCMs

The synthetic hBCMs YPF (f51–53, hBCM3), YPFV (f51–54, hBCM4), YPFVE (f51–55, hBCM5), YPFVEP (f51–56, hBCM6), YPFVEPI (f51–57, hBCM7), YPFVEPIP (f51–58, hBCM8), YPFVEPIPY (f51–59, hBCM9), and bBCMs YPF (f60–62, bBCM3), YPFP (f60–63, bBCM4), YPFPG (f60–64, bBCM5), YPFPGP (f60–65, bBCM6) and YPFPGPI (f60–66, bBCM7) were purchased from GenScript (Piscataway, NJ, USA).

2.7. Identification and quantification of bBCMs and hBCMs by UPLC/HR-MS

Peptides were separated with an Acquity UPLC module (Waters, Milford, MA, USA) on an Aeris PEPTIDE XB-C18 column (150×2.1 mm, 1.7 µm) (Phenomenex, Torrance, CA, USA) kept at 40 °C. Eluents were: 0.1 mL 100 mL^{-1} formic acid (FA) in MilliQ-treated water (solvent A) and 0.1 mL 100 mL^{-1} FA in acetonitrile (solvent B). For the UPLC separation, a linear elution gradient was applied (14% to 28% of solvent B in 14 min) at a flow rate of 0.3 mL min^{-1}. The LC eluate was analysed by HR-MS on a Q Exactive instrument (Thermo Fisher Scientific, San Jose, CA, USA) interfaced through an HESI-II probe for electrospray ionization (Thermo Fisher Scientific). Targeted selected ion monitoring (t-SIM) and data dependent tandem MS analysis (ddMS^2) method with an inclusion list, containing hBCMs and bBCMs exact masses, was applied. The Xcalibur software (version 3.0, Thermo Fisher Scientific) was used for processing HR-MS data. Peak areas were calculated from extracted t-SIM chromatograms of target peptides (hBCMs and bBCMs) with a 3 ppm mass tolerance. Quantification was performed with an external standard 5-point calibration using the synthetic hBCMs and bBCMs peptides. Analyses were performed in triplicate and mean values ± SD were reported.

2.8. Statistical analysis

The significance of the results was analyzed by one-way analysis of variance followed by a Bonferroni *post hoc t* test with with the programming language for statistical computing R version 3.6.0 and the free and open-source integrated development environment RStudio Version 1.1.463 available at http://www.r-project.org. Differences of $P < 0.05$ were considered significant.

3. Results and discussion

3.1. Heat damage of fortifiers, PDHM and fortified PDHM samples

In the present work, three commercial fortifiers (NF, BF, PF) intended for fortification of PDHM for preterm infant nutrition were considered. The NF and BF fortifiers contained low quantity (20–25% g 100 g^{-1}) of proteins and high amount of carbohydrates as an energy supplement (Table 1). The PF contained (82% g 100 g^{-1}) CN and hydrolyzed whey proteins, and it was used to finely adjust the protein supply of PDHM once it has been fortified with NF or BF. The amount of PF added to PDHM was much lower than that of NF and BF fortifiers to comply with the osmolality threshold (400 mOsm L^{-1}). The manufacturing process of fortifiers usually includes severe heat treatments that ensure microbiological safety of the final product, but also can translate in glycation (when sugars are present) and cross-linking phenomena involving the hydrolyzed protein components. Based on this, we assessed the heat damage of the studied fortifiers by determining FUR, PYR and LAL levels. As a control, the same analytical indexes were firstly determined in HM prior to and after Holder pasteurization (Table 2). The Holder treatment determined an increase (about 30%) of the FUR level in PDHM in comparison to the unprocessed sample. As expected for Holder treatment, LAL and PYR levels of PDHM were under the limit of detection, both in unprocessed and Holder-pasteurized samples. NF and BF fortifiers contained two times more FUR than the PF fortifier (Table 2). Indeed, PF sample was richer in proteins, but poorer in reducing sugars (i.e. maltodextrins and lactose) than NF and BF fortifiers (Table 1). All the fortifiers showed FUR levels in lowest range of values reported for liquid infant formulas addressed to nutrition of term infants (Cattaneo, Masotti, & Pellegrino, 2009). Overall, fortification contributed to about 90% of the final FUR level of PDHM, even if less than 5 g 100 g^{-1} of fortifiers were added to PDHM (Table 2). The absence (or low levels) of PYR in the studied fortifiers can be explained by a low extent of the advanced stage of MR. These levels were in the range (traces–12.2 mg kg^{-1}) reported by (Hellwig & Henle, 2012) for

242 milk and whey powders. The highest levels of LAL were revealed in BF and PF samples, which
243 contained CN (Table 2), from which LAL mainly arises (Maga, 1984). These LAL levels were
244 similar to those reported for UHT milk, but lower than those characterizing liquid infant
245 formulas (Cattaneo et al., 2009). The final LAL and FUR levels of fortified PDHM were
246 consequently determined by both the type of fortifier and the adopted fortification mode
247 (Table 2).

248

249 **Table 2**

250 Contents (mg 100 g^{-1} protein) of furosine (FUR), lysinoalanine (LAL) and pyrraline (PYR) in donor
251 human milk (DHM), pasteurized DHM (PDHM), fortifiers (BF, NF and PF) and fortified PDHM; nd,
252 not detected, below 0.2 mg 100 g^{-1} protein.

Sample	FUR	LAL	PYR
DHM	13	nd	nd
PDHM	17	nd	nd
BF	220	8.1	0.81
NF	203	1.6	0.75
PF	120	8.9	nd
PDHM fortified with BF and PF	115	5.1	nd
PDHM fortified with NF and PF	111	3.3	nd

253

254 No data concerning the levels of the studied heat indexes are available for fortifiers and
255 fortified PDHM. Despite this, different authors recommended the content of LAL in infant
256 formulas to be monitored and limited due to the implication of this compound in the
257 decreasing of protein digestibility (Sarwar Gilani, Wu Xiao, & Cockell, 2012) and in the onset of
258 nephrocytomegaly in rats (Friedman, 1999). Nonetheless, the effect of LAL on renal function of
259 premature infants fed with infant formulas was not directly associated with significant
260 physiological deficits, but a role of LAL and MR products in the impairment of kidney tubules
261 cannot be disregarded (Langhendries et al., 1992). However, these results are to be interpreted
262 in the light of the short period of administration of infant formulas to tested subjects and,

therefore, to the impossibility of establishing the effects of LAL on the kidneys at a chronic level, as demonstrated by (Finot, Mottu, Bujard, & Mauron, 1978). Anyway, the response of premature infants to LAL exposure has not been elucidated yet.

Overall, the highlighted numbers evidenced the negligible effect of Holder pasteurization in promoting the heat damage. Contrarily, protein supplementation highly affected the final heat damage of the fortified PDHM. It is worth noting that about half of protein nitrogen in fortified PDHM is ensured by fortification. As demonstrated in the present work, protein/peptide fraction is characterized by severe chemical modifications, which should be taken into account as an additional parameter to assess the overall nutritional properties of fortified PDHM.

3.2. Occurrence of bBCMs in (un)digested fortifiers for PDHM

The enzymatic hydrolysis of milk proteins can potentially release BAPs during manufacturing of fortifiers. For this reason, the undigested fortifiers were assessed for the presence of bBCMs. To this purpose, the three fortifiers were firstly dissolved in UF permeate of PDHM. This experimental approach was addressed to have a matrix background, and hence an analytical "noise" during UPLC/HR-MS, as similar as possible to that of fortified PDHM. Moreover, the use of UF permeate guaranteed a comparable digestion environment during further *in vitro* SGID.

Before dissolution of fortifiers, bBCMs 3 to 7 were searched in the UF permeate. They were not revealed by UPLC/HR-MS (Table 3). Subsequently, the three fortifiers were dissolved in PDHM permeate and subjected to the same analysis. The undigested BF and PF samples contained all searched bBCMs, with the exception of bBCM5 (Table 3). The revealed amounts were quite similar in the two fortifiers, being the bBCM6 the most abundant in both samples.

Only the bBCM7 was found in undigested NF, and its presence was likely related to the hydrolysis of residual proteose peptones normally present in ultrafiltered whey proteins. These proteoses derive from the plasminolysis of bovine β CN naturally occurring in the mammary gland and during milk storage. Similarly, (Fiedorowicz et al., 2016) found 33 μg L^{-1} of bBCM7 in an undigested liquid infant formula, for term infants, exclusively made from hydrolyzed bovine whey proteins.

To the best of our knowledge, no research studies regarding the identification and quantitation of bBCMs in undigested commercial milk fortifiers are available. The presence of bBCM7 in HM (and hence in UF permeate) has been reported by (Jarmołowska et al., 2007) and (Fiedorowicz et al., 2016) at 0–2.8 mg L^{-1} and 0.6–0.7 μg L^{-1}, respectively. Moreover, (Jarmołowska et al., 2007) found bBCM5 up to 10.6 mg L^{-1} in the same HM samples. According to (Fiedorowicz et al., 2016), the presence of bBCMs in HM was the result of their transfer from mother's sera (blood) into HM. In this regard, (Kost et al., 2009) found immunoreactive substances against bBCM7 in blood plasma of breast-fed infants.

As expected, no bBCMs 3 to 7 were revealed in the digestate of UF permeate from PDHM (Table 3). The fortifiers dissolved in permeate were then submitted to *in vitro* SGID. In the BF and PF digestates, the levels of bBCMs 3, 4 and 6 were approximately similar to the undigested counterparts, whereas a great increase of bBCM7 was observed after *in vitro* SGID. The highest quantity of this peptide was found in PF digestate. The bBCM7 was also revealed in digested NF with a slight increase as compared to the undigested sample. A similar amount (0.31 mg L^{-1}) of bBCM7 was found in the *in vitro* digestate of a liquid formula for term infant based on hydrolyzed whey protein by (Fiedorowicz et al., 2016). The different release of bBCM7 in digestates of BF and PF was likely the result of the diverse CN content of the two fortifiers, but it also could be affected by the ratio among the different genetic variants of β CN present in the same samples. Indeed, it has been demonstrated that low (or unquantifiable) amount of

bBCM7 releases during enzymatic hydrolysis of the A2 genetic variant of β CN in comparison to the A1 type (De Noni, 2008). The A1 and A2 are the most widespread β CN variants among Holstein Friesian cows and, likely, they characterized the starting milk used for the manufacturing of the studied fortifiers. However, it was not possible to ascertain the type and ratio of β CN variants present in fortifiers, as the intact CNs were not revealed by both SDS-PAGE and HPLC analyses (data not shown).

Table 3

Levels (mg L^{-1}) of bBCMs in UF permeate from PDHM and in fortifiers (dissolved in UF permeate from PDHM) undigested and after *in vitro* SGID. Values are presented as means ± SD (n = 3); nd, not detected. Letters indicate differences ($P < 0.05$) among fortifiers in UF permeate, for each bBCM.

Sample		bBCM3	bBCM4	bBCM5	bBCM6	bBCM7
UF permeate from PDHM	undigested	nd	nd	nd	nd	nd
	digested	nd	nd	nd	nd	nd
NF in UF permeate	undigested	nd	nd	nd	nd	0.16 ± 0.03^{e}
	digested	nd	nd	nd	nd	0.28 ± 0.05^{e}
BMF in UF permeate	undigested	0.38 ± 0.05^{a}	0.47 ± 0.07^{b}	nd	1.47 ± 0.17^{d}	0.28 ± 0.05^{e}
	digested	0.46 ± 0.05^{a}	0.46 ± 0.06^{b}	0.02 ± 0.01^{c}	1.31 ± 0.12^{d}	4.59 ± 0.16^{f}
PEX in UF permeate	undigested	0.36 ± 0.02^{a}	0.48 ± 0.03^{b}	nd	1.85 ± 0.13^{d}	0.33 ± 0.06^{e}
	digested	0.38 ± 0.06^{a}	0.46 ± 0.10^{b}	0.02 ± 0.01^{c}	1.67 ± 0.14^{d}	6.28 ± 0.06^{g}

3.3. Occurrence of hBCMs and bBCMs in (un)digested PDHM and fortified PDHM

Human BCMs possess a structure similar to bBCMs and, in particular, hBCM3 and bBCM3 share the same aminoacidic sequence. Human BCMs encompass the structural requirements to exert opioid-like activity, i.e. the presence of a Tyr residue at the N-terminus followed by another aromatic residue at the third or fourth position of the peptide sequence. In fact, the investigations on the potential biological action of hBCMs paralleled those regarding

bBCMs and date back to the research studies of (Brantl, 1984) and (Koch et al., 1985), who demonstrated the opioid activity of hBCMs 4, 5, 7 and 8. To date, other hBCMs such as hBCM 9 to 11 are hypothesized to play biological functions as intact peptides or as precursors of the most studied hBCMs 4, 5, 7 and 8 (Hernández-Ledesma, Quirós, Amigo, & Recio, 2007; Tsopmo et al., 2011).

In the present work, no hBCMs were detected in undigested PDHM sample and in the derived UF permeate (Table 4). Differently, other studies identified hBCMs 4, 5, 7 and 8 as possible endogenous peptides in HM as the result of the enzymatic breakdown of β CN in the human mammary gland (Nielsen, Beverly, & Dallas, 2017). In this regard, the plasmin activity in preterm milk has been reported to be higher than in term milk (Armaforte et al., 2010). More recently, (Deglaire et al., 2019) confirmed plasmin to be the most active proteolytic enzyme in undigested PDHM. Consequently, the presence of hBCMs in undigested HM can be related to the endogenous lysis of β CN by plasmin in conjunction with the activity of endopeptidase and/or exopeptidases. Nonetheless, (Ferranti et al., 2004) reported in HM only the presence (not the amount) of hBCM8, despite several precursors of hBCMs were revealed. (Enjapoori et al., 2019) identified (but not quantified) hBCMs 8 to 11 in both preterm and term undigested HM samples. Precursors of hBCMs along with hBCM9 were also found by (Wada & Lönnerdal, 2015) in undigested HM without any quantitation of this peptide. Overall, the few literature data support the potential presence/absence of hBCMs in undigested HM. However, the physiological factors affecting their occurrence and fate in HM are still far to be fully explained.

Following *in vitro* SGID, hBCMs 3, 7, 8 and 9 were released in PDHM (Table 4). As reported by (Deglaire et al., 2019), most of the peptides originated during *in vitro* dynamic SGID of PDHM, mimicking the preterm digestive conditions, were released from β-CN. As previously mentioned, bovine and human BCM3 have the same primary sequence. Our data showed that SGID of fortified PDHM released roughly the same amount of BCM3 found in digested PDHM.

Based on this, it can be argued that the human β CN was the main protein precursor of the released BCM3 also in the fortified PDHM samples. To date, the BCM3 unlikely seems to act as an opioid peptide (Lister, Fletcher, Nobrega, & Remington, 2015). The hBCMs 4, 5 and 6 were not revealed, while the forms 7, 8 and 9 released in the digestates of (fortified) PDHM (Table 4). As found for undigested HM, (Wada & Lönnerdal, 2015) identified hBCM9 in the *in vitro* digested counterpart and suggested this peptide to be an opioidergic peptide like bBCMs. They also found truncated forms (51–59, 52–59, 53–59 and 54–59) of hBCM9, which can potentially elicit immunomodulation (peptide 54–59) (Migliore-Samour & Jollès, 1988) or, as evidenced for hBCM9, inhibit prolyil endopeptidase (peptides 52–59, 53–59, and 56–59) (Asano, Nio, & Ariyoshi, 1991). Recently, (Deglaire et al., 2019) identified the peptide 54–59 in an *in vitro* digested PDHM. In the same digestate, the hBCM8 was also revealed. (Righard, Carlsson-Jonsson, & Nyberg, 2014), using a radioimmunoassay, identified hBCM8 in plasma and milk of lactating women at femto- and pico-molar ranges, respectively.

The highest total amounts of hBCMs characterized PDHM fortified with BF and PF. Overall, the amounts of hBCMs 3, 7 and 8 were higher in PDHM fortified with BF than in the PDHM fortified with NF. In particular, the content of hBCM7 in PDHM fortified with BF almost doubled those of PDHM and PDHM fortified with NF and PF. (Brantl, 1984) and (Koch et al., 1985) showed synthetic hBCM 7 and 8 to display *in vitro* opioid activity in the guinea-pig ileum longitudinal muscle/myenteric plexus. In particular, hBCM8 was two times more active than hBCM7, and both hBCMs were about five time less effective than the bovine counterparts. (Brantl, 1984) and (Koch et al., 1985) also studied the affinity of the same BCMs for the different opioid receptor (μ, λ and κ) in rat brain homogenates. The hBCM7 and hBCM8 especially bound MOR, with less affinity than bBCMs. It is also worth noting that, as reported by (Duraffourd et al., 2012), hBCM7 has been shown to regulate intestinal gluconeogenesis and satiety in rats and mice. In detail, the authors found that hBCM7 infused in animals acted as an

agonist of MOR of the mesenteric-portal area and determined an increase in food intake. Indeed, the gut-brain neural circuit for controlling intestinal gluconeogenesis, and hence food intake, is regulated by MOR. In this regard, (Kost et al., 2009) found immunoreactive molecules against the hBCM7 in the plasma of children (< 1 year) fed mother's milk.

Table 4

Levels (mg L^{-1}) of hBCMs in UF permeate, PDHM and fortified PDHM. Values are presented as means ± SD (n = 3); nd, not detected. * Bovine and human BCM3 have the same primary sequence. # represents a statistical difference ($P < 0.05$) in comparison to digested PDHM; letters indicate differences ($P < 0.05$) between PDHM fortified with NF and PF and PDHM fortified with BF and PF, for each hBCM.

Sample		h/bBCM3*	hBCM4	hBCM5	hBCM6	hBCM7	hBCM8	hBCM9
UF permeate from PDHM	undigested	nd	nd	nd	nd	nd	nd	nd
	digested	nd	nd	nd	nd	nd	nd	nd
PDHM	undigested	nd	nd	nd	nd	nd	nd	nd
	digested	3.46±0.02	nd	nd	nd	0.74±0.01	2.46±0.01	2.69±0.16
PDHM fortified with NF and PEX	digested	2.96±0.12[a]	nd	nd	nd	0.65±0.11[c]	0.50±0.07[#e]	2.42±0.18[g]
PDHM fortified with BMF and PEX	digested	3.69±0.16[b]	nd	nd	nd	1.37±0.10[#d]	1.33±0.17[#f]	3.70±0.72[h#]

Bovine BCMs were not found in PDHM, despite Duarte-Vasquez and co-workers found about 6.9 µg L^{-1} of bBCM7 in HM (Duarte-Vázquez, García-Ugalde, Villegas-Gutiérrez, García-Almendárez, & Rosado, 2017) (Table 5). Bovine BCMs released during SGID and their amounts in digested fortified PDHM were lower than those recorded in SGID of the single fortifiers dissolved in UF permeate (Tables 3 and 5). This finding was due to the low total protein content of UF permeate that implies a higher enzyme-to-protein ratio during *in vitro* SGID of fortifiers. Overall, the amount of bBCMs released upon digestion depended on the bovine CN content of the fortifiers. The fortification with PF, to adjust the protein content to the target value, led to

403 the release of additional bBCMs in the digestate of PHDM fortified with the CN-free NF. The
404 bBCM7 contents of digested PDHM fortified with BF or NF was almost similar due to the
405 simultaneous fortification with PF, which attenuated the difference in the peptide content
406 observed in the digestate of NF and BF fortifiers alone (Table 3). Additionally, the presence of
407 PF reduced the potential influence on bBCM7 release exerted by the possible diverse CN
408 genotype of the two other fortifiers.

409 To the best of our knowledge, the release and the amount of bBCMs in fortifiers and/or
410 fortified HM has not been reported in literature yet. For this reason, the results of the present
411 study can be compared only with data from literature concerning the release of bBCMs during
412 digestion of HM or infant formulas. These studies were conducted using different digestion
413 protocols not tailored for mimicking preterm infant digestion. Moreover, most of them focused
414 only on the release of bBCM7, which has been considered since a long time as the most
415 biologically active among bBCMs. In this regard, using only pepsin and pancreatin as digestive
416 enzymes, (Fiedorowicz et al., 2016) studied the release of bBCM7 in digestates of infant
417 formulas. They reported the release of 0.31–4.79 mg L^{-1} of bBCM7 in digestates, i.e. values
418 overlapping those found in the present study. (De Noni, 2008) evaluated the release of bBCM 5
419 and bBCM7 during SGID of commercial infant formulas. He did not adopt an *in vitro* digestion
420 protocol intended for infants, but he took into account diverse pH values (2–4) during the
421 gastric phase, in order to consider the physiological conditions of infant's stomach. Despite of
422 the pH value, no BCM5 formed at end of the intestinal phase of SGID, while 0.02–0.29 mg L^{-1} of
423 bBCM7 were found. These amounts are lower than those highlighted in the present work, likely
424 as a consequence of the strong activity of pepsin and intestinal proteolytic enzyme preparation
425 used for SGID by (De Noni, 2008). Indeed, in the present work higher pH values (4–6) for the
426 gastric step and reduced enzyme activities were adopted for both gastric and intestinal
427 digestion phases.

To the best of our knowledge, to date no information is available concerning the potential biological role of hBCMs and/or bBCMs in preterm infants. Similarly, a minimum amount of h/bBCM7 capable to exert physiological effects has not been established. According to (Jarmołowska, Kostyra, Krawczuk, & Kostyra, 1999), 0.05% bBCM7 could potentially cause intestinal contractions in human. In the present work, given the amount of bBCM7 found in fortified PDHM digestate and the volume (135–200 mL kg^{-1} d^{-1}) of fortified PDHM ingested by preterm infant, this (and likely others) biological activity would be excluded. Nonetheless, the potential biological effects of fortified PDHM in preterm nutrition deserve further investigations.

Table 5

Levels (mg L^{-1}) of bBCMs in undigested PDHM and digested fortified PDHM. Values are presented as means ± SD (n = 3); nd, not detected. Letters indicate differences ($P < 0.05$) between PDHM fortified with NF and PF and PDHM fortified with BF and PF, for each bBCM.

Sample		h/bBCM3	bBCM4	bBCM5	bBCM6	bBCM7
PDHM	undigested	nd	nd	nd	nd	nd
	digested	3.46 ± 0.02	nd	nd	nd	nd
PDHM fortified with NF and PEX	digested	2.96 ± 0.12[a]	0.18 ± 0.03[c]	nd	0.37 ± 0.04[e]	1.40 ± 0.12[g]
PDHM fortified with BMF and PEX	digested	3.69 ± 0.16[b]	0.68 ± 0.04[d]	nd	1.07 ± 0.08[f]	1.51 ± 0.08[g]

4. Conclusions

To conclude, the present study demonstrated that fortifiers accounted for the overall heat damage and for the occurrence of bBCMs in fortified (digested) PDHM. To control these phenomena, heat treatment and enzymatic hydrolysis of fortifiers should encompass low thermal damage and controlled release of particular (bioactive) peptides. The present results

also showed that hBCMs were released during SGID. According to literature, some hBCMs can potentially elicit biological activities, which could enforce and/or counteract those potentially exerted by bBCMs deriving from hydrolyzed fortifiers themselves and/or from their digestion.

To the best of our knowledge, the present study is the first investigation dealing with the chemical characterization of the fortifiers used in the preparation of HM for preterm infants. Moreover, it is the first research study providing quantitative data regarding the occurrence of both human and bovine BCMs in (digested) fortified PDHM. Despite the role played by the studied chemical protein artifacts and BCMs in preterm nutrition is far to be elucidated, and so far even poorly investigated, these (quantitative) data could better address further studies for ascertaining the nutritional features of protein fortifiers and fortified PDHM in preterm nutrition. In this regard, it will be paramount to implement *in vitro* SGID models that more in-depth mimic *in vivo* conditions of the digestive tract of premature infants. Anyway, the obtained results bring additional knowledge into the field of preterm infant nutrition and provide supplementary evidence for the manufacturing of more tailored protein fortifiers for PDHM.

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Conflict of interest

The authors declare no conflict of interest.

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604 **Figure caption**

605

606 **Fig. 1.** Preparation scheme of samples submitted to *in vitro* SGID.

Figure(s)
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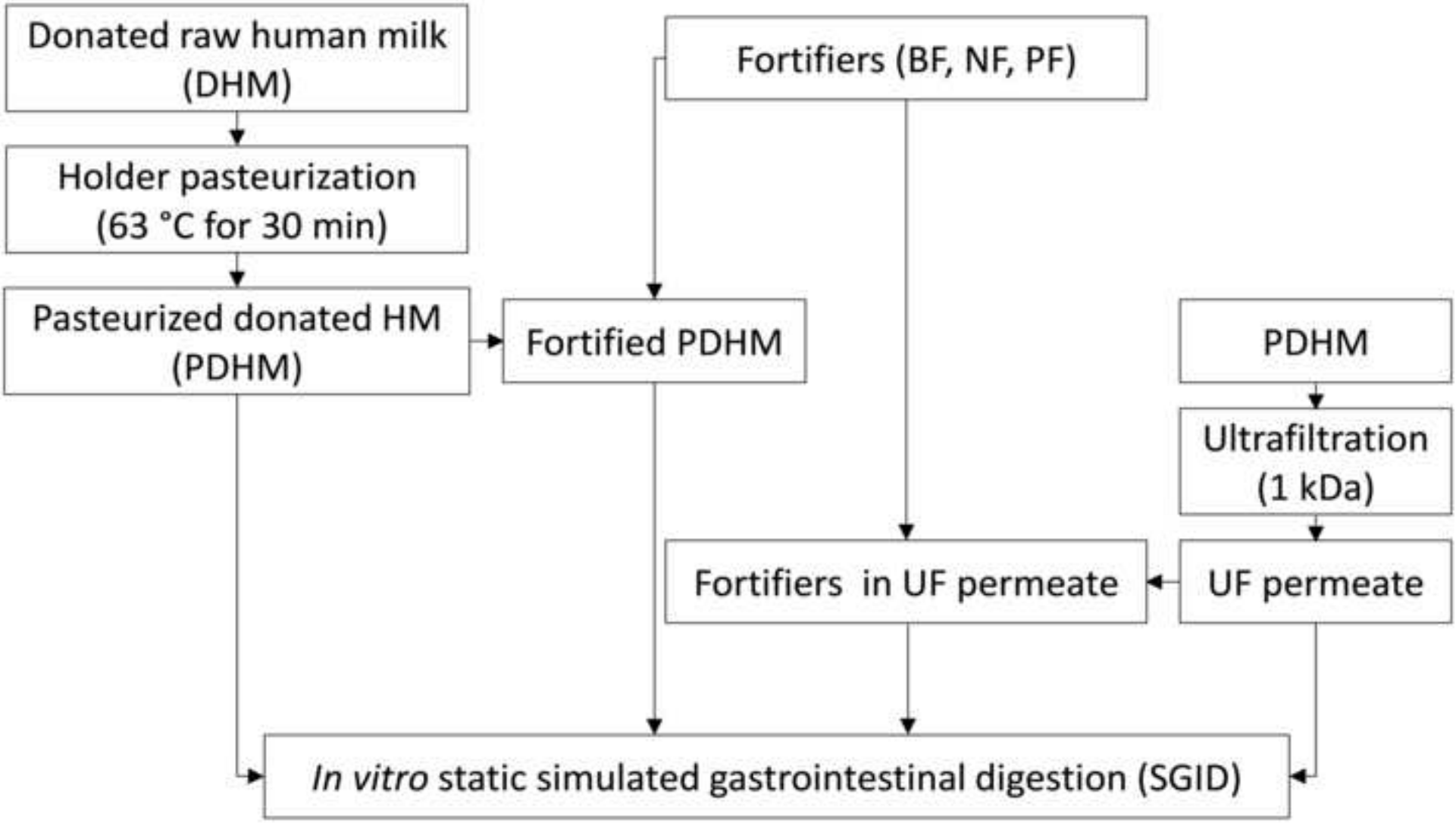

Fig. 1.

***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: