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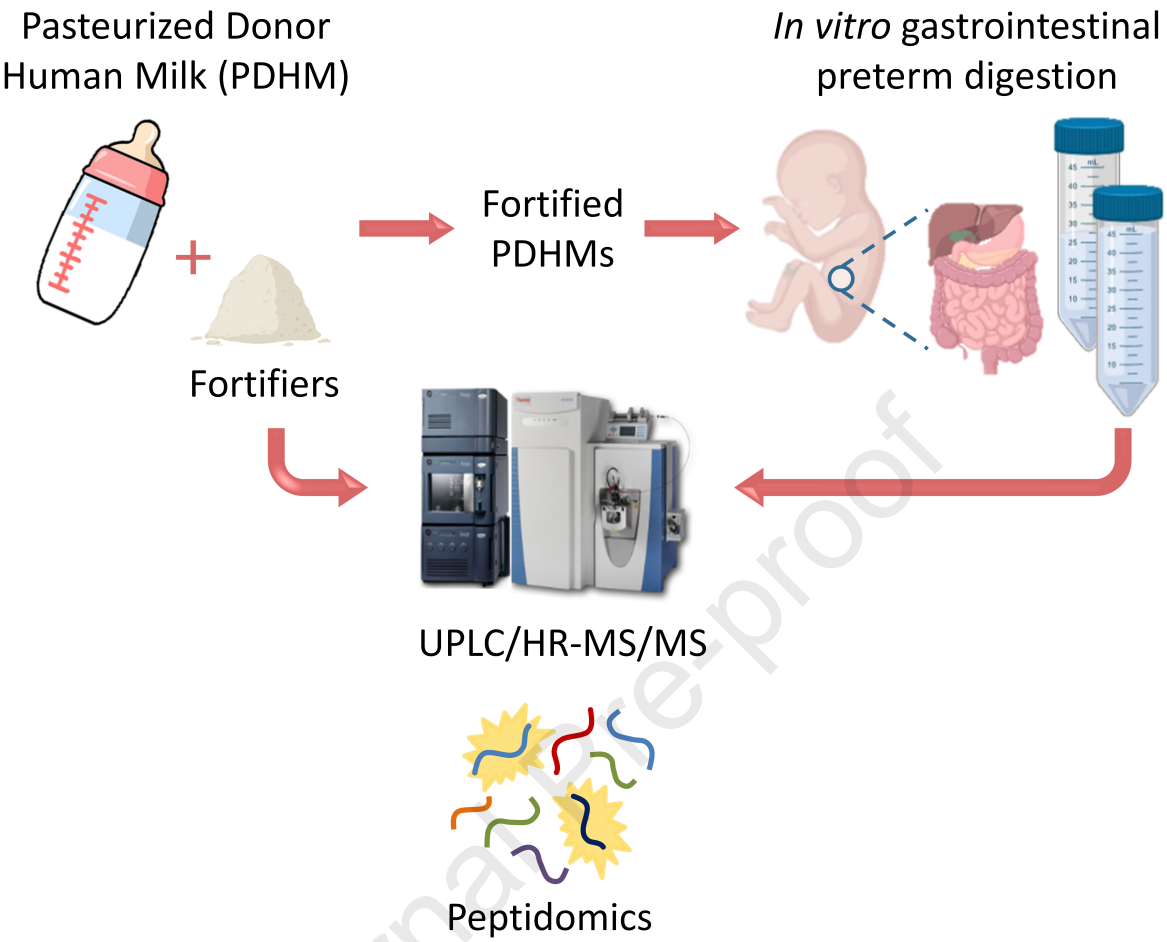

Bovine milk fortifiers and fortified pasteurized donor human milk for premature infant nutrition. Peptidomic overview

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ABSTRACT

Human milk (HM) is the best source of nourishment for infants, but it must be fortified for optimal growth of premature babies. Hydrolyzates of bovine milk proteins are used for fortification of pasteurized donor HM (PDHM). In the present work, peptidomic approach was applied to characterize three commercial fortifiers. These products, intended for premature infant nutrition, are commonly used in neonatal intensive care units. Besides intact fortifiers and PDHM, digests of (un)fortified PDHMs (after preterm *in vitro* static gastrointestinal digestion, SGID) were investigated. Specifically, 186 unique peptides were revealed among the three fortifiers, 21 potential bioactive peptides (BPs)

among them. After SGID of fortified PDHMs, 515 unique peptides were identified. They originated mostly from human β-casein; 15 were homologous to potential BPs of human and bovine milk protein origin. This work represents one of the few peptidomic investigations on fortifiers and fortified PDHM after a simulated preterm infant digestion.

Keywords: human milk; preterm; fortifier; peptidomics; high-resolution mass spectrometry

Abbreviations

HM, Human milk; PDHM, Pasteurized donor human milk; BPs, Bioactive peptides; MBPDB, Milk Bioactive Peptide Database; SGID, *In vitro* static gastrointestinal digestion; CN, Casein; α-LA, α-lactalbumin; β-LG, β-lactoglobulin; ACE, Angiotensin-converting enzyme

1. Introduction

Human milk (HM) is the best source of nourishment for term infant, providing health benefits both in the short and long-term (Victora et al., 2016). Infants born prematurely (gestational age < 37 weeks) and with birth weight less than 1500g (very low birth weight infants) require special attention while fed with the usual feeding volume, to reduce birth-related complications and under-nutrition. HM remains the best source of nutrition even for preterm babies. Pasteurized donor human milk (PDHM), from term delivering mothers, represents a proper alternative, when mother's own milk is not available. Nonetheless, HM does not contain enough nutrients to satisfy the special nutritional requirement of preterm infants, especially proteins, hence fortification is needed for optimal growth. Nutrient requirements are defined as intakes that enable the infant to grow at the same rate as the

fetus (Ziegler, 2014). Therefore, HM is typically fortified to meet protein needs, in the range of 3.1 to 4.1g protein $100kcal^{-1}$ for very low birth weight infants (Arslanoglu et al., 2019). "Standard fortification" is the most applied method and consists of a standard amount of fortifier added to HM, according to the manufacturer's instructions (Arslanoglu et al., 2019).

Available fortifiers differ considerably by nutrient composition (multi-nutrient fortifiers or supplements of proteins, lipids, carbohydrates) and by the origin of milk used. To date, most of the commercially available fortifiers derive from bovine milk, except for the one derived from HM (Prolacta Bioscience, Duarte, CA, USA). A donkey milk-derived fortifier is under experimental evaluation (Bertino et al., 2019; Coscia et al., 2018). Moreover, powdered fortifiers are mostly used as compared to liquid fortifiers, to avoid dilution of HM (Ziegler, 2014). Commercial fortifiers could be partially or extensively hydrolyzed, to reduce the antigenicity and allergenicity of milk proteins, as well as to improve their digestibility. They could also be made of intact milk proteins (Sherriff & McLeod, 2013). The degree of hydrolysis and the composition of peptides formed also depend from the proprietary manufacturing conditions such as type of protease(s), temperature, hydrolysis time, enzyme/protein ratio (Bu, Luo, Chen, Liu, & Zhu, 2013). Consequently, industrial hydrolysis may modify the digestive trajectory of milk proteins/peptides in the gastrointestinal tract in different ways.

To the best of our knowledge, the peptidomic profile of bovine milk fortifiers alone has been investigated only by (Nielsen, Beverly, Underwood, & Dallas, 2018). However, the authors limited their research to a single fortifier without specifying its composition, casein-to-whey protein ratio, degree of hydrolysis, and information regarding extent/frequency of use. Nowadays, this topic should be considered and deepened for actually and widely used fortifiers especially because these products are addressed to a very risky population. Moreover, a deep insight could also demonstrate the presence of bioactive peptides (BPs), which could affect the biological value of fortified HM. (Beverly,

Underwood, & Dallas, 2019) ascertained the peptidomic profile of (un)fortified HM from preterm-delivering mothers and of gastric samples of related preterm infants. Nonetheless, they did not focus neither on the peptidomics of fortifiers alone nor on the intestinal phase, during which further hydrolysis of peptides occurs. Both, human and bovine milk proteins encrypt a variety of BPs (Nielsen, Beverly, Qu, & Dallas, 2017). Some could be potentially present in the undigested fortifiers as a result of the hydrolysis process. Therefore, it is likely that infants fed fortified PDHM will be influenced also by bovine milk protein-derived BPs, differently from those fed HM only. Moreover, BPs could remain intact due to the low proteolytic activity elicited in the gastrointestinal tract of preterm infants (Shani-Levi et al., 2017). To the best of our knowledge, little is known about the peptide composition of fortifiers addressed to HM supplementation. Only few studies investigated the fate of the fortified HM during an *in vivo* gastric digestion in preterm infants (Beverly et al., 2019; Demers-Mathieu, Qu, Underwood, & Dallas, 2018; Nielsen et al., 2018). Moreover, only a single study referred to preterm gastrointestinal digestion *in vitro* (Cattaneo et al., 2020).

On these bases, exploiting the technology of UPLC/HR-MS/MS, this work aimed to: (i) characterize peptidomic profile of targeted commercial fortifiers commonly used for premature infant nutrition in neonatal intensive care units; (ii) determine peptidomic profile of fortified PDHMs, after a preterm *in vitro* static gastrointestinal digestion (SGID); (iii) search for BPs in fortifiers and in digests of the fortified PDHMs.

2. Materials and methods

2.1. Pasteurized donor human milk (PDHM)

A total of 20L of PDHM from term-delivering mothers was collected by Donor Human Milk Bank of "Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico" (IRCCS) (Milan, Italy). The study was approved by the Ethics Committee of the same institution [Approval No. 289-2017]. Lactating women (healthy donors) enrolled for the present study signed the informed consent. HM samples were collected using an electrical breast pump, Holder pasteurized (63°C for 30min) and stored at -24°C until the day of fortification and analysis. Protein content of the pooled PDHM was 1.3g $100mL^{-1}$.

2.2. Fortifiers of PDHM

Three commercial powdered fortifiers from bovine milk were provided and daily used by the Donor Human Milk Bank at IRCCS for "Standard fortification" of PDHM. They were the Aptamil BMF and Aptamil ProExpert PS fortifiers from Nutricia (Danone, Paris, France) and the FM 85 fortifier from Nestlé (Nestlé, Vevey, Switzerland). Hereafter they are named as BF, PF and NF, respectively. Table 1 reports the protein composition of BF, PF and NF, as declared on their labels. The three fortifier samples were prepared for analyses dissolving 5.47g of BF, 1.67g of PF or 6.83g NF, respectively, in 100mL of permeate deriving from ultrafiltration (UF) of PDHM. The permeate was previously prepared using an Amicon stirred cell 8050 (Merck, Darmstadt, Germany) equipped with an Ultracel (Merck) membrane (cut-off 1kDa). Each sample of dissolved fortifier presented the same final protein content. The same amount of bovine milk proteins was also present in fortified PDHM samples (described subsequently, subsection 2.3.).

2.3. Fortification of PDHM

116 To achieve the target protein content of 4.1g 100kcal^{-1}, two different fortification models were
117 established, using BF or NF as the main fortifiers and PF for protein fine adjustment. The 4.1g 100kcal^{-1}
118 threshold was set according to the nutritional program adopted at the IRCCS. In detail, 4.40g BF and
119 0.24g PF, or 5.00g NF and 0.36g PF were dissolved in 100mL of PDHM, previously thawed in the
120 refrigerator at 4°C. The two fortification modalities (BF+PF or NF+PF) of PDHM fortification reflect
121 those adopted at the neonatal intensive care unit and Donor Human Milk Bank at the IRCCS.
122
123 *2.4.* In vitro *digestion of PDHM and fortified PDHM samples*
124
125 An in-house digestion protocol was developed according to literature data (Bourlieu et al.,
126 2014; de Oliveira et al., 2016; Poquet & Wooster, 2016), and recently published by (Cattaneo et al.,
127 2020). Briefly, 10mL of fortifiers dissolved in PDHM permeate, PDHM or fortified PDHMs were
128 digested. The *in vitro* SGID of preterm infants was carried out using 3mL of simulated gastric fluid,
129 pepsin (120U mL^{-1}) and gastric lipase (8.6U mL^{-1}) for the gastric phase and 4mL of simulated intestinal
130 fluid, bile salts (1.6mM), trypsin (395BAEEU mL^{-1}) and pancreatic lipase (59U mL^{-1}) for the intestinal
131 phase. Samples were incubated, under stirring at 37°C, for 3h per phase, adjusting the pH value after
132 each hour for the gastric phase (1sth pH6.0, 2ndh pH5.0, 3rdh pH4.0, using HCl) and setting to pH7.0 for
133 the intestinal one. Subsequently, the digests were cooled on ice, and their pH was set to 5.5 with 6N
134 HCl. Finally, each sample (final volume of 25mL adjusted with MilliQ-treated water), was ultrafiltered
135 using an Omega modified polyethersulfone UF membrane (cut-off 10kDa) in a Nanosep Advance
136 device (Pall, Port Washington, NY, USA) and frozen at -24°C. The enzymes were from Sigma-Aldrich
137 (St. Louis, MO, USA).
138

139 *2.5. UPLC/HR-MS/MS analysis*

140

141 Five µL of 10-kDa-ultrafiltered samples were separated with an Acquity UPLC module (Waters,
142 Milford, MA, USA) on an Aeris PEPTIDE XB-C18 column (150×2.1mm, 1.7µm) (Phenomenex, Torrance,
143 CA, USA) kept at 50°C. Eluents were: 0.1mL 100mL^{-1} formic acid (FA) in MilliQ-treated water (solvent
144 A) and 0.1mL 100mL^{-1} FA in acetonitrile (solvent B). For the UPLC separation, a linear elution gradient
145 was applied (2% to 55% of solvent B in 35min) at a flow rate of 0.3mL min^{-1}. The LC eluate was
146 analysed by HR-MS/MS on a Q Exactive instrument (Thermo Fisher Scientific, San Jose, CA, USA)
147 interfaced through a HESI-II probe for electrospray ionization (Thermo Fisher Scientific). The ion
148 source and interface conditions were set as previously reported (Cattaneo et al., 2020). The LC eluate
149 was analyzed by MS using full scan and data dependent tandem MS analysis (ddMS^2) of the ten most
150 intense ions (Top10). Mass spectra were acquired over m/z range from 100–1500; the ten most
151 intense 1^+–8^+-charged ions detected in each spectrum underwent HCD fragmentation (ddMS^2, data
152 dependent scan acquisition mode). The resolution was set at 70000 and 17500 for full scan and
153 ddMS^2 scan types, respectively. The AGC targets were 1×10^5, and maximum ion injection times were
154 110ms.

155

156 *2.6. Peptide identification and peptidomic data analysis*

157

158 Mass spectrometry data were processed, and peptides were identified using the Proteome
159 Discoverer 1.4 software (Thermo Fisher Scientific) and indications of (Dallas & Nielsen, 2018; Nielsen
160 et al., 2018). Automatic peak detection was performed with a setting of signal-to-noise ratio of 4 as
161 suggested by (Mangé, Bellet, Tuaillon, Van de Perre, & Solassol, 2008). Peptide sequences were

identified from MS/MS spectra using SequestHT against the HM protein library created by Dallas and coworkers (available at www.dallaslab.org), and the database of *Bos taurus* (UniProt taxon ID 9913) considering their genetic variants (Farrell et al., 2004). A non-specific enzyme cleavage pattern was defined, and 12 missed cleavage sites (maximum allowed for the algorithm) were set. Phosphorylation of serine and threonine, deamidation of asparagine, glutamine and arginine, oxidation of methionine and cyclisation of an N-terminal glutamine to pyro-glutamic acid were selected as dynamic modifications. Mass error tolerance for precursor ions was 5ppm and for fragment ions was 0.02Da. A strict false discovery rate of peptide identification was set (FDR=0.01). Each sample was digested in duplicate, and two injections of each digest were performed during UPLC/HR-MS/MS analysis. Undigested samples were acquired in duplicate. Acquisitions were processed with Proteome Discoverer 1.4 software by merging the analyses data outputs, in order to obtain multiconsensus reports with an absolute number of peptides that was found in each sample.

Peptides identified in all samples were examined for homology using the Milk Bioactive Peptide Database (MBPDB, http://mbpdb.nws.oregonstate.edu/ accessed October 2019) (Nielsen et al., 2017), a comprehensive collection of milk BPs reported in literature. The search was performed setting the following parameters: “100” for similarity threshold, “identity” for amino acid scoring matrix and “yes” for getting extra output.

3. Results and discussion

3.1. Peptidomic profile of undigested fortifiers BF, PF and NF

The selected fortifiers, BF, PF and NF, had a different protein composition (Table 1). As declared on their labels, both BF and PF contained hydrolyzed casein (CN) and hydrolyzed whey proteins, while only whey protein hydrolyzate was present in NF. Preliminary SDS-PAGE did not reveal any intact proteins in all the fortifiers (data not shown). The powders were firstly dissolved in HM permeate to simulate the matrix effect and the analytical noise during the UPLC/HR-MS/MS of fortified PDHMs. Peptidomic profile of undigested samples was firstly investigated (Fig. S1). A different number of peptides characterized undigested BF, PF and NF. The three fortifiers shared 11 of the 186 total identified peptides (Fig.3). Most peptides in BF and PF (54% and 57%, respectively) were in the MW range of 1–2kDa, with 4–23 amino acid residues; in NF peptides smaller than 1kDa prevailed (62%), 6–17 amino acids long. Peptides bigger than 3kDa were absent in all samples. BF and PF contained a similar number of peptides (n=134 and n=141, respectively), while 37 were found in NF. Conversely, (Nielsen et al., 2018) reported a greater number of peptides (n=538) in an undigested bovine milk protein-based fortifier powder (Abbott Similac HMF composed of nonfat milk and whey protein concentrate) using different sample preparation and analytical conditions.

BF and PF showed a similar peptidomic profile (Fig. S1) and a similar number of peptides deriving from the same parent proteins (Fig. 1). This could be explained by the fact that both contained hydrolyzed CN together with hydrolyzed whey proteins. BF and PF shared 108 peptides (81% and 77% of total peptides for BF and PF, respectively), especially from bβ-CN and bβ-LG (Fig. 2A and Fig. 3). Fig. 2A shows that peptide profiles of both fortifiers largely overlapped. Only 11 peptides were in common among the three fortifiers (7 from bβ-CN, 2 from bovine serum albumin and 1 from bβ-LG) (Fig. 3). Peptides identified in NF also derived from the same protein region of BF and PF fortifiers, but in a lower extent. Despite the composition, NF contained most unique peptides (n=20) from bβ-CN. Their presence was likely related to the hydrolysis of proteose peptones [bβ-CN(1–105)

and bβ-CN(1–107)], which are normally retained in ultrafiltered whey proteins and derive from the plasminolysis of bβ-CN. In fact, all bβ-CN peptides (except bβ-CN(193–209), a fragment of another product of bβ-CN plasminolysis) derived from bβ-CN(57–92) region (Fig. 2A). Moreover, contamination with CN peptides in partially hydrolyzed whey protein-based infant formula was also reported by (Wada & Lönnerdal, 2015a). Anyway, bβ-CN released the highest number of peptides in each sample.

Search of the identified peptides against the MBPDB revealed 21 peptides that were identical to known BPs (Table 2). Whether these peptides exert their biological effects depends on their amount and capability to avoid further breakdown and reach the site of action intact, without being hydrolyzed. Several studies identified BPs that may be relevant for the gut, such as those with antimicrobial and mucin-stimulatory properties (Nielsen et al., 2017). The bioactive peptide bβ-CN(193–209) having angiotensin-converting enzyme (ACE) inhibitory, antimicrobial, antithrombin and immunomodulatory activities and present in all the studied samples, was identified also by (Nielsen et al., 2018) in their HM fortifier. The opioid-acting bovine β-casomorphins (bBCMs) are among the most studied BPs of bovine milk proteins, as they have been suggested (but not proven) to exert opioid activity and contribute to an increased risk for certain non-communicable diseases (Summer, Di Frangia, Ajmone Marsan, De Noni, & Malacarne, 2020). In all the three fortifiers, we found the most biologically relevant and the most studied bBCM7 [bβ-CN(60–66)]. Moreover, BF and PF contained also bBCM4 [bβ-CN(60–63)], bBCM6 [bβ-CN(60–65)] and bBCM9 [bβ-CN(60–68)] (Table 2). In partially and extensively hydrolyzed commercial infant formulas, (Wada & Lönnerdal, 2015a) reported the presence of PEP-inhibitor [bβ-CN(59–66)], ACE-inhibitor [bβ-CN(59–68)], an opioid peptide [bβ-CN(60–65)] and an immuno-regulatory peptide [β-CN(63–68)], which we also found in the studied fortifiers. In both, BF and PF, we identified four different phosphorylated peptides. Phosphorylation is

a post-translational modification, which involves CNs mostly at serine residues (Farrell et al., 2004). Caseinophosphopeptides are capable of binding divalent cations, such as Ca^{2+}, and affecting their bioavailability (Bouhallab & Bouglé, 2004). The two fortifiers shared the peptides bα_{S1}-CN(41–52)2P and bβ-CN(7–16)1P. The peptides bα_{S1}-CN(42–52)2P and bβ-CN(8–15)1P were only present in BF, while bβ-CN(33–39)1P and bα_{S1}-CN(41–53)2P were found in PF. The studied fortifiers contained several peptides coming from the bα_{S1}-CN N- and C-termini and bβ-CN C-terminus (Fig. 2A). These proline-rich regions are hydrophobic and are known to be the precursor sequence of bitter peptides (Murray et al., 2018). Bitter taste of milk protein hydrolyzates is common and it depends mainly on the molecular weight and hydrophobicity profile of the derived peptides, and hence on the specific enzyme used for hydrolysis, the degree to which the protein is hydrolyzed (Leksrisompong, Miracle, & Drake, 2010).

3.2. Peptidomic profile of undigested PDHM

In undigested PDHM, we identified 70 unique and different peptides. They derived from hβ-CN, hα_{S1}-CN, polymeric immunoglobulin receptor, osteopontin and hκ-CN (Fig. 4). Peptides from hβ-CN were the most abundant (n=34), followed by hα_{S1}-CN, polymeric immunoglobulin receptor, osteopontin and hκ-CN. These findings were previously reported by (Dallas et al., 2015; Dingess et al., 2017; Guerrero et al., 2014), who also evidenced that the identified peptides derived from proteins (hα_{S1}-CN, osteopontin, polymeric immunoglobulin receptor and hκ-CN), which are not the most abundant in HM (Donovan, 2019). No fragments from hα-lactalbumin (hα-LA), the primary protein in HM, were present. It is worth noting that other research studies report extremely variable number of peptides found in undigested HM, as it depends not only on milk itself (time of parturition, stage of

lactation, individual donor variability), but also on sample preparation and analysis (sampling procedures, sample preparation, analytical conditions and data elaboration) (Beverly et al., 2019; Dallas et al., 2014, 2015; Deglaire et al., 2019; Dingess et al., 2017; Guerrero et al., 2014; Nielsen et al., 2018; Wada & Lönnerdal, 2015b).

3.3. Peptidomic profile of digested PDHM and fortified PDHM digests

To mimic preterm infant nutrition, SGID was applied to fortified PDHMs (PDHM fortified with BF+PF and PDHM fortified with NF+PF). Peptidomic analysis identified 515 unique peptides deriving from human (n=431) and bovine (n=70) milk proteins. Fourteen peptides derived from protein regions with identical sequence; therefore, it was not possible to establish whether they were of human or bovine milk origin (h/bα-LA, h/bβ-CN, h/b serum albumin). In the premature infant stomach, after ingestion of fortified HM, (Nielsen et al., 2018) found a greater number of peptides (n=1720). This could be attributed to different HM and fortifier samples used and diverse digestion model (*in vivo* gastric instead of *in vitro* gastrointestinal). PDHM fortified with BF+PF had a similar number of unique peptides compared to the NF+PF fortification (n=411 and n=420, respectively). Peptides of HM origin were the most abundant (84% and 87% in PDHM with BF+PF and NF+PF, respectively) (Fig. S3), and β-CN prevailed both in human and in bovine proteins, as also reported by (Beverly et al., 2019; Nielsen et al., 2018) in the stomach of premature infants. It has been suggested that the endogenous proteolysis of raw HM and *in vivo* HM digestion at infant's stomach are protein-selective, since hβ-CN-derived peptides have been found as the most numerous also in the raw HM and in HM gastric digestates *in vivo* (Dallas et al., 2014, 2015; Guerrero et al., 2014). Both types of fortification released a comparable number of peptides from the main parent proteins, as shown in Fig. 4 and Fig. S3.

According to (Dallas et al., 2014), peptides from lactotransferrin, bile salt-activated lipase and hα-LA were not present in the HM, but they were released in the term infant stomach. Our data support these findings, even using a simulated preterm SGID. After digestion of fortified PDHMs, peptides from human milk proteins increased as compared to undigested PDHM. Meanwhile peptides from bovine milk proteins were fewer than those identified in the undigested fortifiers (Fig. 2B). Protein fortification accounted for a half of total protein content in both fortified PDHMs. Nevertheless, human-derived peptides were released to a greater extent, compared to the bovine-derived ones. Likely, hydrolyzed CN and whey proteins of fortifiers could have been further hydrolyzed by digestive proteases, leading to the formation of single amino acids or very short peptides, which could not have been detected by the UPLC/HR-MS/MS analysis.

The two digests of fortified PDHM shared 316 unique peptides. As expected, most of them (88%) were of human origin, being hβ-CN the most represented precursor protein. Among bovine-derived peptides (10%), those coming from bβ-CN were the most abundant.

To determine the effect of fortification on peptide release after SGID, peptidomic profiles of the two fortified PDHMs were compared with those of (non-fortified) PDHM (Fig. S2). Total peptide count was higher in the PDHM digest (n=509) than in the fortified samples (n=411 and n=420 for PDHM fortified with BF+PF and NF+PF, respectively). This difference is likely due to the diverse enzyme-to-protein ratio occurring during SGID of fortified and unfortified PDHM (Beverly et al., 2019).

Finally, we searched for the potential BPs in MBPDB database. Fifteen potential BPs were revealed in fortified PDHMs (Table 2). Among them, only five were of HM protein origin. Meanwhile, we identified a higher number of bovine milk-derived BPs. As the bioactivity of bovine milk (compared to HM) has been more extensively studied through years, this result may not represent the biological reality (Nielsen et al., 2018). The bovine milk-derived peptides bβ-CN(193–209), bβ-CN(59–67), bβ-

CN(59–68) and bβ-LG(46–55) and hβ-CN(136–144), hβ-CN(120–132) and hβ-CN(169–175) of human origin were previously reported as BPs found in the stomach of premature infants fed with fortified HM (Nielsen et al., 2018). Probably they formed during the gastric phase of digestion and remained unaffected by intestinal proteases, since we found them after SGID. The opioid peptide bBCM7, identified in all the fortifiers, was still present after SGID of PDHM with both types of fortification (Fig. 2B). PDHM with BF+PF and NF+PF digests also contained bBCM4 and bBCM6, previously identified in the undigested BF and PF fortifiers (Fig. 2B). Opioid peptides of human milk origin, hBCM7 [hβ-CN(51–57)], hBCM8 [hβ-CN(51–58)] and hBCM9 [hβ-CN(51–59)], identified and quantified in our previous study (Cattaneo et al., 2020), were also present in the digests of fortified PDHM. The BPs hBCM8 and hBCM9 were not reported in Table 2, since they are not included in the MBPDB; however, their potential bioactivity has been previously demonstrated (Enjapoori, Kukuljan, Dwyer, & Sharp, 2019). It is worth noting that some bovine peptides identified in the undigested fortifiers were still found in the digests of fortified PDHM, while others almost disappeared likely due to enzymatic hydrolysis. We also found some human and bovine BPs, previously reported by (Wada & Lönnerdal, 2015b, 2015a), in both fortified PDHMs. In particular, they studied the effects of pasteurization on the occurrence of HM BPs and, after digestion, the authors reported hβ-CN(50–58) and hβ-CN(52–59) as PEP-inhibitors, hBCM9 as an opioid peptide, hβ-CN(105–117) as an immunomodulatory peptide and hβ-CN(154–160) and hβ-CN(167–173) as antioxidants. They concluded that pasteurization does not affect the release of BPs after SGID of HM. As concerned bovine BPs, (Wada & Lönnerdal, 2015a, 2015b) found bβ-CN(59–66) and (59–67) as a PEP-inhibitor; bβ-CN(59–68) as an ACE-inhibitor and bβ-CN(60–68) as an opioid peptide in hydrolyzed infant formulas subjected to SGID.

4. Conclusions

The present research demonstrated that the studied HM commercial fortifiers contained a different number and type of peptides, some of which could potentially exert biological activity. After SGID of fortified PDHMs, most of the peptides were released from HM proteins, but some bovine BPs persisted. To the best of our knowledge, peptidomic profile of these fortifiers remained uncharacterized. For this reason, we shed some light on these products, intended for premature infant nutrition.

Nonetheless, further research is needed to better mimic *in vitro* the digestive process of preterm infants, and to ascertain the biological role the bovine milk peptides to the overall health outcome of premature infants fed fortified PDHM.

Overall, this work represents one of the few peptidomic investigations on fortifiers used for premature infant nutrition in neonatal intensive care units and the first peptidomic evaluation of the fortified PDHM composition upon *in vitro* simulated preterm gastrointestinal digestion.

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438 **Figure captions**

439

440 **Fig. 1.** Number of peptides from bovine (b) milk proteins, identified in undigested BF (dark gray), PF
441 (light gray) and NF (black) fortifiers. bα_{S1}-CN, α_{S1}-casein; bα_{S2}-CN, α_{S2}-casein; bβ-CN, β-casein; bκ-CN,
442 κ-casein; bα-LA, α-lactalbumin; bβ-LG, β-lactoglobulin; bSA, serum albumin.

443

444 **Fig. 2.** Visual representation of peptides derived from major bovine milk proteins bα_{S1}-CN, bβ-CN, bβ-
445 LG and bα-LA and identified in (A) undigested BF (dark gray), PF (light gray) and NF (black) fortifiers
446 and in (B) digested PDHM fortified with BF + PF (dark gray) and NF + PF (black).

447

448 **Fig. 3.** Venn diagram indicating numbers of the unique peptides identified in undigested BF, NF and PF
449 fortifiers.

450

451 **Fig. 4.** Number of peptides from human and bovine milk proteins, identified in undigested (UD) PDHM
452 (white), digested (SGID) PDHM (light gray) and digested PDHM fortified with BF + PF (dark gray) and
453 NF + PF (black). Human (h) and bovine (b) proteins are: β-CN, β-casein; hTRFL, lactotransferrin; hCEL,
454 bile salt-activated lipase; α_{S1}-CN, α_{S1}-casein; α-LA, α-lactalbumin; hOSTP, osteopontin; hPIGR,
455 polymeric immunoglobulin receptor; κ-CN, κ-casein; SA, serum albumin; α_{S2}-CN, α_{S2}-casein; β-LG, β-
456 lactoglobulin.

Table 1

Protein composition of the commercial fortifiers (as declared on their labels) intended for protein fortification of PDHM for nutrition of preterm infants.

Fortifier	**Protein composition (g 100 g^{-1})**
BF	Hydrolyzed caseins (12.5) and hydrolyzed whey proteins (12.5)
PF	Hydrolyzed caseins (41.0) and hydrolyzed whey proteins (41.0)
NF	Hydrolyzed whey proteins (20.0)

Table 2

Potential BPs identified from MBPDB in undigested BF, NF and PF fortifiers and in digests of PDHM fortified with BF + PF and PDHM fortified with NF + PF.

Peptide	Protein				BF	NF	PF	PDHM BF+PF	PDHM NF+PF	Function
GVSLPEW	bα-LA	20	–	26	×		×	×		ACE-inhibitory
RPKHPIKHQGLPQEVLNENLLRF	bα_{S1}-CN	1	–	23	×		×			Antimicrobial, immunomodulatory
VYPFPGPI	bβ-CN	59	–	66		×		×	×	PEP-inhibitory
VYPFPGPIP	bβ-CN	59	–	67					×	PEP-inhibitory
VYPFPGPIPN	bβ-CN	59	–	68	×	×	×	×	×	ACE-inhibitory, antioxidant
YPFP	bβ-CN	60	–	63	×		×	×	×	Anticancer, opioid
YPFPGP	bβ-CN	60	–	65	×		×	×	×	DPP-IV Inhibitory, opioid
YPFPGPI	bβ-CN	60	–	66	×	×	×	×	×	ACE-inhibitory, anticancer, anxiolytic, immunomodulatory, increases jejunal mucus secretion, increases MUC2, MUC3 and MUC5A expression, opioid, reduces pancreas MDA level, satiety
YPFPGPIPN	bβ-CN	60	–	68	×		×	×		ACE-inhibitory, DPP-IV inhibitory
PFPGPI	bβ-CN	61	–	66		×				Cathepsin B inhibitory
PGPIPN	bβ-CN	63	–	68		×				Anticancer, immunomodulatory
TQTPVVVPPFLQPE	bβ-CN	78	–	91	×					Antioxidant
HKEMPFPK	bβ-CN	106	–	113	×					Antimicrobial
EMPFPK	bβ-CN	108	–	113			×			ACE-inhibitory, antimicrobial, bradykinin-potentiating, increase MUC4 expression
MPFPKYPVEP	bβ-CN	109	–	118	×		×			ACE-inhibitory
SKVLPVPQ	bβ-CN	168	–	175	×		×			ACE-inhibitory
KVLPVPQ	bβ-CN	169	–	175	×					ACE-inhibitory
YQEPVLGPVRGPFPIIV	bβ-CN	193	–	209	×	×	×	×	×	ACE-inhibitory, antimicrobial, antithrombin, immunomodulatory
QEPVLGPVRGPFPIIV	bβ-CN	194	–	209	×		×			ACE-inhibitory
LKPTPEGDL	bβ-LG	46	–	54	×		×			DPP-IV inhibitory
LKPTPEGDLE	bβ-LG	46	–	55	×		×	×	×	DPP-IV inhibitory
LKPTPEGDLEIL	bβ-LG	46	–	57	×		×			DPP-IV inhibitory
YPFVEPI	hβ-CN	51	–	57				×	×	Anticancer, opioid
SPTIPFFDPQIPK	hβ-CN	120	–	132				×	×	Stimulates proliferation
LENLHLPLP	hβ-CN	136	–	144				×	×	ACE-inhibitory
WSVPQPK	hβ-CN	169	–	175					×	ACE-inhibitory, antioxidant
QVVPYPQ	hβ-CN	182	–	188				×	×	Antioxidant

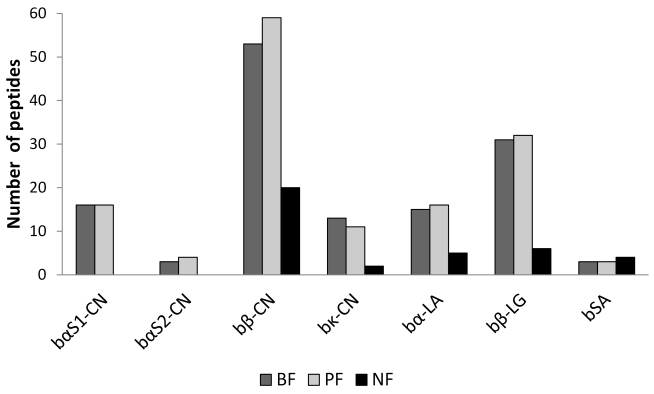
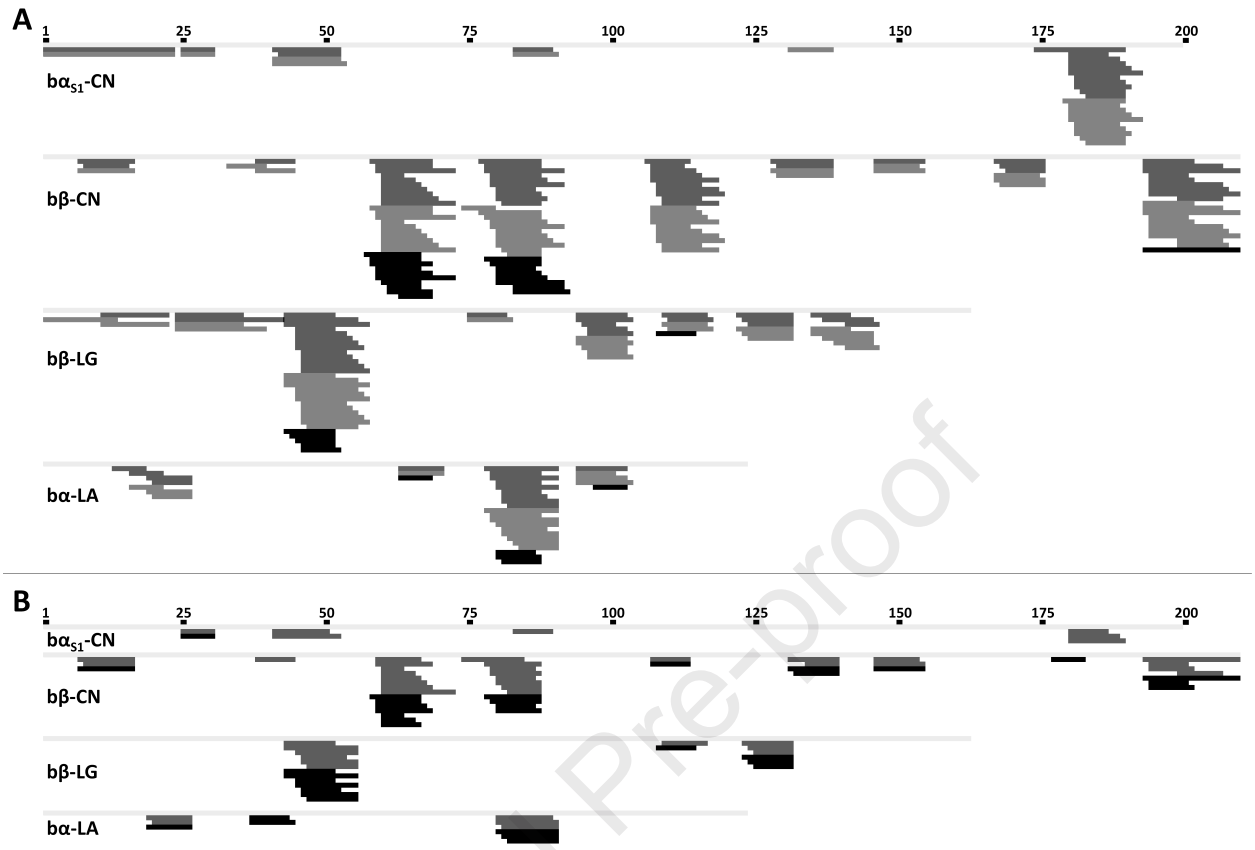


Fig. 1



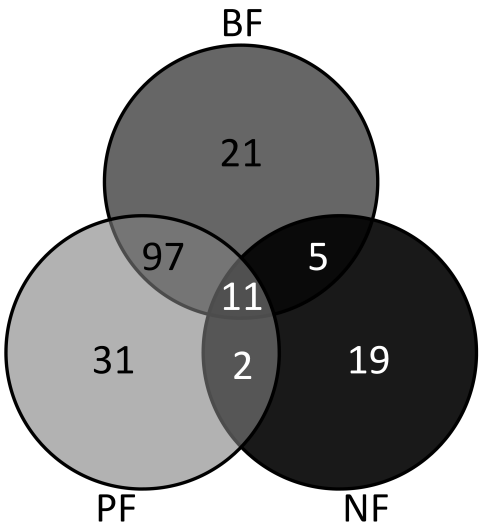


Fig. 3

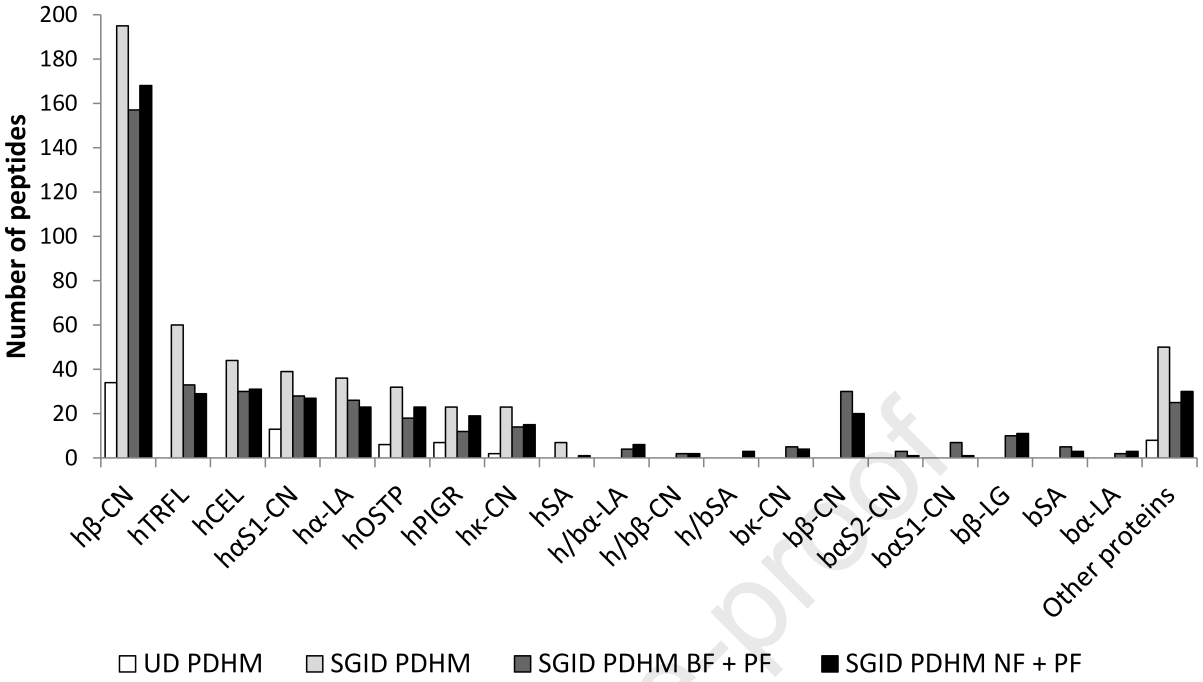


Fig. 4

Highlights

- Pasteurized donor human milk (PDHM) is fortified for premature infant nutrition
- Hydrolyzed bovine milk proteins are used for fortification of PDHM
- Peptidomic characterization of three commercial bovine milk fortifiers
- Peptidomic investigation of the fortified PDHMs after an *in vitro* preterm digestion
- Intact fortifiers and digests of fortified PDHMs contained bioactive peptides

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: