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Simulated dynamic digestion reveals different peptide releases from human milk processed by means of holder or high temperature-short time pasteurization

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ABSTRACT

High Temperature-Short Time (HTST) pasteurization was proposed as an alternative to Holder pasteurization (HOP) to increase the retention of specific human milk (HM) bioactive proteins. The present study explored whether HTST and HOP differently affect peptide release during simulated preterm infant gastrointestinal digestion. Raw (RHM), HOP- and HTST- pasteurized HM were digested using an *in vitro* dynamic system, and the identified peptides were analyzed by mass spectrometry and multivariate statistics. Before digestion, 158 peptides were identified in either RHM, HTST- or HOP- HM, mostly (84.4%) originating from β-casein (CASB). During gastric digestion, HOP-HM presented a greater number and more abundant specific CASB peptides. A delayed release of peptides was observed in RHM during the intestinal phase, with respect to both pasteurized HM. Although limited to gastric digestion, the HM peptidomic profile differed according to the pasteurization type, and the pattern of the HTST peptides showed a greater similarity with RHM.

1. Introduction

The increasing diffusion of Human Milk Banks throughout the world is a clear sign of the efficacy of the WHO strategy on breastfeeding, which was launched in the 1980s (WHO/UNICEF, 1981). Although perplexities were frequent in the past, as regards the adequacy of donor human milk (HM) for the nutrition of preterm and very low birthweight infants, cumulative clinical evidence in recent years has clarified that a mother's own milk is the gold standard for feeding all infants, and that donor HM is the second best feeding choice (PATH, 2013; WHO, 2003). Donor HM, as well as mother's own milk, should be pasteurized in specific clinical situations to inactivate life-threatening viral and bacterial agents. A pasteurization process at 62.5 °C for 30 min (holder pasteurization - HOP) is currently recommended in almost all international guidelines for the constitution of HM banks (Moro et al., 2019), because such a treatment is able to retain many of the beneficial and protective effects of mother's own milk (Moro et al., 2019; Peila et al., 2016; Picaud & Buffin, 2017), including the reduction of the risk of necrotizing enterocolitis (Cossey et al., 2013; Dicky et al., 2017).

However, the awareness that some important nutritional and non-nutritional biological factors, such as immunological factors and bioactive proteins, are denatured by HOP (Peila et al., 2016; Picaud & Buffin, 2017) has encouraged researchers throughout the world to develop novel HM processing methods that can ensure microbial and viral inactivation, while improving the preservation of its nutritional, immunological and functional constituents (Moro et al., 2019; Peila

Abbreviations: ACE, angiotensin converting enzyme; BAL, bile acid lipase; BT, butyrophilin; FASN, fatty acid synthase; GRAVY, grand average of hydropathy value; HTST, high temperature-short time pasteurization; HOP, HOlder pasteurization; HM, human milk; LTF, lactoferrin; OPN, osteopontin; PEP, prolyl endopeptidase; PIGR, polymeric immunoglobulin receptor; PCA, principal component analysis; RHM, raw human milk; XDH, xanthine dehydrogenase; LALBA, α-lactalbumin; CASA, α_{s1}-casein; CASB, β-casein; CASK, κ-casein.

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et al., 2017; Wesolowska et al., 2019). Although there is universal agreement about the effects of HOP on HM, only a few reports have addressed the study of whether, how, and to what extent, such differences can influence HM digestion. Major ethical concerns have emerged regarding *in vivo* trials conducted to study infant digestion, and there is a limited availability of relevant animal models (Sangild et al., 2013). Alternative *in vitro* models that can be used to simulate both term and preterm gastrointestinal digestion have been proposed (De Oliveira et al., 2016; Hur et al., 2011; Ménard et al., 2014), and these have provided important evidence about the different digestion patterns of raw vs. HOP-HM. It has in particular been demonstrated that HOP affects the gastric proteolysis of some major proteins, such as for β-casein (CASB), as observed *in vitro* at the term stage (de Oliveira et al., 2016), and lactoferrin (LTF), as shown *in vitro* (de Oliveira et al., 2016; De Oliveira et al., 2016) and *in vivo* (De Oliveira et al., 2017). HOP further modulates the kinetics of the peptides released during gastrointestinal digestion (Deglaire et al., 2016; Deglaire et al., 2019). In addition, it tends to reduce the degree of lipolysis and/or the release of specific fatty acids (de Oliveira et al., 2016; De Oliveira et al., 2016).

High Temperature-Short Time (HTST) pasteurization, which is performed by heating the milk at 72 °C for 15 s, followed by a fast cooling, is one of the most promising alternative heat processing treatments for HM. We have recently (Nebbia et al., 2020) conducted the first comparative study on *in vitro* dynamic digestion, at the preterm stage, of DM pasteurized by either HOP or HTST, using a benchtop HTST pasteuriser that had previously been patented and validated by our group (Giribaldi et al., 2016). Overall, the HOP and HTST processing of HM resulted in similar proteolysis and lipolysis kinetics during simulated gastrointestinal digestion (Nebbia et al., 2020). Nevertheless, the study highlighted small, but significant differences between the two treatments. One specific protein electrophoresis band, containing immunoglobulins and lactoferrin, was slightly more resistant to proteolysis in HTST treated HM than in HOP-HM. In addition, the HTST pasteurization of HM resulted in a higher intestinal release of the total and essential free amino acids than HOP, at a comparable level to that of RHM (Nebbia et al., 2020).

Therefore, we hypothesized that the application of different pasteurization methods could result in different peptide release patterns, including those of bioactive peptides, during digestion. The purpose of this study has therefore been to compare the peptide profile of raw, HOP and HTST treated HM during digestion, by means of a comprehensive peptidomic investigation.

2. Methods

2.1. Human milk sampling, pasteurization and in vitro dynamic digestion

Milk samples were collected, pasteurized and digested, by means of DIDGI®, as previously described (Nebbia et al., 2020). Briefly, mature HM (1–3 months after delivery) from 5 healthy donors was collected and stored (frozen at −20 °C for < 4 months) by the HM bank at the Regina Margherita Children's Hospital in Turin (Italy). The HM was thawed, pooled, and either stored without further processing (RHM, at −20 °C) or pasteurized beforehand by means of HOP (62.5 °C for 30 min) or HTST (72 °C for 15 s). HOP was performed using the donor HM bank equipment (Metalarredinox, Verdellino, BG, Italy). HTST was performed using a patented proprietary small-scale device (Giribaldi et al., 2016). Each milk donor involved in the research signed a written consent form, which stated that protection of the mothers' and infants' data was ensured.

RHM, HOP and HTST samples were subjected to *in vitro* digestion using the DIDGI® bi-compartmental dynamic system (Ménard et al., 2014), to simulate the digestion of a preterm newborn at a postnatal age of four weeks (De Oliveira et al., 2016). The specific digestion parameters are detailed elsewhere (Nebbia et al., 2020). Digestion experiments were performed in triplicate for each matrix over a period of three hours. Aliquots were collected before digestion and at 30, 60, and 90 min after the beginning of the digestion in both the gastric and intestinal compartments. Additional samples were collected from the intestinal compartment at 120 and 180 mins. Protease inhibitors, namely 10 μL of pepstatin A (0.72 mM) per mL of gastric digesta or 50 μL of pefabloc (0.1 M) per mL of intestinal digesta, were added immediately after sampling, before storage at −20 °C, until analysis.

2.2. Peptide identification

Mass spectrometry (MS) analysis was conducted as previously described (Deglaire et al., 2016; Deglaire et al., 2019). Briefly, a nano-RSLC Dionex U3000 system, fitted to a Q-Exactive mass spectrometer (Thermo Scientific, San Jose, The USA), equipped with a nano-electrospray ion source, was used. Digesta samples were diluted 200 times in the injection buffer and filtered (0.45 μm cut-off) before being concentrated on a PepMap100 μ-precolumn (C18 column, 300 μm i.d. × 5 mm length, 5 μm particle size, 100 A pore size; Dionex, Amsterdam, The Netherlands) and separated on a PepMap100 RSLC column (C18 column, 75 μm i.d. × 150 mm length, 3 μm particle size, 100 A pore size; Dionex). Peptide separation was performed at a flow rate of 0.3 μL. min^{-1} using solvents A [2% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) trifluoroacetic acid in HPLC gradient grade water] and B [95% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) trifluoroacetic acid in HPLC gradient grade water]. The elution gradient first rose from 5 to 35% in solvent B over 85 min, then up to 85% in solvent B over 5 min before column re-equilibration. The mass spectra were recorded in positive mode using the m/z 250–2000 range. The resolution of the mass analyzer for an m/z of 200 atomic mass units was set in acquisition mode to 70 000 for MS and 17 500 for MS/MS. The ten most intense ions were selected for MS/MS fragmentation for each MS scan, and were excluded from fragmentation for 15 s. Peptides were identified from the MS/MS spectra using X!TandemPipeline software (Langella et al., 2017) against an HM protein database (Molinari et al., 2012), to which the common Repository of Adventitious Protein (http://thegpm.org/crap) had been added. The possible post-translational modifications were serine or threonine phosphorylation, methionine oxidation, lysine or arginine lactosylation, and cyclisation of glutamine or glutamic acid into pyroglutamic acid. Any potential protein modifications as a result of the use of AEBSF (Pefabloc) as a serine protease inhibitor were also considered: amino-ethylbenzenesulfonylation of histidine, lysine, tyrosine and serine. Peptides identified with an e-value < 0.01 were automatically validated, and this resulted in a false discovery rate of the peptides of<0.4%.

2.3. Quantification of the peptides

Each identified peptide was quantified, by means of label-free MS, using the MassChroQ software (Valot et al., 2011). A m/z width of 10 ppm was used to obtain extracted ion chromatograms of the peptides in time-aligned chromatograms, and the area under the curve was then quantified. When a peptide was measured with several charge states, all the ion intensities were summed.

2.4. Biochemical characteristics of the peptides

Several biochemical characteristics were determined for each peptide: the amino acids at the cleavage sites in positions $P1$ (C-term end) and $P1'$ (N-term end), the number of essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine), the isoelectric point, the molecular weight and the Grand Average of Hydropathy Value (GRAVY), as defined by the ExPASy "ProtParam tool" (Gasteiger et al., 2005). A positive GRAVY corresponds to a hydrophobic peptide and a negative one to a hydrophilic peptide (Kyte & Doolittle, 1982). Bioactive peptides were searched within the BIOPEP (Minkiewicz et al., 2008) and MBPDB (Nielsen et al., 2017)

databases, both of which were accessed in June 2020. Only an exact matching between sequences was considered.

2.5. Statistical analyses

Data analyses were performed using PAST software, version 3 (Hammer et al., 2001). Before the analysis, the MS/MS abundances of each peptide were corrected for the digestion time-specific dilution factors, resulting from the dilution caused by the progressive gastric/intestinal juice release and emptying within the DIDGI® system. The peptide abundances were then transformed by means of log-scaling [log10(abundance)], and any missing data were given an arbitrary value of 1 when necessary (multivariate and univariate statistics).

A Venn diagram distribution of the peptides was built for each digestion time. Sporadic peptides, detected in only one sample replicate, were not considered.

An overall multivariate analysis of variance (Principal Component Analysis - PCA) was conducted separately on the peptides from gastric digestion (n = 395), and on the intestinal peptides (n = 856), by considering each peptide [$\log_{10}$(abundance)] as a variable and the sampling time of each digestion replicate and each meal as individual. For intestinal peptides, PCA, excluding the peptide abundances at 30 min of digestion from all samples, was also performed (n = 822). Sporadic peptides, present in only 1 sample replicate, were not included in the analysis. PCA was conducted by giving a different color code to each processing treatment, and by setting each digestion time point as a group. Data were automatically auto-scaled before analysis by means of PAST software.

Two types of clustering analysis were conducted, taking into consideration only peptides that were detected in at least two gastric times and three intestinal digestion ones. The abundance of each peptide was expressed for each time point as the relative ratio with respect to the maximum value of the same peptide during digestion, and was averaged over the three digestion replicates at each time point for each processing treatment. Relative abundance data were used for an ascending hierarchical clustering, on the basis of Ward's agglomeration of the minimum within-cluster variance in PAST. The number of clusters was determined from the bar heights at one of the most marked jumps. A k-means non-hierarchical clustering was also performed by setting the number of target clusters according to Ward's clustering results. The two clustering methods were compared in order to obtain the best overlap between the two methods. The data included in the clustering procedure were visualized by means of PCA, whereby the peptides were set as samples, the cluster numbers as groups and the relative abundance as variables. The association of the qualitative and quantitative characteristics with each cluster was checked with Fisher's test and the Kruskal-Wallis test, respectively.

Univariate statistical analyses were conducted separately on the peptides present in the undigested milk, during gastric digestion, or during intestinal digestion. An analysis of variance (ANOVA) was conducted on the [$\log_{10}$(abundance)] of the peptides present in at least one replicate in all the treatments (n = 70 for undigested milk, n = 117 for gastric peptides, n = 194 for intestinal peptides), and in at least 2 gastric times or 3 intestinal digestion ones. One-way ANOVA (for undigested milk, with processing treatment as the factor) and two-way ANOVA (for gastric or intestinal peptides, with processing treatment, time and their interaction as factors) were fitted to the log10 abundances using PAST software. When statistical significance was reached for a treatment (p-value < 0.05), post-hoc tests were run (Tukey's or Dunn's test for one-way ANOVA, depending on the normality of the residuals; Tukey's test for two-way ANOVA).

An in-house program allowed the peptides and their average cumulative abundances to be mapped onto the parent protein sequence. The abundances of the peptides were averaged, for each type of milk and each digestion phase (milk prior to digestion, gastric or intestinal phases), over the different digestion times of the corresponding phase, and were summed amino acid by amino acid, and finally $\log_{10}$- transformed. Any missing abundances were set at 0. Log_{10}.and the transformed abundances were plotted on the amino acid sequence of the protein.

3. Results

3.1. Identified proteins and peptides

Twelve human milk proteins were found to be at the origin of all the identified peptides (n = 1196). The latter contained from 6 to 51 amino acids and had a molecular weight that ranged from 636 to 5651 Da. The detected modifications were: methionine oxidation (n = 47), glutamine cyclization (n = 29), aminoethylbenzenesulfonylation (n = 28), arginine lactosylation (n = 6) and cysteine acetylation (n = 5). Modified peptides (n = 101) were often detected as unmodified (74%). Of all the detected peptides, 42 were only found sporadically (only in 1 or 2 samples through digestion) and were therefore not considered for the subsequent statistical analyses. The majority of the peptides originated from β-casein (CASB – 48.3%), lactoferrin (LTF – 10.3%), bile salt-stimulated lipase/bile acid lipase (BAL – 9.0%) and α_{s1}-casein (CASA – 7.5%), and the other parent proteins accounted for<5% of the detected peptides. The mean peptide molecular weight derived from CASB was 2091 Da, and this was followed by those from CASA (1658 Da), while the peptides from α-lactalbumin (LALBA – 3.8%) displayed the lowest mean molecular weight (1193 Da).

3.2. Human milk proteolysis prior to digestion

Before digestion, 158 peptides were identified in either RHM, HTST or HOP, and almost all were derived from CASB (84.8%). The remaining peptides were derived from CASA (4.4%) and a polymeric immunoglobulin receptor (PIGR – 4.4%). The mean size of the peptides in the undigested milk was 2098 Da, with κ casein (CASK) being at the origin of the largest peptides (about 2472 Da).

Fig. 1 shows a Venn diagram distribution of the peptides before digestion (Fig. 1A) of the differently processed HM samples. Of the exclusive peptides in the undigested HM, 9 were from RHM, 42 from HOP (38 from BCN) and 6 from HTST (Supplementary table 2A). One specific HTST peptide (CASB 54–59) displayed an immunomodulatory capacity. Shared peptides were 70 among RHM, HOP and HTST (all from CASB, 2 with antimicrobial and 2 DNA-synthesis stimulating bioactivity), 9 in both HOP and HTST (including CASB 154–160 with antioxidant bioactivity), 8 in both RHM and HTST (including CASB 161–166 with ACE-inhibitory bioactivity), and 14 in both RHM and HOP (Fig. 1A).

One-way ANOVA revealed a significant difference in the abundances of 20 CASB peptides, the majority of which (75%) were located at the protein C-term (Supplementary table 2A). Eighteen peptides were more abundant in HOP than in either HTST alone (n = 7), in both RHM and HTST (n = 8), or in RHM alone (n = 3). No significant difference was observed between HTST and RHM for 17 peptides. On the other hand, two bioactive peptides, showing a DNA-synthesis stimulating bioactivity or antimicrobial activity, were significantly more abundant in HOP than in RHM or in HTST, respectively.

3.3. Multivariate profiling of the peptide abundances during digestion

A limited fraction of peptides was shared by both digestive compartments (17% in RHM, 14% in HTST and 16% in HOP), and all the subsequent gastric and intestinal peptide analyses were therefore conducted separately. Fig. 2 shows graphical multivariate representations of the $\log_{10}$ transformed abundances of all the peptides obtained from each processing treatment during gastric digestion (G30; G60; G90). Each time point is grouped separately from the others, and the first principal components, representing the ongoing time of gastric digestion, are shown from left to right (Fig. 2). The second principal

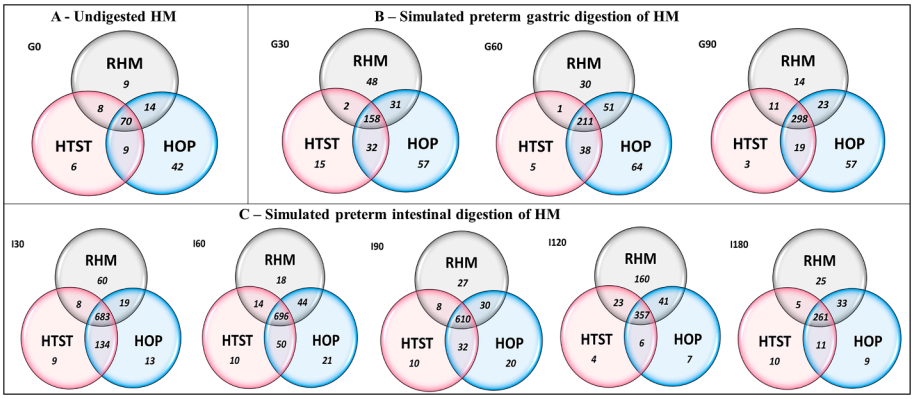

Fig. 1. Venn diagram of the peptides detected during the *in vitro* dynamic digestion of human milk (HM) both raw (RHM – grey) and processed, by either holder (HOP – blue) or high temperature-short time (HTST – pink) pasteurization, in undigested milk (A), gastric (B) and intestinal (C) compartments at different times. G0: undigested HM; G30-G90: 30–90 min of gastric digestion; I30-I180: 30–180 min of intestinal digestion. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

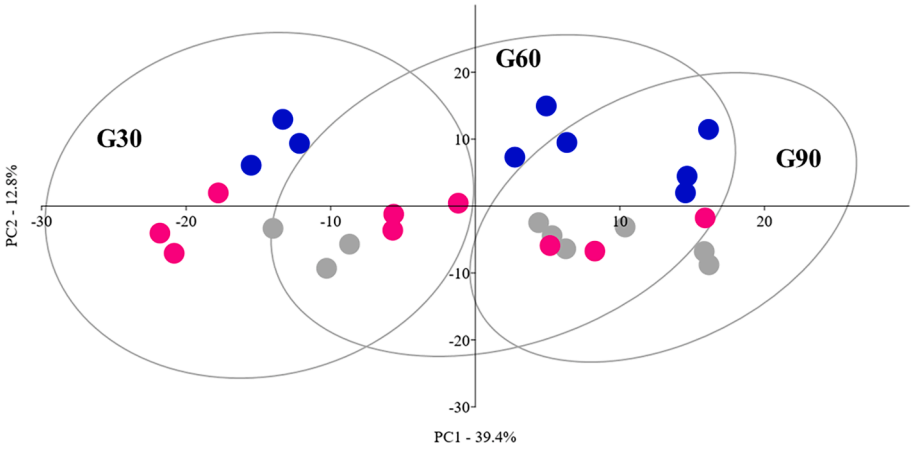

Fig. 2. Principal component analysis (PCA) of the $\log_{10}$ transformed abundance of peptides during *in vitro* dynamic digestion of human milk both raw (RHM – grey dots) and processed, by either holder (HOP – blue dots) or high temperature-short time (HTST – pink dots) pasteurization, in gastric compartment at 30, 60 and 90 min (G30, G60, G90). Ellipses represent 95% confidence intervals. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

component in PCA, which accounts for 12.8% of the total variability, confirms the grouping between the different treatments, with RHM and HTST showing similar behavior to HOP. Fig. 3 reports the same analysis for the peptides released at the intestinal level. The time-dependent grouping along the first principal component (Fig. 3A) was complicated by the out-grouping behavior of RHM at I30. By excluding all of the I30 samples from the PCA (Fig. 3B), the time trend became more visible on the first PC. No clear difference was seen for digestion when considering the different pasteurization methods.

3.4. Peptide clustering during digestion

The clustering procedure on the peptides released during simulated dynamic digestion allowed five gastric peptide clusters and four intestinal ones to be discriminated. Regarding the gastric phase (Fig. 4 and Supplementary Figure S1), two clusters (n°3 and n°4) showed an increasing abundance trend during digestion and grouped separately on the second PC with respect to clusters showing a slightly decreasing trend (n°2) or steady trend (n°1 and n°5). The major differences between the different treatments were seen in clusters 3 and 4 (n = 170 peptides), with RHM and HOP displaying higher abundances than HTST, and in clusters 1 and 2 (n = 131 peptides), with the peptides displaying a higher abundance in HOP than in either RHM or HTST. An association of gastric clusters with specific characteristics (Supplementary Table 1) revealed that cluster 1 was characterized by smaller, acidic and more hydrophilic peptides, with a lower occurrence of essential aminoacids, while cluster 2 was characterized by peptides from CASB; on the other hand, cluster 4 was specifically associated with peptides derived from LTF and BAL, and to less peptides from CASB; cluster 5 was associated with C-terminal peptides.

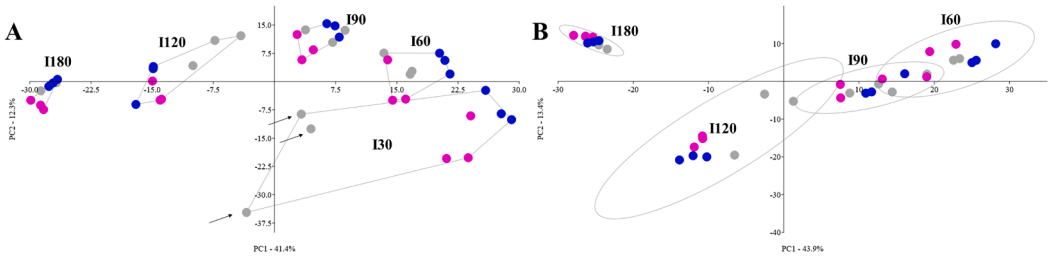

Fig. 3. Principal component analysis (PCA) of the $\log_{10}$ transformed abundance of peptides detected during *in vitro* dynamic digestion of human milk both raw (RHM – grey dots) and processed, by either holder (HOP – blue dots) or high temperature-short time (HTST – pink dots) pasteurization, in the intestinal compartment at 30, 60, 90, 120 and 180 min. The arrows indicate RHM at 30 min. A: PCA for the first and second PC ellipses represent 95% confidence intervals. B: PCA for first and second PCs, without time 30. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

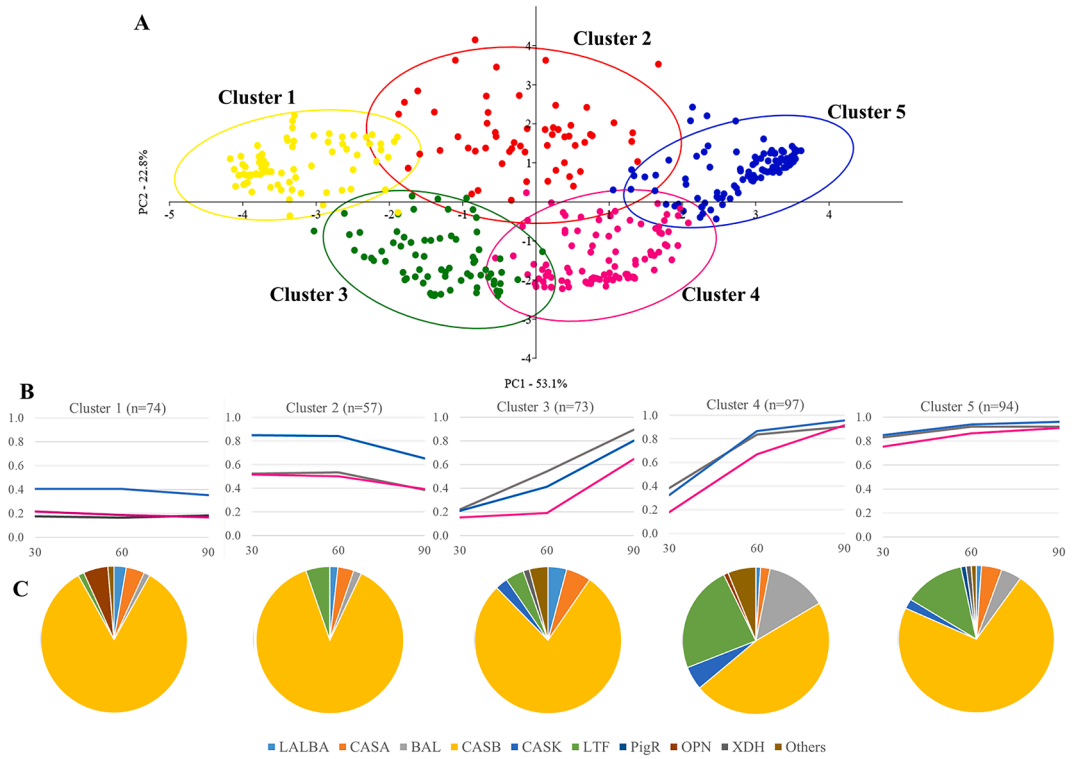

Fig. 4. A) Principal Component Analysis of the $\log_{10}$ transformed abundance during gastric digestion of human milk (HM) coupled with k-means non-hierarchical clustering analysis. Peptides detected in at least 2 gastric digestion times were considered. Different spots represent the relative abundance of single peptides (1: maximum value during digestion). Different colored spots identify different clusters. B) The number of peptides and average relative abundance trend in each cluster and for each HM processing (raw HM: gray line; holder pasteurization: blue line; high temperature-short time pasteurization: pink line). C) Pie charts representing the relative weight of the single HM proteins in each cluster. α-lactalbumin: LALBA; α_{s1}-casein: CASA; bile acid lipase: BAL; β-casein: CASB; κ-casein: CASK; lactoferrin: LTF; Polimeric immunoglobulin receptor: PigR; osteopontin: OPN; xanthine dehydrogenase: XDH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

As far as the intestinal phase is concerned (Fig. 5 and Supplementary Figure S2), all the clusters showed a delayed initial peptide increase (I30), due to the slower release of peptides in RHM, with respect to both types of pasteurized HM. Two clusters (n° 1 and n° 2) showed a pronounced decrease at the end of intestinal digestion, while cluster 3 showed an almost steady trend. Cluster 4 displayed major variations over the intestinal digestion for all the treatments, with a marked higher abundance of RHM peptides at I120. An association of intestinal clusters with specific characteristics (Supplementary Table 1) revealed that cluster 1 was specifically associated with the LTF peptides, which were instead underrepresented in clusters 2 and 3. Clusters 3 and 4 were both associated with smaller and slightly more acidic peptides.

3.5. Individual peptide release during gastric digestion

The number of released peptides progressively increased from 343 to 425 during gastric digestion. The relative proportion of peptides common to all the treatments increased from 46% to 70% (Fig. 1B). The largest peptide release was from CASB (above 70%), at all the digestion times, followed by LTF (above 10%). HOP presented the highest number of unique peptides for all the time-points, while the prevalence of RHM decreased markedly during gastric digestion (from 20% to 4%) (Fig. 1B). HTST always showed just a few unique peptides. The characteristics of the unique peptides are reported in Supplementary Table 3A. The mean size of the peptides released by gastric proteases was about 2100 Da. The origin of the unique peptides differed according to the processing treatment: CASB peptides, such as those derived from osteopontin (OPN), were more frequently detected in HOP; the unique RHM peptides originated from LTF and from fatty acid synthase (FASN) more frequently than from the pasteurized samples; RHM and HTST both generated specific peptides from CASA, although those detected in HTST were shorter. Overall, five bioactive peptides were released from CASB gastric digestion: one with antimicrobial activity, two with DNA synthesis stimulating activity, one with antioxidant activity and one with ACE-inhibitory activity. The ACE-inhibitory peptide (CASB 161–166) was only detected in HTST at G30. The antioxidant peptide (CASB 154–160) was detected in both types of pasteurized HM, from the first sampling time until the end of the gastric phase, while it was only present at the end of the gastric phase in RHM (Supplementary Table 3A).

Two-way ANOVA revealed a significant difference in the abundance of 75 peptides, according to the processing treatment, the majority of which(n = 58) were from CASB (Supplementary Table 3B). Most of these peptides (n = 52) were also affected by the digestion time, but only 13 showed a significant interaction between time and processing. Twenty-seven peptides were released by gastric digestion of the caseins (CASA, CASB and CASK) in similar amounts in RHM and HOP, and less abundant in HTST. Thirteen peptides (7 from CASB, 4 from LTF, 1 from BAL and 1 from PIGR) were significantly different in all the samples; 9 of these were significantly more abundant in HOP. Eleven peptides (9 from CASB and 2 from LTF) were released in similar amounts in RHM and HTST, but significantly more so in HOP. Five CASB and 1 butyrophilin (BT) peptides were released, by gastric digestion, in the same way after both pasteurization methods, compared to RHM. Other CASB peptides were significantly less abundant in HTST than in HOP (n = 6) or RHM (n = 6). Finally, 3 LTF, 2 CASB and 1 BAL peptides were released more by HOP than by RHM during gastric digestion.

3.6. Individual peptide release during intestinal digestion

The number of detected peptides progressively decreased during

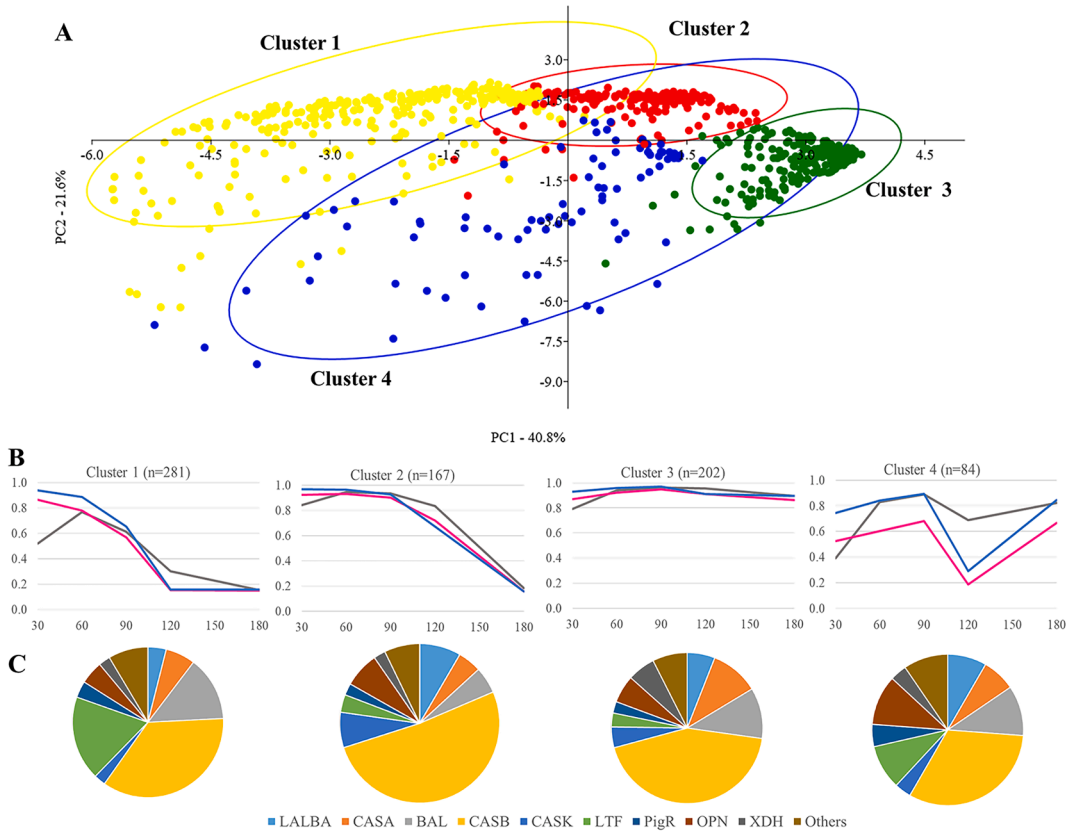

Fig. 5. A) Principal Component Analysis of the $\log_{10}$ transformed abundance during intestinal digestion of human milk (HM) coupled with k-means non-hierarchical clustering analysis. Peptides detected in at least 3 intestinal digestion times were considered. Different spots represent the relative abundance of single peptides (1: maximum value during digestion). Different colored spots identify different clusters. B) The number of peptides and average relative abundance trend in each cluster and for each HM processing (raw HM: gray line; holder pasteurization: blue line; high temperature-short time pasteurization: pink line). C) Pie charts representing the relative weight of the human milk proteins in each cluster. α-lactalbumin: LALBA; α_{s1}-casein: CASA; bile acid lipase: BAL; β-casein: CASB; κ-casein: CASK; lactoferrin: LTF; Polimeric immunoglobulin receptor: PigR; osteopontin: OPN; xanthine dehydrogenase: XDH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

intestinal digestion from 926 at 30 min of intestinal digestion to 354 at 180 min of intestinal digestion. The mean size of the released peptides was smaller than those in the gastric phase and decreased from about 1550 Da to about 1270 Da; the detected peptides also showed a more acidic pI, that is, from 5.6 to 5.2 at I180. The relative proportion of peptides common to all the treatments was always above 73%, with the exception of I120, which was below 60% (Fig. 1C). The peptides detected in the intestine were mostly derived from CASB (about 40% of the peptides at each time point), BAL (10–11% at all the digestion times), and LTF (from 10% at I30 to 7% at I180). At the beginning of intestinal digestion, about 15% of the detected peptides were characteristic of pasteurization, as detected after both processing treatments but not in RHM. The unique peptides in RHM reached their highest value at I120 (about 27%), while very few unique peptides were found in either of the pasteurized milk types (about 1–2% at each time point - Fig. 1C). The characteristics of the unique peptides are reported in Supplementary Table 4A. The unique peptides originating from CASB in HTST were longer in size than those in both RHM and HOP. Nine bioactive peptides (2 with ACE-inhibitory activity, 2 stimulating proliferation, 1 with immunomodulatory activity, 1 antioxidant, 1 with antimicrobial activity, 1 with opioid activity and 1 with PEP inhibiting activity) were found at the beginning of intestinal digestion. After 180 min, 7 bioactive peptides were detected in all three samples. The antimicrobial peptide (CASB 182–198) was only detected in the pasteurized HM samples at I30 and I60.

Two-way ANOVA revealed a significant difference in the treatment factor for the abundance of 98 peptides (Supplementary Table 4B). Most of these peptides (n = 81) were also affected by the digestion time, although only 12 showed a significant interaction between time and processing. More than half of the significantly different peptides (n = 52) were released by the intestinal digestion of CASB. Forty peptides, 37 of which were derived from caseins, were detected at significantly lower levels in HTST than in either RHM or HOP. Sixteen peptides (10 from caseins, 3 from PIGR, 2 from xanthine dehydrogenase - XDH - and one from OPN) were released in similar amounts in RHM and HTST, and significantly more in HOP. Twelve peptides were differentially released by intestinal digestion in the same way after both pasteurization methods, compared to RHM: 4 from CASB were released more by RHM, while 6 from BAL and 2 from LTF were more abundant after both pasteurization processes. Eight peptides were significantly different in all the samples, with the BAL peptides (n = 3) always being released more according to a HOP > HTST > RHM trend, while the others showed a HOP > RHM > HTST trend. Finally, 14 peptides (9 from CASB) were significantly less abundant in HTST than in HOP. One bioactive opioid peptide (CASB 51–58) was significantly lower in HTST than in RHM. Another three bioactive peptides were found to be significantly different, by means of two-way ANOVA, and were more abundant in HOP, followed by RHM and finally by HTST.

3.7. Peptide mapping onto the parent protein sequences

The first six proteins at the origin of the identified peptides are indicated in Fig. 6, which shows the average cumulative peptide abundances on the protein sequence during digestion. The sequence was

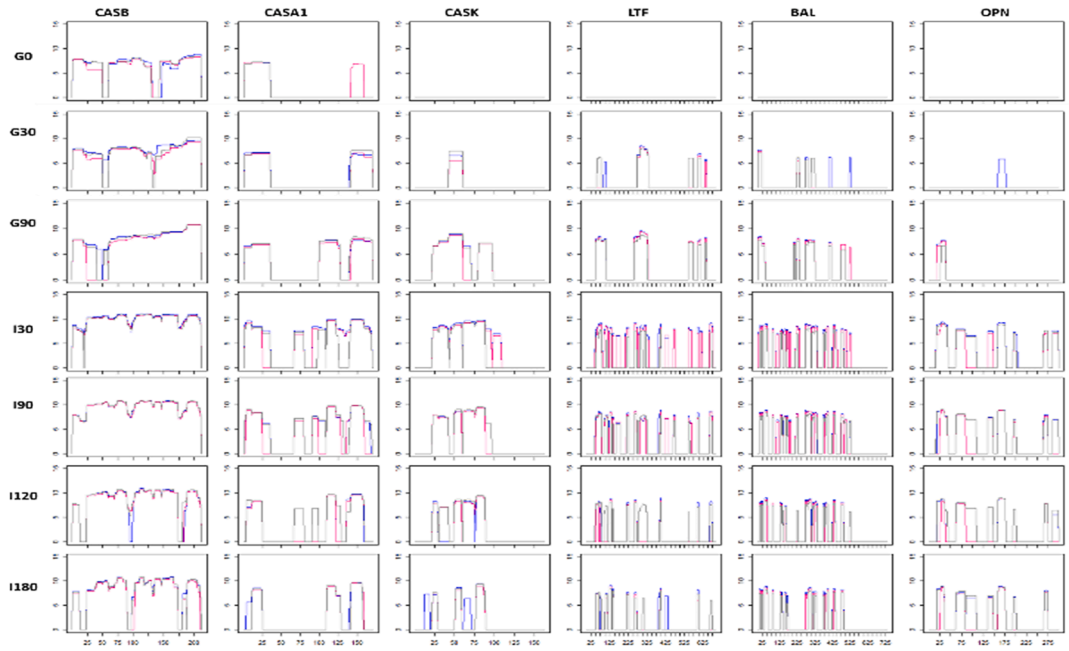

Fig. 6. Mapping of the cumulative aminoacid abundance for the first 6 most abundant human milk (HM) (G0) proteins during gastric digestion (G30-G90) and intestinal digestion (I30-I180) for each HM processing (raw HM: gray line; holder pasteurization: blue line; high temperature-short time pasteurization: pink line). β-casein: CASB; α_{s1}-casein: CASA1; κ-casein: CASK; lactoferrin: LTF; bile acid lipase: BAL; osteopontin: OPN. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

100%covered by peptides during the entire digestion process only for CASB. During the gastric phase, CASB presented peptides that were more abundant for HOP on several parts of the sequence, although the abundances on the sequence were very similar for all the processing treatments at G90. The CASK sequence was covered slightly less in HTST in the gastric phase, but not in the intestinal phase. It should be noted that it was not possible to identify peptides from the C-terminal part of CASK with the method used in this study, due to the high number of glycosylation sites present on this side of the protein. The intestinal digestion patterns of the more abundant HM proteins were differently shaped over time, according to the processing technique. This was in particular evident for LTF, BAL and OPN, with their sequences mostly being covered by both pasteurization methods up to I90, while this occurred after 120 min in RHM.

4. Discussion

The present analysis represents the first peptidomic investigation of the effects of different HM pasteurization methods on the kinetics of peptide release in a simulated dynamic gastrointestinal digestion of preterm newborns. A selective impact of the heat treatment has been demonstrated, depending on the origin of the protein.

Before digestion, the peptidomic mapping revealed a high level of similarity with previous findings on HOP pasteurized term and preterm milk (Deglaire et al., 2016; Deglaire et al., 2019). The same number of peptides was in fact detected in both raw and pasteurized term milk (Deglaire et al., 2016), and the relative proportion of release from CASB, CASA and PIGR was similar. A higher number of proteolytic fragments were reported in undigested preterm milk (Deglaire et al., 2019), and this is generally attributed to an evolutional adaptation of mother's own milk aimed at providing the preterm neonate with a more readily available nitrogen source than intact proteins (Armaforte et al., 2010). The presence of peptides in undigested milk may also be attributed to the activity of proteolytic bacteria possibly contaminating the starting milk pool, although the majority of them are compatible with the activity of endogenous milk proteases (Deglaire et al., 2016; Deglaire et al., 2019). Nielsen and colleagues (Nielsen et al., 2017) also found a higher number of pre-digestive peptides in unpasteurized term HM, using the Orbitrap Fusion technology, likely due to the higher sensitivity of the equipment, and to the different sample purification procedures. Consistently with previous observations (Deglaire et al., 2016; Deglaire et al., 2019), the HOP pasteurized milk presented a greater number of specific CASB peptides, as well as more abundant peptides, than the raw and HTST pasteurized milk, particularly in the 75–100, 110–125 and 200–211 regions. The 75–100 and 110–125 regions include cleavage sites for plasmin, i.e. lysine or arginine residues (Deglaire et al., 2016). HOP pasteurization may have activated the milk plasmin system, through an inactivation of the plasminogen activator inhibitors and plasmin inhibitors (Ismail & Nielsen, 2010). LTF, a natural inhibitor of plasminogen activation (Zwirzitz et al., 2018), was more severely denatured in HOP than in HTST (67% vs. 25% of denatured lactoferrin, as reported elsewhere (Nebbia et al., 2020)), and was thus likely to have had a more reduced inhibitory activity in HOP than in HTST. The 200–211 region was shown to be particularly prone to cytosol aminopeptidase hydrolysis (Deglaire et al., 2016), and may thus be indicative of a greater activity in HOP milk. Peptide mapping thus points toward a high similarity of RHM and HTST milk, in terms of released peptides at a gastric level, thus confirming that the HTST treatment is better able to retain the original RHM profile than HOP, as previously demonstrated for other parameters (Giribaldi et al., 2016). Nevertheless, the higher release of peptides following HOP may represent a nutritional advantage, in terms of providing more readily available nitrogen sources to preterm infants (Armaforte et al., 2010). However, whether this initial advantage results in an overall higher absorption of these proteolytic fragments is still a matter of debate.

In the present study, the kinetics of peptide release during digestion showed differential trends in the gastric and intestinal phases, which were therefore analysed separately to obtain a better distinction. This approach indicated that the overall variability between the gastric and intestinal peptides was high, with respect to the variability between the processing treatments. Furthermore, previous reports (Deglaire et al., 2016; Deglaire et al., 2019) also indicated that the peptide release trends were characterized by different dynamics in the two phases, thus enforcing the idea that separate analyses may provide a more detailed

picture of the differential trends of processing treatments.

During the gastric phase, the time course evolution of the peptide release revealed an increasing number of detected peptides that were common to all the processes. On the other hand, the number of shared peptides was much higher for the two pasteurization methods at the beginning of the intestinal phase, with RHM showing an enhanced number of peptides at 120 min of intestinal digestion. Multivariate analyses allowed such differential trends to be visualized more clearly using PCA. A slightly more similar pattern of proteolysis was found during gastric digestion between HTST and RHM. To the best of our knowledge, this is the first report that has indicated that the HTST method affects the proteolytic digestion of HM in a more similar way to RHM than HOP, although this observation is limited to the gastric phase. Clustering analysis identified two specific clusters of peptides, mainly containing peptides derived from CASB and short acidic peptides, which were more abundant (about 2-fold) after HOP processing throughout gastric digestion. Other peptide clusters showed a slightly delayed increasing trend in HTST, compared to RHM and HOP, mainly due to the LTF and BAL peptides. The present findings on HOP confirm previous observations (Deglaire et al., 2016) on term HM, pertaining to the release of CASB peptides during gastric digestion. Overall, we have observed, for the first time, a lower gastric peptide release from CASB following HTST, than for HOP, which is perhaps due to the lower exposition of specific cleavage sites in HTST. Such a difference was not detected when protein profiling was used in a paired approach (Nebbia et al., 2020), or when mapping the CASB sequence covered by peptides during gastric digestion. The present peptidomic approach has revealed a delayed peptide release from LTF and BAL, two important bioactive proteins for infants, in HM pasteurized by HTST, during the gastric phase, although this was not visible at the whole protein level (Nebbia et al., 2020). This is likely due to a lower level of protein denaturation in HTST than in HOP, as observed for LTF (25% vs. 67% of denatured lactoferrin (Nebbia et al., 2020)). Previous *in vivo* observations on the gastric digestion of raw term HM (Beverly et al., 2019) also reported similar peptide release trends, in particular for LTF. A small number of peptides owing to milk fat globule membrane proteins, such as butyrophilin and fatty acid synthase, were detected during gastric digestion, albeit almost exclusively in RHM. In a recent report (Nebbia et al., 2020), we observed an alteration of fat globule membrane associated proteins following both HOP and HTST. The protein aggregation on the fat globule surface in the pasteurized samples, as observed by means of confocal microscopy and electrophoresis, may have reduced enzyme accessibility, at least in the gastric phase, toward fat globule membrane associated proteins.

As already mentioned, the overall release of peptides showed that the effect of pasteurization was predominant during intestinal digestion, irrespective of what technology was used. RHM did not group with the pasteurized HM samples at the beginning of intestinal digestion, as shown by means of the clustering approach, which revealed a delayed increase in intestinal peptides originating from RHM in all the clusters. The cluster that contained most of the intestinal peptides derived from LTF showed higher abundances in both pasteurized HM, as was also observed for the LTF sequence coverage of the intestinal digestion over time. The peptide release trend reflected the protein trend, as already described (Nebbia et al., 2020), with LTF being barely detectable in the pasteurized samples, already after 30 min of intestinal digestion. This ultimately seems to result in a higher intestinal digestion of this protein, following pasteurization. An almost overlapping trend was observed for BAL during intestinal digestion of the pasteurized vs. raw HM. The BAL sequence also resulted to be digested more in the early intestinal phase, when pasteurized. Considering that both LTF and BAL are bioactive proteins, the delay in their intestinal digestion in RHM may be of particular relevance for preterm infants, whose digestive system is often immature, since such a delay may indicate a prolonged supporting activity to digestion (BAL) or even a prolonged protection (LTF). The latter possibility has still not been adequately investigated, although the intact protein has been detected in the stools of breastfed infants (Lönnerdal, 2013), and protection is known to also be conveyed by specific LTF bioactive peptides. Nevertheless, it should be mentioned that, in the present analysis, no bioactive peptides from LTF were identified at any time or for any milk type, perhaps due to the specific sample preparation method used in the present experiment, including the strict parameters considered to match the sequences (100% identity).

Very few peptides from immunoglobulin subunits were found in all the samples during digestion. This could be due to the ability of secretory IgAs to survive digestion (Demers-Mathieu et al., 2018), as demonstrated by their detection in the stools of breastfed infants (Schanler et al., 1986), since this characteristic is directly related to its function as a passive immunity system for infants (Lönnerdal, 2013).

Finally, as already observed in the gastric phase, more than half of the peptides derived from milk fat globule membrane proteins (FASN, XDH, BT) were detected exclusively in RHM during intestinal digestion, thus enforcing the hypothesis that the pasteurization of HM, irrespective of the processing method, may lead to a shielding effect over the surface of fat globules, thus reducing the proteolysis of some embedded proteins in the milk fat globule membrane (Nebbia et al., 2020).

The present study has indicated, for the first time, that the digestion of HM proteins does not follow the same pattern as that of peptide release, when different pasteurization methods are used. Our results show that, at least at a gastric level, HTST pasteurization is able to retain a closer peptide release pattern to that of raw milk than HOP. The peptide release pattern of HM proteins during the intestinal phase, showed different characteristics from RHM for both pasteurization methods. Whether or not this could ultimately affect the nitrogen availability in some way warrants further *in vivo* investigation, in which other omics approaches, such as metabolomics, should be included.

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CRediT authorship contribution statement

Marzia Giribaldi: Conceptualization, Data curation, Project administration, Writing – original draft. **Stefano Nebbia:** Investigation, Methodology, Writing - review & editing. **Valérie Briard-Bion:** Investigation, Methodology, Writing - review & editing. **Julien Jardin:** Investigation, Methodology, Writing - review & editing. **Chiara Peila:** Resources, Methodology, Writing - review & editing. **Alessandra Coscia:** Resources, Methodology, Writing - review & editing. **Didier Dupont:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing. **Laura Cavallarin:** Conceptualization, Supervision, Project administration, Writing - review & editing. **Amélie Deglaire:** Conceptualization, Supervision, Methodology, Data curation, Project administration, Writing – original draft.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marzia Giribaldi, Laura Cavallarin, and Alessandra Coscia have competing interests, since they are the inventors of a patent pertaining to the HTST human milk pasteuriser used in the study (Patent no. EP2974603 A1). No conflict of interest exists for the remaining authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130998.

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