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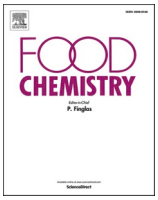

Peptidomic profile of human milk as influenced by fortification with different protein sources: An *in vitro* dynamic digestion simulation

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ABSTRACT

Fortification of human milk (HM) is often necessary to meet the nutritional requirements of preterm infants. The present experiment aimed to establish whether the supplementation of HM with either an experimental donkey milk-derived fortifier containing whole donkey milk proteins, or with a commercial bovine milk-derived fortifier containing hydrolyzed bovine whey proteins, affects peptide release differently during digestion. The experiment was conducted using an *in vitro* dynamic system designed to simulate the preterm infant's digestion followed by digesta analysis by means of LC-MS-MS. The different fortifiers did not appear to influence the cumulative intensity of HM peptides. Fortification had a differential impact on the release of either donkey or bovine bioactive peptides. Donkey milk peptides showed antioxidant/ACE inhibitory activities, while bovine peptides showed opioid, dipeptil- and propyl *endo*- peptidase inhibitory and antimicrobial activity. A slight delay in peptide release from human lactoferrin and α-lactalbumin was observed when HM was supplemented with donkey milk-derived fortifier.

1. Introduction

Human milk (HM) is considered the best feeding choice for all infants, in particular for preterm newborns and very low birth weight infants, as stated by the World Health Organization (WHO/UNICEF, 1981) and by the American Academy of Pediatrics (Meek & Noble, 2022). Under specific clinical conditions, HM alone may not be enough to meet the increased nutritional needs of preterm newborns. The suggested strategy in Neonatal Intensive Care Units is to fortify HM with additional proteins to meet the target requirements for neonatal nutrition (Arslanoglu et al., 2019; Fabrizio et al., 2020). Commercially available fortifiers are commonly derived from either intact or hydrolyzed bovine milk (BM) proteins. Clinical studies have shown that the fortification of HM with BM-based fortifiers may result in gastrointestinal intolerance episodes (Kreissl et al., 2013; Rochow et al., 2011). Feeding interruptions resulting from intolerance phenomena are common in preterm infants, and may negatively impact their growth and nutrition, also being a clinical indicator for the risk of developing necrotizing enterocolitis. Signs and symptoms of feeding intolerance are various: diagnosis can be made by measuring gastric residual volume, either coupled to abdominal distension or not, or observing abdominal distension and gastrointestinal symptoms (including vomiting, bilious

Abbreviations: ACE, Angiotensin Converting Enzyme; ANalysis Of Variance, ANOVA; Bile salt-stimulated lipase, CEL; Bovine milk, BM; Donor human milk fortified with bovine milk-derived fortifier, BMF; Donkey milk, DM; Donor human milk fortified with donkey milk-derived fortifier, DMF; Grand Average of Hydropath, GRAVY; High Performance Liquid Chromatography, HPLC; Human Milk, HM; Isoelectric point, pI; Lactoferrin, TrFL; LC-MS-MS, Liquid Chromatography Tandem Mass Spectrometry; Molecular mass, Mr; Osteopontin, Ostp; Principal Component Analysis, PCA; α-lactalbumin, LAlbA; α_{s1}-casein, CasA1; α_{s2}-casein, CasA2; β-casein, CasB; β-lactoglobulin, LacB; κ-casein, CasK.

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vomiting and blood in stool), and by a combination of the above mentioned factors (Weeks et al., 2021). The reason underlying the occurrence of feeding intolerance episodes might partly rely in the well-known differences between BM and HM proteins, in terms of both relative abundance and molecular structure. One of the believed factors that seems to play a role in the onset of feeding intolerance to BM proteins is the higher hardness of the curd formed in the stomach, which might increase the persistence of BM proteins in the digestive system (Roy et al., 2020). However, milk from monogastric animals (donkey, mares) has been shown to contain proteins that have a similar profile to that of HM, in terms casein-to-whey-protein ratio - 1.26 for donkeys, 0.67 for humans, and 4.69 for bovines (Tidona et al., 2014) - and in the quantity of specific protein fractions. The major equine milk casein constituent, unlike BM, is β-casein, followed by αs1-casein, while αs2-casein is present in trace amounts in equine milk, and absent in HM (Barłowska et al., 2011; Bertino et al., 2010; Tidona et al., 2014).

Donkey milk (DM) has been proposed as an alternative to BM for infant nutrition, due to its close protein and lipid profile to HM (Bertino et al., 2010; Gastaldi et al., 2010; Valle et al., 2018). DM fat contains higher proportions of mono- and poly-unsaturated fatty acids, and a higher ratio of ω-6 to ω-3 fatty acids, with respect to BM, and more similar to HM. Also, DM has the closest proportion of palmitic acid located at the sn-2 position to that of HM (Roy et al., 2020). Antioxidant, Angiotensin Converting Enzyme (ACE)-inhibitory and antimicrobial peptides are released during *in vitro* static gastrointestinal digestion of DM proteins (Piovesana et al., 2015; Tidona et al., 2011). Furthermore, it was shown, in a murine model, that feeding a diet containing DM decreased the deposition of body lipids and affected the glucose and lipid metabolism in a manner more similar to HM than to BM (Lionetti et al., 2012; Trinchese et al., 2015, 2018). Finally, because of the differences with BM protein structure, DM has also been demonstrated to be alternative for children with severe BM protein allergies (Monti et al., 2007; Sarti et al., 2019): most of bovine linear epitopes on caseins have a low resemblance to that of HM and DM; despite a high level of identity (> 70%) among BM, HM and DM, the linear epitopes of bovine α-lactalbumin and serum albumin show high difference with the corresponding domains present in the donkey's homologues. β-lactoglobulin, the most recognized allergen of bovine whey, shares low sequence identity (< 56% of identity) with respect to its equine counterparts, whereas it is absent in HM, also occurring mainly as dimers in BM, whereas it has a monomeric structure in DM (Cunsolo et al., 2017).

To date, few investigations have been carried out on the impact of HM fortification on preterm infant digestion. Fortification with a BM-based fortifier, containing intact whey proteins, was found to limit the release of peptides from HM proteins (Beverly et al., 2019; Nielsen et al., 2018), and similar results were observed when using an *in vitro* static digestion and an highly hydrolyzed whey protein fortifier (Pica et al., 2021). In a clinical trial conducted by the authors (Coscia et al., 2018), preterm newborns and very low birth weight infants received an isocaloric and isoproteic supplementation of HM with either a BM-based fortifier, containing highly hydrolyzed whey proteins, or an experimental DM-based fortifier, containing whole milk proteins. We found that the use of DM-based fortifier significantly reduced the occurrence of feeding intolerance, feeding interruptions, bilious gastric residuals, and vomiting (Bertino et al., 2019; Cresi et al., 2020), and that it led to similar auxological and neurodevelopmental outcomes to the BM-based counterpart (Peila, Spada, Bertino, et al., 2020; Peila, Spada, Deantoni, et al., 2020). The use of BM- and DM-based fortifiers also showed similar impact the development of allergic manifestations in the first 6–8 years of life (Peila et al., 2024). We hypothesized that the observed different biochemical quality of the two fortifiers may have resulted in an overall different digestion behavior of the fortified HM, and that such a difference ultimately led to the observed diversity in terms of feeding tolerance. We therefore designed an experiment to assess the digestion kinetics of lipids and proteins of HM samples fortified with BM or DM fortifiers using an *in vitro* dynamic digestion model designed to simulate preterm infant digestion conditions. We found that both fortifiers resulted in a similar net degree of proteolysis and lipolysis at the end of digestion, but a different profile of released free fatty acids and free amino acids (Nebbia et al., 2022).

In the present study, we have performed a comprehensive peptidomic investigation of HM supplemented with isoproteic and isocaloric BM-based or DM-based fortifier, and digested using *in vitro* dynamic digestion at the preterm stage, to gain further insight into the fate of HM proteins, as affected by the type of fortification, during preterm digestion.

2. Material and methods

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

Milk samples were collected, fortified and digested, by means of DIDGI® (DIgesteur Dynamique Gastro-Intestinale), as previously described (Nebbia et al., 2022). Preterm HM (35–37 weeks gestational age) was collected 1 to 3 months after delivery from 5 healthy donors (age 27–35 years), stored at −20 °C for 1–6 months, pooled and pasteurized (Metalarredinox, Verdellino, Italy) (62.5 °C for 30 min) at the donor HM bank of the Regina Margherita Childrens' Hospital in Turin (Italy). The present study (ISRCTN70022881) was conducted in accordance with the Declaration of Helsinki. Each milk donor involved in this research signed a written consent form in which the data protection of the parents and infant was assured. Moreover, the donors were informed that only milk samples stored in excess would be used for research purposes.

HM was fortified either with an experimental multi-component fortifier powder from DM (Coscia et al., 2018), or with a commercially available multi-component fortifier powder (FM85 Nestlé, Switzerland) containing extensively hydrolyzed bovine whey proteins. The experimental DM-based product was produced by ultrafiltration of pasteurized DM in a pilot stainless steel plant. Retentate from the ultrafiltration process was then pasteurized and aseptically lyophilized and packed, after assessment of compliance to the microbiological and chemical requirements and safety criteria by Italian legislation. The two fortifiers were added to 100-ml pooled donor HM to achieve isoproteic (as nitrogen content) and isocaloric fortified meals, which included a protein supplementation of 1 g and 18 kcal *per* 100 mL of HM, thought provided as intact proteins for DM, and extensively hydrolyzed whey proteins for BM. From now on, the terms DMF and BMF will be used as acronyms for the respective dietary types, *i.e.* DMF - donor HM fortified with DM-derived protein fortifier, BMF - donor HM fortified with BM-derived protein fortifier.

The DIDGI® system (Ménard et al., 2014) was used to simulate the digestion of a preterm newborn at a postnatal age of four weeks, as previously detailed (De Oliveira et al., 2016; Nebbia et al., 2020). Digestions were performed in triplicate, and aliquots (20 mL) were collected before digestion (G0), after 30 and 90 min in the gastric phase (G30 and G90), and after 30, 60, 90 and 180 min in the intestinal phase (I30, I60, I90 and I180). Following subsampling, enzymes were inhibited with 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, before storage at −20 °C.

2.2. Identification and quantification of the peptides

Mass spectrometry (MS) analysis was conducted as previously described (Deglaire et al., 2016; Giribaldi et al., 2022). A nanoRSLC U3000 system (Dionex, Amsterdam, The Netherlands), fitted to a Q-Exactive mass spectrometer (Thermo Scientific, San Jose, The USA), equipped with a nanoelectrospray ion source, was used for this purpose. Digesta samples were thawed, 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) 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 A solvents [2% (*v*/v) acetonitrile, 0.08% (v/v) formic acid, and 0.01% (v/v) trifluoroacetic acid in HPLC gradient grade water] and B solvents [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, and then then up to 85% 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 amu 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 of each MS scan, and were excluded from fragmentation for 15 s. Peptides were identified from the MS/MS spectra using X!TandemPipeline software, version 0.2.38 (Langella et al., 2017). Sequences were identified using a *Equus asinus* database (135 reviewed proteins and 1389 unreviewed proteins from UniprotKB, https://www.uniprot.org/) for DM peptides, a HM protein database (Molinari et al., 2012) for HM peptides, and a homemade database composed of major BM proteins (4654 reviewed proteins) for BM peptides, while possible non-specific sequences were targeted using the common Repository of Adventitious Protein (http://thegpm.org/crap).

The possible post-translational modifications were serine or threonine phosphorylation, methionine oxidation, lysine or arginine lactosylation, and the cyclisation of glutamine or glutamic acid into pyroglutamic acid. 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%.

Each identified peptide was quantified, by means of label-free MS, using MassChroQ software (Valot et al., 2011). An 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.

2.3. Biochemical characteristics of the peptides

The peptides were characterized for their biochemical features: the isoelectric point (pI), the molecular mass (Mr), the peptide length, the aliphatic index, and the Grand Average of Hydropathy Value (GRAVY), as defined by the ExPASy "ProtParam tool" (Gasteiger et al., 2005). In this way, a positive GRAVY corresponded to a hydrophobic peptide and a negative one to a hydrophilic peptide (Kyte & Doolittle, 1982). Human and bovine bioactive peptides were searched for within the BIOPEP database (Minkiewicz et al., 2008) and the MBPDB database (Nielsen et al., 2017), both of which were accessed in April 2024. The exhaustive review of Guha et al. ((Guha et al., 2021) was used to search for the bioactive peptides of DM. Only an exact matching between sequences was considered.

2.4. Statistical analyses

The data analysis was performed using PAST software, version 4 (Hammer et al., 2001), on a subset of unique peptide sequences, *i.e.,* peptides detected in more than one protein database (human, donkey or bovine) were not considered. When not stated differently, statistical analyses, counts, histograms and pie charts were obtained using Excel software functions, and assembled into Figures using a combination of PowerPoint and Paint utilities. The MS/MS abundance of each peptide was corrected for digestion time-specific dilution factors, which resulted 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. Histograms reporting the number of peptides detected at the different digestion times and grouped by organism (human, donkey or bovine) were created using Excel utility, as were pie charts representing the original protein source in terms of percentages.

Different overall multivariate analyses of variance (Principal Component Analysis - PCA) were conducted separately on all the peptides (no. = 2206), on all the human peptides (no. = 1251), on DMF and BMF peptides detected in gastric digestion (no. = 752), or only on gastric HM peptides (no. = 436), and on DMF and BMF intestinal peptides (no. = 1621), or only on intestinal HM peptides (no. = 896), by considering each peptide [log10(abundance)] as a variable and the sampling time of each digestion replicate and each fortification as an individual. Sporadic peptides that were only present in 1 sample replicate were considered to be absent and were given an arbitrary value of 1 for [log10(abundance)] in the analysis. Any missing peptide values, which were absent in 1 out of 3 replicates of a sample, were coded with a "?", and the PCA was run using the iterative imputation option for missing value handling. PCA was conducted by giving a different color code to each fortification, and by setting each digestion time point as a group. Data were automatically auto-scaled before analysis by means of PAST software.

The peptide clustering procedure was performed separately on only the DM and HM peptides, because of the low number of detected bovine peptides. Two types of clustering analysis were conducted, taking into consideration only peptides that were detected at least at two gastric and three intestinal digestion times. 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, at a first instance, for 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 then performed by setting the number of target clusters according to Ward's clustering results. The results of the two clustering methods were compared to obtain the best overlap between the two methods. The data included in the clustering procedure were visualized by means of PCA, wherein the peptides were set as samples, the cluster number as a group, and the relative abundance as a variable. The association of the qualitative and quantitative characteristics with each cluster was checked by means of Fisher's test and the Kruskal-Wallis test, respectively.

Univariate statistical analyses were conducted separately on the peptides present during gastric digestion or during intestinal digestion, or during both. An analysis of variance (ANOVA) was conducted on the [log10(abundance)] of the peptides present in at least one of three replicates at all the considered time points. Two-way ANOVA (for gastric or intestinal HM peptides, considering the fortification type, time and their interaction as factors) and one-way ANOVA, or Kruskal-Wallis, in the case of non-normal residues (for DM and BM peptides, considering time as the factor) 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). A fortification factor was considered significant when the difference between the mean peptide abundance of the two different fortification types (BMF and DMF) was > ± 10% and the p-value was <0.05. A digestion time factor was considered significant when statistical significance was reached (p-value <0.05), and a difference > ±10% was found between the minimum and maximum abundances. An additional two-way ANOVA was fitted to the cumulative [log10(abundance)] of the HM peptides detected in each digestion replicate at each time, by setting the type of fortification, considering the time and their interaction, as factors.

3. Results

3.1. Biochemical characteristics and origin of the peptides

The numbers of HM, BM, and DM peptides detected at each sampling time for each replicate digestion are reported in Supplementary Table 1. Overall, for the gastro-intestinal digesta, 1254 HM peptides were identified from 27 HM proteins, with only 13 and 16 human peptides being exclusively identified in the DMF and BMF fortified meals, respectively (Fig. 1A). Most of the detected HM peptides were derived from β-casein

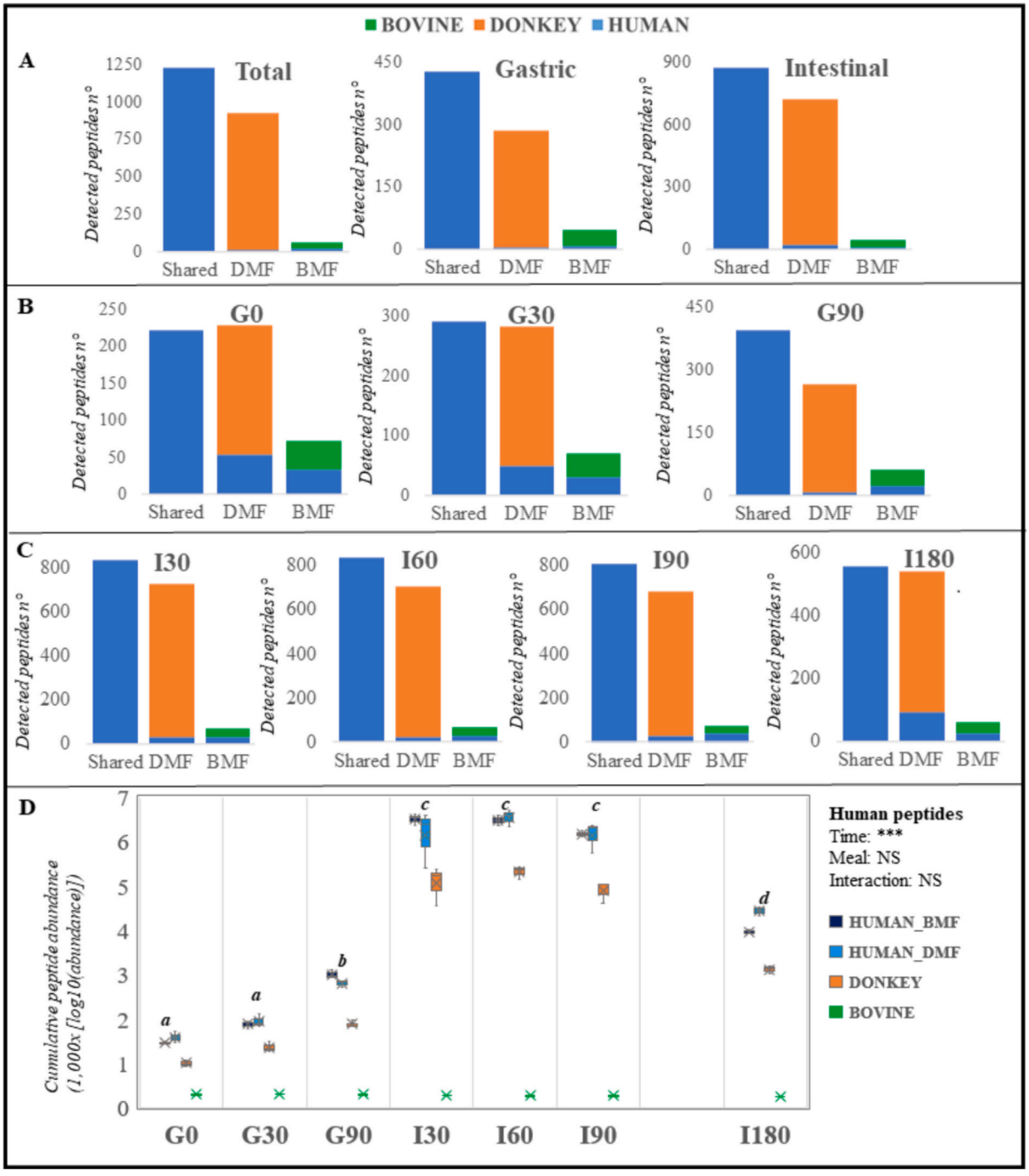

Fig. 1. Graphical representation of the number and abundance of the peptides detected in human milk fortified with a commercial protein fortifier derived from bovine milk (BMF) or with an experimental protein fortifier derived from donkey milk (DMF). Different colors code for different source organism of the peptides: blue for human origin, orange for donkey origin, green for bovine origin. A) Number of peptides detected overall (total), or only during gastric/intestinal digestion. B) Number of peptides detected at gastric digestion times of 0, 30 and 90 min (G0, G30, G90). C) Number of peptides detected at intestinal digestion times of 30, 60, 90 and 180 min (I30, I60, I90 and I180). D) Cumulative abundance of the peptides detected at each digestion time. The graphs represent box plot distribution, and bars represent minimum and maximum values. Human_BMF (dark blue): human peptides in milk fortified with BMF; Human_DMF (light blue): human peptides in milk fortified with DMF; Donkey (orange): donkey milk peptides; Bovine (green): bovine milk peptides. Two-way ANOVA of the human peptide abundance: time: *** ($p < 0.001$); meal: not significant ($p > 0.05$); no interaction. Post-hoc classes (for time) are labelled with different bold italic letters. (The reader can refer to the web version of this article to interpret the references to color). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(CasB_HM, 49.9%), lactoferrin (TrFL, 11.5%), bile salt-stimulated lipase (CEL, 6.9%), osteopontin (Ostp, 6.2%), and αs1-casein (CasA1_HM, 5.0%). The identified peptides were 6 to 50 amino acid long, and their size ranged from 597 to 6399 Da. A further 914 peptides were identified in the DMF samples as originating from DM-specific proteins (Fig. 1A). The majority of the DM peptides were released by β-casein (CasB_DM digestion, 41.4%), and these were followed by those originating from β-lactoglobulin (LacB_DM, 20.4%), αs1-casein (CasA1_DM, 16.2%) and αs2-casein (CasA2_DM, 13.6%). The number of amino acid residues from the DM peptides ranged from 6 to 50, and the mass ranged from 627 to 6394 Da. Only 46 peptides from BM proteins were identified in the BMF samples (Fig. 1A), with the majority being released by β-casein (CasB_BM, 45.7%) and β-lactoglobulin (LacB_BM, 26.1%). The number of amino acid residues in the BM-derived peptides ranged from 6 to 15, and the mass from 627 to 1691 Da. The peptides released during gastric digestion from the BM proteins were on average shorter (about 958 Da *vs.* 2457 Da and 2577 Da for donkey and human peptides, respectively), and more acidic (5.2 *vs.* 6.9 of pI); the mean biochemical characteristics of the peptides originating from BM remained almost unaffected during intestinal digestion, while the mean size of the DM and HM peptides decreased (about 1500 Da) and pI decreased (about 5.7) in comparison to the gastric phase.

Before digestion (Fig. 1B), 308 HM peptides were detected; 72.1% of which were common to both DMF and BMF. Moreover, 175 additional peptides, originating from DM proteins, were identified in DMF. Forty peptides originating from BM proteins were identified in the G0 (undigested) BMF. About half of the peptides detected in the undigested samples were from CasB_HM, and these were followed by CasB_DM (22.6%), CasA1_DM (4.4%), and CasA2_DM (3.1%) (Fig. 2B). In general, casein isoforms originated the majority (above 87%) of the peptides detected at G0, regardless of the milk origin.

The number of peptides from HM and DM increased during the gastric phase (Fig. 1B), while the specific peptides from the BM remained stable in number. Furthermore, 45% and 55% of the peptides were common to the two meals at 30 and 90 min, respectively (Fig. 1B), and originated mostly from CasB_HM (80% and 70% of the shared peptides at G30 and G90), and from human TrFL (8% and 11% of the shared peptides at G30 and G90). As far as the unique peptides are concerned, gastric digestion mainly originated peptides from CasB_DM (44% and 52% of the unique peptides at G30 and G90), CasA1_DM (9% and 10% of the unique peptides at G30 and G90), and CasB_HM (15% and 8% of the unique peptides at G30 and G90). At the end of gastric digestion, only 8 HM peptides were exclusive in the DMF fortified meal, and 22 in BMF (Supplementary Table 2A).

The variability of the parent protein of the detected peptides across both meal types and across all species of origin increased from the beginning of intestinal digestion, with casein isoforms originating about 60% of the peptides (Fig. 2C). The numbers of the HM and DM peptides were higher in the intestinal phase than in the gastric phase, while the number was almost steady for peptides originating from BM proteins (Fig. 1C). About half of the peptides were common to the two meals for all the intestinal digestion times, and mostly originated from human CasB (38–42% of the shared peptides), TrFL (9–12%), Ostp (8–10%), and CEL (8%). As for the unique peptides, most of the DM-derived peptides in the intestinal digest were from CasB_DM (27–30% of the unique peptides), LacB_DM isoforms (17–21%), and from CasA1_DM and CasA2_DM (about 11–15% and 10–13%, respectively). With the exception of I180, the proportion of shared and exclusive peptides in DMF and BMF was stable throughout the intestinal phase (Supplementary Table 3A).

Two-way ANOVA was performed on the cumulative abundances of the HM peptides that were common to the two fortification types, and it revealed that the overall digestibility of HM proteins was unaffected by the fortification type, along digestion, while time was a significant factor in modifying the abundance of HM peptides (Fig. 1D). The peptides from DM followed a similar release trend over time to that of HM, while the BM peptides showed a steady cumulative abundance over time.

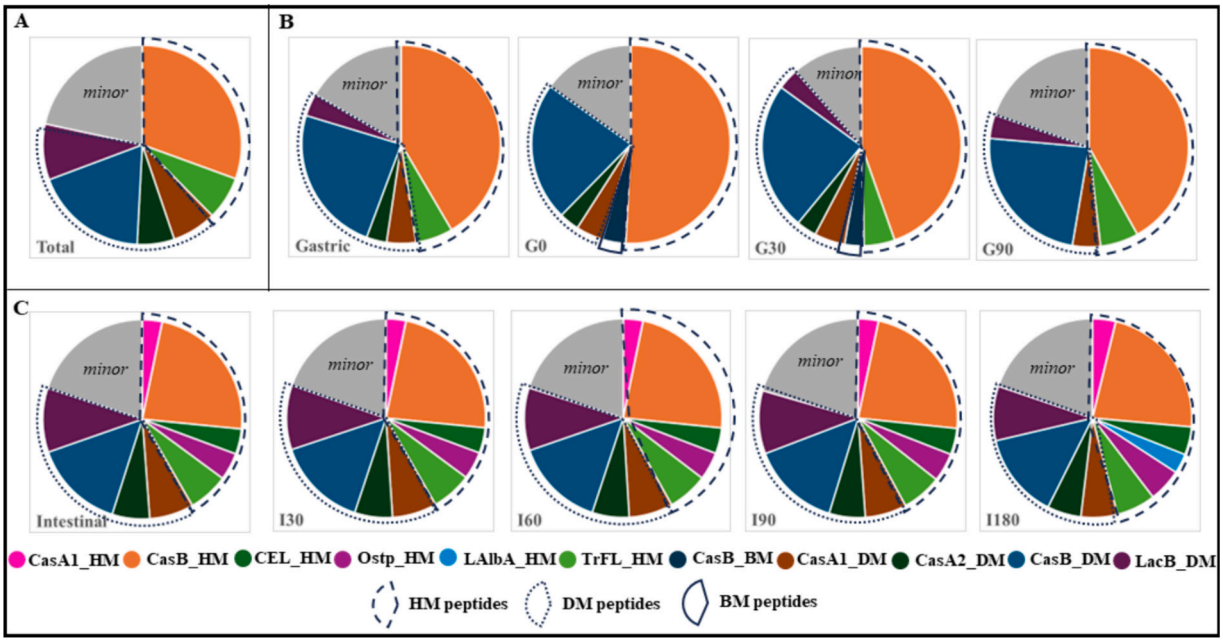

Fig. 2. Pie charts representing the source protein of the peptides detected in fortified human milk using a bovine- or a donkey- derived fortifiers. The percentages refers to the total of peptides detected at each digestion time, irrespective of the origin. The different origins are highlighted by different edge lines (continuous: bovine; dotted: donkey; dashed: human). A) Pie charts representing percentages over total detected peptides. B) Pie charts representing percentages of overall detected gastric peptides, and gastric peptides at digestion times of 0, 30 and 90 min (G0, G30, G90). C) Pie charts representing percentages of overall detected intestinal peptides, or intestinal peptides at digestion times of 30, 60, 90 and 180 min (I30, I60, I90, I180). Protein origin: αs1-casein: CasA1; β-casein: CasB; bile salt-stimulated lipase: CEL; osteopontin: Ostp; α-lactalbumin: LAlbA; lactoferrin: TrFL; αs2-casein: CasA2; β-lactoglobulin: LacB. (The reader can refer to the web version of this article to interpret the references to color).

3.2. Multivariate profiling of the peptide abundances during digestion

The multivariate profiling (Fig. 3) of the abundances of peptides of human origin in gastric and intestinal phases (Fig. 3D and F) allows a distribution of different digestion sampling time along the first principal component (PC1), with respect to the multivariate profiling including peptides from BM and DM (Fig. 3C and E). When peptides of different origin were considered throughout digestion (Fig. 3A), the second principal component (PC2), which accounted for about 18% of the variability, displayed separate profiling of the intestinal samples (positive values on PC1) from the differently fortified meals (negative values on PC2 for peptides from BMF). This separation was not observed when only human peptides were considered (Fig. 3B). A similar behavior was observed when the gastric peptides were considered (Fig. 3C): the PC2, which accounted for about 32% of the variability, showed a separate profiling of the differently fortified meals (negative values on PC2 for peptides from BMF). However, when only human peptides were considered, this separation was reduced (Fig. 3D), and the variability explained by the PC2 decreased to 12%.

A different trend was highlighted when the intestinal peptides were considered: when all the peptides were taken into account (Fig. 3E), including those of bovine and donkey origin, PC1, which represented about 46% of the overall variability, allowed a rough separation to be made of DMF and BMF, while the digestion time was mostly associated with PC2 (26% of the variability). The situation changed when the HM-derived intestinal peptides were considered (Fig. 3F), and, in this case, most of the variability was explained by PC1 (50%) and was associated with the different sampling times. Moreover, the peptide abundances from the first two sampling times (I30-I60) were grouped together.

3.3. Clustering of the human and donkey milk peptides

A non-hierarchical clustering process on the HM peptides (no = 873) highlighted the existence of gastric and intestinal clusters, and the existence of specific grouping features in the intestinal peptides according to the source of fortification (Fig. 4). Six clusters were identified as representative of the main grouping tendencies that emerged from the clustering process (Fig. 4): peptides exclusively detected in the gastric phase are grouped in Cluster A; Cluster B contains peptides that were detected during digestion; Clusters C, D, E and F group the intestinal peptides. Cluster A, which mainly contains gastric peptides, is associated with longer peptides, mainly derived from CasB_HM, which is also positively associated with Cluster B (Fig. 5). Cluster C contains HM peptides that are detected sooner in BMF (G90) that in DMF (I30); cluster D contains HM peptides that are present until I180 in DMF, while not in BMF. Cluster C is associated with acidic peptides, which were significantly more abundantly derived from LAlbA_HM, and Cluster D is significantly associated with higher presence of peptides from TrFL. The remaining clusters, which represent most of the peptides, mainly contain intestinal peptides and show an overlapping trend for both fortified meals.

A non-hierarchical clustering process performed on the peptides

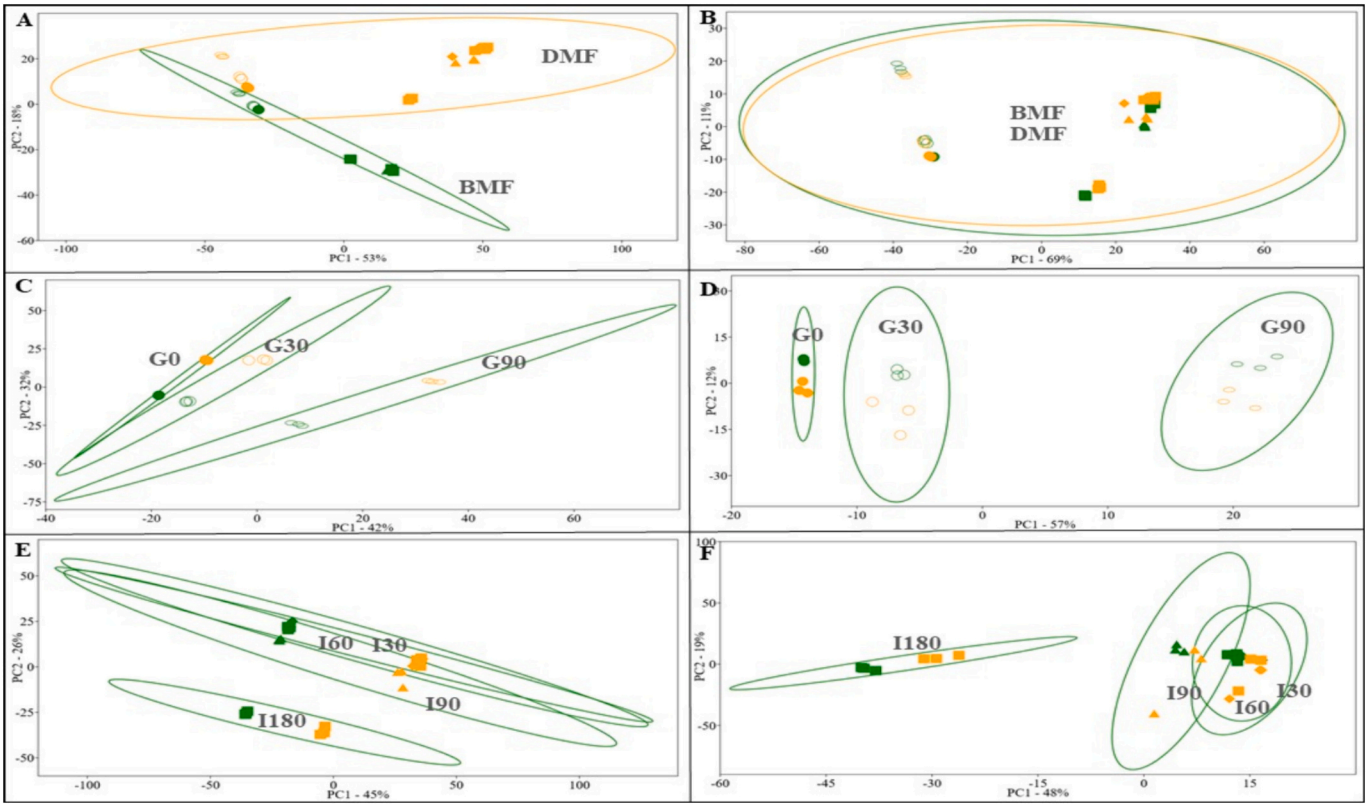

Fig. 3. Principal component analyses (PCA) of the Log10 transformed abundance of peptides during *in vitro* dynamic digestion of human milk fortified with a commercial protein fortifier derived from bovine milk (BMF – in green) or of human milk fortified with an experimental protein fortifier derived from donkey milk (DMF – in orange). Gastric digestion at 0, 30 and 90 min (G0 - ●, G30 - ○, G90 - ○); intestinal digestion at 30, 60, 90 and 180 min (I30 - ▬, I60 - ▲, I90 - ◆, I180 - ■). The ellipses represent 95% confidence intervals. A) PCA of the transformed abundance of the peptides detected during digestion, irrespective of the milk origin; B) PCA of the transformed abundance of human milk peptides detected during digestion; C) PCA of the transformed abundance of peptides detected during gastric digestion, irrespective of the source organism; D) PCA of the transformed abundance of human milk peptides detected during gastric digestion; E) PCA of the transformed abundance of peptides detected during intestinal digestion, irrespective of the source organism; F) PCA of the transformed abundance of human milk peptides detected during intestinal digestion. (The reader can refer to the web version of this article to interpret the references to color). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

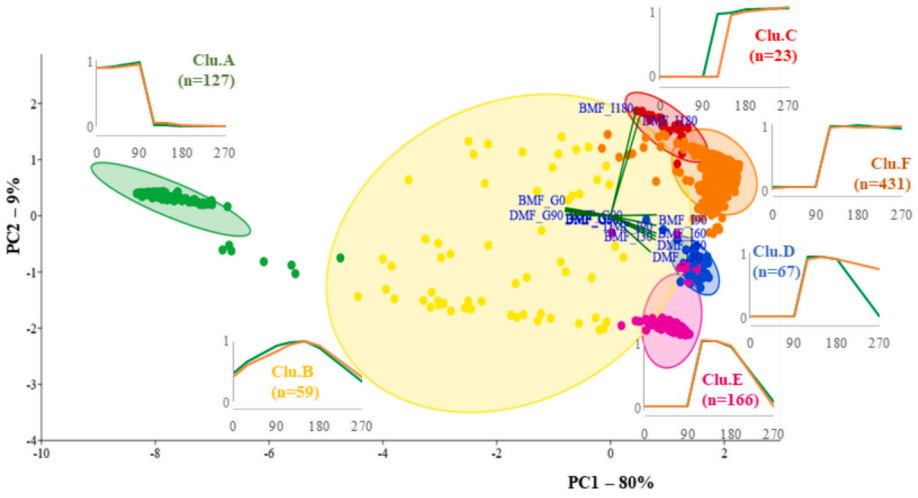

Fig. 4. Principal component analysis (PCA) of the mean scaled Log10 transformed abundance of human peptides during *in vitro* dynamic digestion of human milk fortified with a commercial protein fortifier derived from bovine milk (BMF) or human milk fortified with an experimental protein fortifier derived from donkey milk (DMF), grouped according to the detection profile according to k-means non-hierarchical clustering and Ward's clustering procedure. Peptides that were detected at 3 digestion times at least in both fortified meals were considered. Clu.A-Clu.F: cluster code; the ellipses represent 95% confidence intervals; the green lines represent the biplots on the two main PCs; differently colored spots indicate different clusters. The graphs represent the mean scaled transformed abundance (vertical axis: 0–1) of the peptides in each cluster during digestion, where green indicates BMF and orange indicates DMF. (The reader can refer to the web version of this article to interpret the references to color). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

derived from DM (no = 712) also revealed a differential trend for gastric and intestinal peptides (Supplementary Fig. 1). Four clusters were identified as representative of the main grouping tendencies that emerged from the DM derived peptide clustering process: peptides exclusively detected in the gastric phase are grouped in Cluster J; Cluster K contains peptides that were detected during digestion; Clusters W and Y group the intestinal peptides. Supplementary Fig. 2 represents the relative abundance of the source HM proteins in the different Clusters, and the variables significantly associated with each Cluster. Cluster J, which mainly contains gastric peptides, is associated with longer, less acidic, more hydrophilic peptides, mainly derived from CasB_DM. Similar peptide characteristics are also associated with Cluster K. The two intestinal Clusters are associated with a higher presence of peptides from CasA_DM and CasK_DM in Cluster W, and with shorter peptides in Cluster Y.

The small number of BM peptides (no = 46) was considered insufficient to allow a clustering of the peptide release tendencies during digestion.

3.4. Quantitative variations between samples during digestion

The majority of gastric HM peptides exclusive in either BMF or DMF (Supplementary Table 2A) are represented by CasB_HM peptides (74.7%), followed by TrFL (9.8%); exclusive BMF or DMF peptides are found more at G0 and G30 than at the end of the gastric digestion (17.2%). Two-way ANOVA, performed on the log10-transformed abundances of gastric HM peptides, revealed that 7.5% of the peptides detected in both samples (no =147), all of which were from CasB, were significantly different for the two types of fortification (Suppl. Table 2B). The digestion time resulted to be the factor that significantly affected most of the considered gastric peptides (44.2%). Overall, the CasB_HM peptides that were exclusive or significantly more abundant in BMF during gastric digestion were slightly shorter (2826 *vs.* 3133 Da), and preferentially located at the C- and N-terms of the sequence, while those more abundant in DMF were mainly located in the core.

Most of the exclusive peptides (Suppl. Table 3 A) in the intestinal digestion are represented by CasB_HM peptides (44.7%), followed by TrFL (19.4%), and they were detected more at the end of intestinal digestion (39.1%). Two-way ANOVA, performed on the log10-transformed abundances of intestinal HM peptides, revealed that 10.6% of the peptides detected in both samples (no =528), were significantly different according to fortification (Suppl. Table 3B). The digestion time resulted to be the significant factor that affected most of the analyzed intestinal peptides (41.0%). The significantly different HM peptides between the types of fortification mainly originated from CasB (28.6%), LAlbA (16.1%), CEL (14.3%), and TrFL (10.7%). Overall, the CasB_HM peptides were exclusive or significantly more abundant in DMF than in BMF during intestinal digestion (63.9%). Inversely, the LAlbA peptides, which were influenced by the fortification type in the intestine, were more abundant in BMF, and on average longer than in DMF (1697 *vs.* 1264 Da). About 40.7% of the TrFL exclusive peptides were present at I180, and more often in DMF; in addition, they were also generally more acidic in DMF that in BMF (5.8 *vs.* 7.2). The exclusive/significantly more abundant peptides from Ostp and CasA1_HM were mostly observed in DMF, often at I180, and were generally longer in DMF than in BMF (1630 *vs.* 1002 Da for Ostp; 1427 *vs.* 1053 Da for CasA1_HM).

3.5. Bioactivity

Ten HM peptides, all from CasB, were identical to those reported as bioactive in the literature, and collected in the BIOPEP (Minkiewicz et al., 2008) and MBPDB (Nielsen et al., 2017) databases (Table 1). Two of them were only found before and during gastric digestion and showed proliferative (RETIESLSSSEESITEYK) and antimicrobial activities (QELLLNPTHQIYPVTQPLAPVHNPISV). Two other peptides, with cholesterol binding capacity (VLPVPQ) and propylpeptidase-inhibitory (HLPLPL) bioactivity, were detected during digestion in BMF, and only in the intestinal phase in DMF (Table 1). Six peptides were only detected in the intestinal phase; two of these, with ACE-inhibitory and proliferative activities, significantly increased (NLHLPLP) or decreased (SPTIPFFDPQIPK) during digestion at the end of intestinal digestion (Suppl. table 3). The other bioactive HM peptides were present only in the intestinal phase, and showed opioid, immunomodulatory, and ACE-inhibitory activities (Table 1). Several bioactive peptides were identified as specific from the bovine peptide dataset (Table 1): 11 were specific from CasB_BM sequence, and 3 from LacB_BM. Three bovine bioactive peptides were exclusively detected before and during gastric digestion, one with zinc binding bioactivity from LacB_BM (VEELKPTPEGDLEIL), two from CasB_BM with propylpeptidase-inhibitory activity

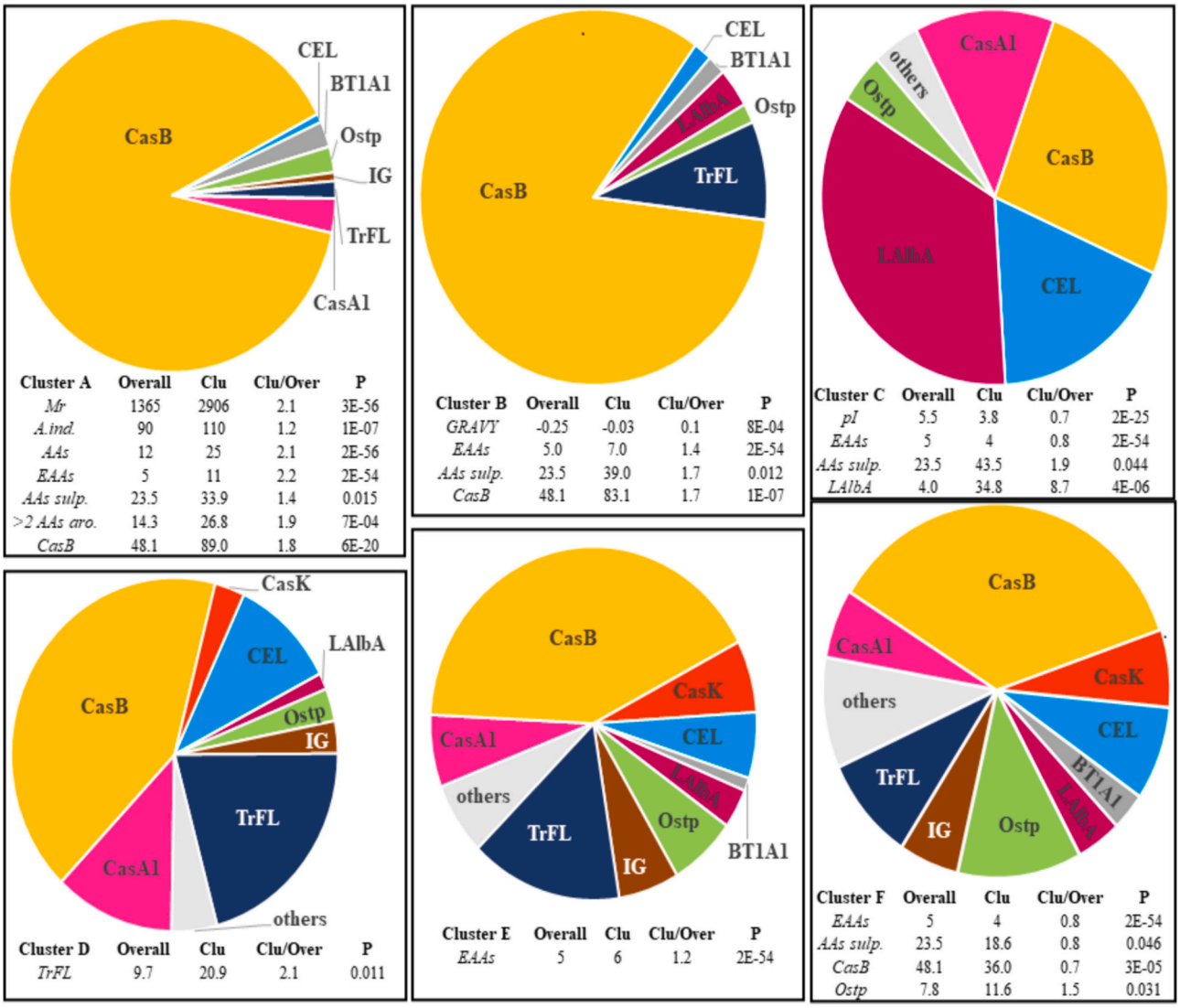

Fig. 5. Pie charts representing the percentages of human proteins in each cluster. The boxed tables report the significantly associated biochemical characteristics for each cluster, on the basis of Fisher's test and the Kruskal-Wallis test for the qualitative and quantitative characteristics. Overall: Occurrence of modality in the overall values; Clu: Occurrence of modality in the cluster; Clu/Over: Occurrence in the cluster/overall modality occurrence; P: Significance of the association with the cluster. Mr.: Peptide mass (Dalton) (median); pI: Peptide isoelectric point (median); GRAVY: Peptide grand average of hydropathy (median); A.ind.; aliphatic index (median); AAs/EAAs: number of total/essential aminoacids (median); AAs sulp.: presence of 1 or more sulphorated AAs; >2 AAs aro.: presence of 2 or more aromatic AAs. BT1A1: Butyrophilin; CasA1: αs1-casein; CasB: β-casein; CasK: κ -casein; CEL: bile salt-stimulated lipase; IG: immunoglobulins; LAlbA: α-lactalbumin; TrFL: lactoferrin; Ostp: osteopontin. (The reader can refer to the web version of this article to interpret the references to color).

(VYPFPGPIP), and ACE-inhibitory/antioxidant activities (YPFPGPIPN). The other BMF peptides were detected at each digestion time, and they showed ACE-inhibitory activity, propylpeptidase- or dipeptidyl peptidase IV- inhibitory activity, opioid activity, and antimicrobial activity (Table 1). Four intestinal and one steady DM peptides were recognized in the DMF as being bioactive, and all showed antioxidant and/or ACE-inhibitory activity. Four of them were released from CasB_DM, one peptide from the LacB sequence (Table 1).

4. Discussion

The present study has been conducted to examine the effects of two types of fortification on HM protein digestion, in the context of improving the quality of clinical nutrition of preterm infants in Neonatal Intensive Care Units. We considered two HM fortifiers that differed in terms of milk origin (bovine *vs.* donkey milk) and of the molecular form of their proteins (extensively hydrolysed bovine whey proteins *vs.* whole DM proteins). We sought to evaluate the effect of the fortification on the release of peptides from human, donkey, and bovine milk after *in vitro* dynamic digestion under conditions designed to simulate the preterm infant's digestion, to highlight whether the difference in the type of protein fortification had an effect on the peptide release kinetics during digestion.

We have recently reported differences in the digestion kinetics of HM protein and lipids and on the release of free amino acids between DMF and BMF (Nebbia et al., 2022). A difference in the number of free AAs was found before and during digestion, due to the originally higher number of free AAs in the BM-based fortifier. However, despite the different protein and lipid compositions of the undigested fortified HM, a similar net overall degree of proteolysis and lipolysis was observed at the end of the intestinal phase for both types of fortified milk.

In the present experiment, we have characterized the peptide release during digestion, from both a quantitative and a qualitative point of view. We found that the number of peptides in undigested preterm HM was similar to what was previously reported for preterm HM (Deglaire et al., 2019a), and higher than the number reported in term HM

Table 1
bioactive peptides found in BMF and/or DMF diets: Protein code and sequence position; amino acid sequence; post translational modifications (position and type); Cluster code (see Fig. 4 and Suppl. Fig. 1 for trend); bioactive function. LacB: Beta lactoglobulin; CasB: Beta casein; BM: bovine milk peptide; DM: donkey milk peptide; HM: human milk peptide; BMF: donor milk fortified with bovine milk-derived fortifier; DPP-IV: dipeptidyl peptidase-IV; ACE: Angiotensin-converting-enzyme; PEP: propylendopeptidase.

Peptide	Amino acid sequence	Modification	Cluster	Activity
LacB_BM (43–57)	VEELKPTPEGDLEIL		BMF	Zinc binding peptide
LacB_BM (46–55)	LKPTPEGDLE		BMF	DPP-IV Inhibitory
LacB_BM (125–135)	TPEVDDEALEK		BMF	Antimicrobial; DPP-IV Inhibitory
CasB_BM (58–66)	LVYPFPGPI		BMF	ACE-inhibitory
CasB_BM (59–66)	VYPFPGPI		BMF	ACE inhibitory; PEP-inhibitory
CasB_BM (59–66)	VYPFPGPIH		BMF	PEP-inhibitory
CasB_BM (59–67)	VYPFPGPIP		BMF	PEP-inhibitory
CasB_BM (59–67)	VYPFPGPIPN		BMF	ACE-inhibitory; Antioxidant
CasB_BM (59–68)	YPFPGPI		BMF	Opioid; ACE-inhibitory; Antioxidant; Induce gut inflammatory immune response; Increase mucin expression; Reduces pancreas malondialdehyde; Satiety; Anxiolytic; Anticancer
CasB_BM (60–66)	YPFPGPIPN		BMF	ACE-inhibitory; Antioxidant; DPP-IV Inhibitory
CasB_BM (60–68)	PFPGPIPN		BMF	ACE-inhibitory
CasB_BM (61–68)	EMPFPK		BMF	ACE-inhibitory; Antimicrobial; Bradykinin-Potentiating; Increase mucin expression
CasB_BM (108–113)	YPVEPF		BMF	Opioid; Antioxidant; DPP-IV Inhibitory; Antimicrobial; Increase mucin expression
CasB_BM (114–119)	HQPHQPLPPT		BMF	ACE-inhibitory
CasB_BM (145–154)	DSESAPLRVY		W	ACE-inhibitory; Antioxidant
LacB2_DM (51–60)	MPFLKSPIVPF		K	ACE-inhibitory
CasB_DM (115–125)	GENLRLPVHL		Y	ACE-inhibitory; Antioxidant
CasB_DM (136–145)	IQPFMHQVPQ		Y	ACE-inhibitory; Antioxidant
CasB_DM (146–155)	VAPFPQPVVP		Y	ACE-inhibitory
CasB_HM (176–185)	RETIESLSSSEESITEYK	8S–10S|9S–10S|8S–9S	–	Stimulates proliferation
CasB_HM (1–18)	RETIESLSSSEESITEYK	8S|10S|9S	A	Stimulates proliferation
CasB_HM (1–18)	YPFVEPIP		F	Opioid
CasB_HM (51–58)	VEPIPY		F	Immunomodulatory
CasB_HM (54–59)	VLPVPQ		B	Inhibition of cholesterol solubility
CasB_HM (75–80)	SPTIPFFDPQIPK		F	Stimulates proliferation
CasB_HM (105–117)	NLHLPLP		F	ACE-inhibitory
CasB_HM (123–129)	HLPLPL		B	PEP-inhibitory
CasB_HM (125–130)	WSVPQPK		F	ACE-inhibitory; Antioxidant
CasB_HM (154–160)	VLPIPQ		F	ACE-inhibitory
CasB_HM (161–166)	QELLLNPTHQIYPVTQPLAPVHNPISV		A	Antimicrobial
CasB_HM (185–211)	QELLLNPTHQIYPVTQPLAPVHNPISV	1Q	A	Antimicrobial
CasB_HM (185–211)				

(Deglaire et al., 2016; Giribaldi et al., 2022). The higher number of peptides found in preterm HM could be due to the higher plasmin activity in preterm HM than in term HM (Dallas et al., 2015), which mostly hydrolyzes CasB, as previously found (Deglaire et al., 2019b). Moreover, CasB was the main DM parent protein in undigested DMF, and the main BM protein in undigested BMF.

In the present experiment, the use of protein fortifiers has not appeared to influence the overall HM peptide release process during digestion, as the release trend and origin of these peptides were found to overlap those reported previously by our group (Giribaldi et al., 2022). Previous reports on peptidomic profiling of HM during digestion reported a lower diversity and lower abundance of HM peptides when

fortified with BM-derived proteins, in comparison to the digestion of non-fortified HM (Beverly et al., 2019; Pica et al., 2021). Although the two fortifiers differed greatly, in terms of molecular form of the nitrogen, a similar trend of digestion of the specific proteins from HM was found for the two fortified HMs (BMF and DMF), as confirmed by means of the presently used peptidomic approach. Overall, the cumulative abundances of HM peptides in the differently fortified meals were not different. Supplementing HM with highly hydrolyzed whey proteins of bovine origin did not generally increase the net peptide release to any great extent with respect to using whole DM proteins. Moreover, the cumulative abundance of the peptides from DM followed a very similar trend to that of the HM peptides, while the peptides from BM showed a steady presence during digestion, thus explaining why a similar net degree of protein hydrolysis had previously been observed (Nebbia et al., 2022). This was also confirmed by profiling the abundance of all the released peptides through multivariate analysis: the differences that were observed between the samples disappeared when only the abundance of the HM peptides was considered. To the best of our knowledge, this is the first report that has indicated that such different fortifiers (whole donkey *vs.* hydrolysed bovine whey proteins) result in limited differences in the release of HM peptides during digestion. Furthermore, a clinical trial conducted on very low birth weight preterm infants fed with DMF *vs.* BMF (Bertino et al., 2019) showed that DM fortification did not seem to affect the overall nutritional efficiency, as demonstrated by the comparable gain in weight and length observed in the two groups at the end of the intervention.

During both gastric and intestinal digestion, most of the identified HM peptides originated from CasB, while only a few peptides were detected from LAlbA, probably due to the presence of four disulfide bonds (Deglaire et al., 2016; Deglaire et al., 2019b; Pica et al., 2021), which resulted in a great resistance to gastric hydrolysis. Indeed, few peptides deriving from LAlbA with a low intensity were found in the gastric phase, and Nebbia and colleagues reported that 100% of the whole protein was still detectable at the end of the gastric phase (Nebbia et al., 2020; Nebbia et al., 2022). The differences that were detected in the release of specific HM peptides were limited to a small peptide cluster that was significantly associated with LAlbA, which included peptides that had already been detected at the end of gastric digestion in BMF, while they were detected from the intestinal digestion in DMF. The other difference was seen in a small intestinal cluster that was significantly associated with TrFL, whose peptides were detected until the end of digestion in DMF, while they were not detected in the final stage in BMF. The longer persistence of these acidic peptides derived from TrFL may indicate a longer persistence of TrFL at the intestinal level in DMF diet than in BMF, although this was not detected when whole protein was profiled, in a coupled experimental approach (Nebbia et al., 2022). A longer preservation of HM TrFL peptides at the gut level is of utmost relevance for the nutrition of preterm infants, because of the well-known biological functions exerted by TrFL, which include antibacterial activity, anti-inflammatory activity, intestinal barrier protection, and immune cell modulation (Liu et al., 2021).

Regarding the peptides derived from DM, those originating from CasB were the most abundant during digestion. Most of them were associated with the gastric phase, when CasB is quickly hydrolysed, in a similar manner to the homologous protein in HM. On the other hand, few of the gastric peptides with a very low abundance were found to derive from LacB, which, although resistant to gastric digestion, was quickly hydrolysed in the intestinal phase, in agreement with observations on intact protein profiling (Nebbia et al., 2022; Tidona et al., 2011). No peptides originating from donkey TrFL were detected, and only a few peptides were detected from LAlbA and lysozyme, although those proteins are present in DM (Tidona et al., 2011; Tidona et al., 2014), but have also been reported as being among those most resistant to digestion.

Recent studies have investigated the release of bioactive peptides in fortified HM using *in vivo* and *in vitro* digestion protocols; the number of BM-derived bioactive peptides was higher than HM-derived ones, probably due to the larger amount of available information on BM bioactivity (Beverly et al., 2019; Demers-Mathieu et al., 2018; Nielsen et al., 2018; Pica et al., 2021). In our study, 14 bioactive peptides from BM were detected, that is, more than those detected by Pica et al. (2021) using static simulated gastrointestinal digestion and the same BM fortifier. Among the 10 bioactive peptides detected by those authors in BM fortified donor HM, opioid peptide BCM-7 was also found. As already reported (Pica et al., 2021), the manufacturing process of BMF, despite being claimed to be produced only from whey proteins, results in contamination from several casein peptides, some of which show bioactivity. Five peptides with ACE-inhibitory and antioxidant activities were found in DMF during digestion, thus supporting the *in vivo* findings on the anti-inflammatory and antioxidant activities of a diet including DM (Lionetti et al., 2012; Trinchese et al., 2015).

5. Conclusion

From a quantitative point of view, in this experiment, the peptide release from HM proteins was similar in both quantity and type of peptides, regardless of the fortification type. Only a few HM proteins showed small differences in the peptide release kinetics at the intestinal level, and these differences depended on the provided fortifier. HM lactoferrin showed more persistent peptides during intestinal digestion when HM was fortified with the DM derived fortifier, and this can result in potential advantages, in terms of gut protection, against infections and inflammation, due to the biological importance of lactoferrin protein and its fragments. A further advantage of fortifying HM with the DM derived fortifier is that additional ACE-inhibitory and anti-inflammatory DM peptides were released during digestion. Both findings are of great interest for the clinical nutrition of preterm infants, who are often prone to intestinal health impairment and inflammation.

The results obtained from the digestomic experiments, together with previous evidence gained in an *in vivo* clinical trial, indicate that the fortification of HM with the DM derived fortifier represents an alternative to fortification with BM derived fortifiers, with possible advantages in terms of bioactive compound provision at the intestinal level.

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Conflict of Interest Disclosure

LC has competing interests, since she is co-inventor of a patent regarding the experimental fortifier derived from donkey milk used in the study (Registered EU patent no. EP 3057450B1). No conflict of interest exists for the remaining authors.

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CRediT authorship contribution statement

Marzia Giribaldi: Writing – review & editing, Data curation, Conceptualization. **Stefano Nebbia:** Writing – original draft, Methodology, Investigation, Data curation. **Valerie Briard-Bion:** Writing –

review & editing, Supervision, Methodology, Investigation, Data curation. **Julien Jardin:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation. **Olivia Ménard:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation. **Didier Dupont:** Writing – review & editing, Funding acquisition, Data curation, Conceptualization. **Alessandra Coscia:** Writing – review & editing, Resources, Methodology. **Francesco Cresi:** Writing – review & editing, Resources, Methodology. **Cristina Lamberti:** Writing – review & editing, Supervision, Data curation. **Laura Cavallarin:** Writing – original draft, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization. **Amélie Deglaire:** Writing – review & editing, Supervision, Project administration, Investigation, Data curation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Laura Cavallarin has patent #Food Composition - EP 3057450 B1 issued to n.a. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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