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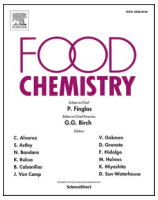

# Supplementing human milk with a donkey or bovine milk derived fortifier: Consequences on proteolysis, lipolysis and particle structure under *in vitro* dynamic digestion

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ABSTRACT

Fortification of human milk (HM) is often necessary to meet the nutritional requirements of preterm infants. This study sought to establish whether HM supplemented with an experimental donkey milk-derived fortifier (DMF) or a commercial bovine milk-derived fortifier (BMF) affected digestion, using an *in vitro* dynamic system at the preterm stage. Particle size in gastric phase was higher in DMF than in BMF, due to protein aggregates surrounding lipid globules. Before digestion, BMF, with its extensively hydrolysed proteins, had a higher degree of proteolysis (30%) than DMF (11%), which contained intact proteins. After digestion, this difference was reduced concomitantly to a similar net degree of proteolysis (33%). DMF, with a higher proportion of ω3, resulted in a lower ω6/ω3 free PUFA ratio than BMF throughout digestion, although the final degree of lipolysis was similar (54%). In summary, DMF could represent a better source of proteins and lipids for the preterm infant.

## 1. Introduction

Very preterm babies (gestational age < 32 weeks) and Very Low Birth Weight Infants (VLBWIs, birthweight < 1500 g) currently account for the majority of infants held in Neonatal Intensive Care Units (NICUs). Good nutrition represents a key factor in ensuring both long-term survival and the development of healthy children. An inadequate nutrition could hinder or prevent the necessary "catch-up" to achieve optimal development, risking permanent negative health consequences. Indeed, infants born early in the third trimester miss the placental transfer of nutrients, which would create stores for use in the postnatal period (Arslanoglu et al., 2019). The mother's own milk is considered as the best feeding choice for all infants, and the benefits of human milk (HM) feeding are especially relevant for premature and underweight babies, as stated by the World Health Organization, and by the American Academy of Pediatrics. However, HM alone, when given to the premature or underweight baby at the appropriate feeding volumes, may not always meet the recommended nutritional needs (Arslanoglu et al., 2019). In NICUs, the most common strategy is supplementing HM with additional proteins to meet the target requirements for neonatal nutrition (Kumar et al., 2017). The most efficient method for stimulating better growth rates for premature and underweight babies is based on the individualized fortification of HM (Arslanoglu et al., 2019; Fabrizio et al., 2020), where protein supplementation is adjusted on the basis of either the protein content of HM, or on the basis of the specific metabolic response of the baby.

Most commercially available HM fortifiers are derived from bovine milk proteins (with different casein/whey protein ratios, and different degree of protein hydrolysis), to which different types of carbohydrate substrates are added (usually maltodextrins) (Arslanoglu et al., 2019). However, some studies have highlighted the risk of adverse effects for infants receiving bovine milk proteins, such as metabolic acidosis

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(Rochow et al., 2011) and the increase of osmolarity, with potential adverse effects on the subsequent gastrointestinal tolerance (Kreissl et al., 2013).

In the past decade, fortifiers produced exclusively by ultrafiltrated pasteurized HM have been made commercially available. Previous studies have demonstrated some beneficial outcomes for those premature babies fed on HM fortified with supplements derived from human sources compared to others fed on HM fortified with supplements from bovine sources. Reported benefits include a reduction of necrotizing enterocolitis (Cristofalo et al., 2013), lower mortality and morbidity rates (Abrams et al., 2014), improved feeding tolerance, and reduced hospitalization costs (Assad et al., 2016). However, recent systematic studies suggest that the claimed benefits of commercial fortifiers derived from HM are based on low-quality evidence, and need to be confirmed by more rigorous clinical trials (Ananthan et al., 2020; Grace et al., 2021).

Based on its physiochemical properties, milk from monogastric animals has been proposed as potentially suitable alternative source for human nutrition (Barłowska et al., 2011). Of particular interest is donkey milk which is known to have a protein and lipid profile close to HM that from ruminants such as bovine milk (Altomonte et al., 2019; Bertino, 2010; Gastaldi, 2010). Donkey milk has an unusual composition that has been previously demonstrated as beneficial in treating children with serious bovine milk protein allergies (Monti et al., 2007). Donkey milk has also been shown to be closer than bovine milk from a functional point of view. The application of murine models have demonstrated that a supplementation of basal diets with donkey milk rather than bovine milk decreases the accumulation of body lipids and affects glucose and lipid metabolism in a manner closer to that of the HM (Trinchese et al., 2015; Trinchese et al., 2018). Furthermore, donkey milk is a potential source of many bioactive peptides, including opioid, angiotensin-converting enzyme (ACE) inhibitory, antimicrobial, antithrombin and immunomodulatory activities (Altomonte et al., 2019). Taking all this into account, we have shown that premature and underweight babies receiving isocaloric and isoproteic food based on HM fortified with either bovine or experimental donkey milk-based supplements (Coscia et al., 2018) lead to similar auxological and neurodevelopmental outcomes (Peila, Spada, Bertino, et al., 2020; Peila, Spada, Deantoni, et al., 2020). Additionally, the donkey milk-based fortifier significantly reduced the occurrence of feeding intolerance, feeding interruptions, bilious gastric residuals and vomiting (Bertino et al., 2019; Cresi et al., 2020). Interestingly, a different urinary metabolomics profile was perceived between the two groups, as a consequence of the different quality of the nutrients used for food fortification (Giribaldi et al., 2020).

The hypothesis behind the present work was that the higher tolerance to the food based on donkey milk supplements observed in trials (Bertino et al., 2019) was the result of a difference in the infant digestion process reflecting the nutrient bioavailability of the donkey and bovine milk derived fortifiers. However it appears that few investigations have been carried out on the impact of supplementing HM with protein fortifiers from non-bovine sources on macronutrient digestion. Studies on HM supplemented with derivatives from bovine milk have so far focused on the peptide release and protein digestion processes observed in infant gastric aspirates (Beverly et al., 2019; Demers-Mathieu et al., 2018; Nielsen et al., 2018) or by using static *in vitro* digestion models (Cattaneo et al., 2020; Pica et al., 2021). Recently, we have evaluated the degree of lipid and protein digestion of HM in preterm infant conditions using an *in vitro* dynamic digestion system (DIDGI®) (de Oliveira et al., 2016; Nebbia et al., 2020). The same digestion device was used in this study for evaluating whether, and to which extent, the isocaloric and isoproteic supplementation of HM with fortification products derived from donkey or bovine milk may differentially affect the subsequent digestion of the macronutrients.

## 2. Materials and methods

All chemicals and enzymes were purchased from Sigma Aldrich (Saint Quentin Fallavier, France), except where otherwise stated.

### 2.1. Collection of donor HM and fortification

Preterm HM was obtained from the donor bank at the Regina Margherita Children's Hospital, Turin (Italy). Each milk donor involved in the research signed a written consent form ensuring confidentiality of data relating to mother and baby. Preterm HM was obtained from 5 selected healthy donors and stored at −20 °C for a maximum of 4 months. Individual samples were thawed and combined before undergoing Holder pasteurization (Metalarredinox, Verdellino, Italy) (62.5 °C for 30 min). The HM samples were fortified either with an experimental multi-component powdered fortifier derived from donkey milk (FPD) (Coscia et al., 2018), or with a commercially available multi-component powder (FM85 Nestlé, Switzerland) derived from bovine milk (FPB); the two resulting infant foods were labelled respectively, DMF and BMF. 4 and 5 g of FPD and FPB respectively were added to 100 mL of HM representing a supplementation of 1 g of protein and about 18 Kcal.

### 2.2. Protein and lipid content of the fortifiers and of the fortified HM samples

Total nitrogen and non-protein nitrogen (NPN) were determined by Kjeldahl method. Total nitrogen was determined for samples of 100 mg of each of the powdered supplements (FPD and FPB) and for 3 mL samples of each of the fortified HM foods (DMF and BMF). NPN was determined after protein precipitation using 40 mL of trichloroacetic acid 15% (p/v). The true protein content was determined by subtracting the NPN value from the total nitrogen. Specific N conversion factors of 6.19 for NPN and 6.38 for protein were used.

Total lipids were extracted from 650 mg samples of each powdered supplement (FPD and FPB) and from 500 μL samples of the two fortified foods (DMF and BMF). Samples were dispersed into 10 mL of a chloroform–methanol mixture (2:1, v/v), and lipid extraction was performed for 1 h whilst shaking. Extracts were washed with 2 mL of KCl 0.8% (w/v) and with 2 mL of chloroform–methanol-KCl 0.8% (3/48/47, v/v/v) after centrifugation at 5000 g for 5 min. The solvent phases were filtered (Whatman filter paper, 2.5 μm, Grosseron, France) and total lipids weighed after evaporation under nitrogen.

### 2.3. In vitro dynamic digestion

DMF and BMF were subjected to simulated gastrointestinal digestion using the bi-compartmental *in vitro* dynamic system DIDGI® (Ménard et al., 2014). The apparatus was set up to simulate the digestion of a premature baby at a postnatal age of four weeks. The specific gastrointestinal parameters are taken from de Oliveira et al (2016) and are summarized in Supplementary data Table 1. The transit time followed Elashoff's equation with $t_{1/2} = 36$ min and $\beta = 1.15$ for gastric emptying and $t_{1/2} = 200$ and $\beta = 2.2$ for emptying of the intestine stage. The pH acidification in the gastric compartment followed a polynomial curve as described previously by Nebbia et al. (2020). The intestinal pH was maintained at 6.2. Gastric enzymes were supplied in the form of rabbit gastric extract (Lipolytech, France) (pepsin and lipase: 120 and 8.6 U/mL of total gastric volume, respectively) and intestinal enzymes were supplied in the form of porcine pancreatin (trypsin and lipase: 1.5 and 59 U/mL of total intestinal volume), in addition to bovine bile salts.

Simulated dynamic digestions were performed in triplicate over three hours for both types of prepared infant food. Sample aliquots were collected before digestion (G0) and at 30, 60 and 90 min after the beginning of the digestion from both the gastric (G30, G60 and G90) and intestinal compartments (I30 and I90). An additional sample at 180 min was collected from the intestinal compartment (I180). Structural

analyses (particle size distribution and confocal microscopy) were performed on the samples at the time of collection, as well as lipid extraction after the addition of lipase inhibitor (50 μL of 4-bromophenylboronic acid (0.1 M) *per* mL of digesta). Protease inhibitors (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 to aliquots of each digested sample before storage at −20 °C.

### 2.4. Particle size distribution of DMF and BMF before and during digestion

The distribution of the particle size was measured both before and during the simulated digestion of the two prepared infant foods using a Mastersizer 2000 (Malvern Instruments, Malvern, UK) laser light scattering equipped with two laser sources. The refractive indexes used were 1.458 for lipid at 633 and 466 nm and 1.333 for water (dispersant phase) in the measurement cell. The mode value for particle diameter, the mean particle diameter D [4,3] and the specific surface area (SS) were deduced from the size distributions measured.

### 2.5. Analysis of the microstructure of the DMF and BMF using confocal microscopy

The microstructure of the two prepared infant foods, and that after 90 min. of simulated gastric digestion, were observed using a Nikon C1Si confocal laser scanning microscopy (CLSM) on inverted microscope TE2000-E (Nikon, Champigny-sur-Marne, France), as previously described by Bourlieu et al. (2015). A 40x oil-immersion objective was used for all images. Three fluorescent dyes, Fast Green®, Rhodamine-PE® and Lipidtox® (Thermo Fisher Scientific, Waltham, MA, USA), were used to simultaneously label the proteins, amphiphilic compounds and apolar lipids, respectively.

### 2.6. Protein analysis

#### 2.6.1. Determination of the degree of protein hydrolysis (DPH)

The overall and net DPH were calculated from the measurement of primary amines ($NH_2$) present before digestion, and those released during digestion, by using the o-phtaldialdehyde (OPA) method. Briefly, 50 μL of each sample were mixed with 100 μL of the OPA reagent (0.5% w/v SDS, 0.25 mg/mL OPA, 7 mM DTT, 20 mM sodium tetraborate) in a 96-well microtiter plate (Greiner Bio-One, Courtaboeuf, France) and the absorbance at 340 nm was measured after 10 min with a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific). A methionine standard solution (0 to 2 mM) was used as calibration curve. The total releasable primary amines, [$NH_2$ (total)] expressed in mg/L of infant food sample, were determined after total acid hydrolysis in 6 N HCl at 110 °C for 24 h. The overall and net (i.e. digestion-specific) DPH were calculated as follows:

$$\text{overall DPH}\,(\%) = 100\,\text{x}\,\frac{[\text{NH}_2(\text{t})\,] - [\text{NH}_2(\text{secretions})\,]}{[\text{NH}_2(\text{total})\,]\,\text{x S}} \quad (1)$$

$$\text{net DPH}\,(\%) = 100\,\text{x}\,\frac{([\text{NH}_2(\text{t})\,] - [\text{NH}_2(\text{secretions})])\,\text{x}\,1/\text{S} - [\text{NH}_2(\text{t0})]}{[\text{NH}_2(\text{total})] - [\text{NH}_2(\text{t0})]} \quad (2)$$

where [$NH_2$(t0)] and [$NH_2$(t)] are the concentrations of primary amines (as mg/L) before and after t min of digestion. S was the estimated ratio of infant food within the digesta (L of food/L of digesta) determined from Elashoff's equation and from the secretion flows within the dynamic digestion system. The term [$NH_2$(secretions)] is the concentration of primary amines arising from bile and pancreatin (mg/L of intestinal digesta).

#### 2.6.2. Protein profile analysis of fortifiers and of DMF and BMF by LDS-PAGE

Samples (3 μg of proteins in the gastric phase and 30 μg in the intestinal phase) were solubilized in a NuPAGE® LDS sample buffer (Thermo-Fisher Scientific), and electrophoresis run in reducing conditions (0.5 M DTT) on 4–12% gradient NuPAGE® Novex Bis-Tris precast gels (Thermo-Fisher Scientific) as previously described by Nebbia et al. (2020). Samples were loaded onto the gels taking into account the dilution of the gastric and intestinal juices at the different sampling times. The gels were then stained with Coomassie Blue (Biorad, Hercules, CA). Gel images were acquired using an Image Scanner III (GE, Healthcare) at a 300 dpi resolution. Densitometric analysis was performed using an Image Quant TL™ (GE Healthcare). Protein bands were identified by comparing the observed migration to data published in previous papers (Bertino, 2010; Nebbia et al., 2020). During gastric and intestinal digestion, the abundance of the major proteins, at each digestion time, was calculated from the grey band intensity observed with that measured before digestion; the result is expressed as a percentage.

#### 2.6.3. Amino acid profile of undigested DMF and BMF and quantification of free amino acids released during intestinal digestion

Free amino acids (AAs) were quantified as detailed previously (de Oliveira et al., 2016). Briefly, samples (1 mL) were treated with 50 mg of sulfosalicylic acid, and incubated for 1 h at 0 °C. The mixtures were centrifuged (5000 *g*, 15 min at 4 °C) and the supernatants were filtered through a 0.45 μm pore-size membrane (Sartorius, Palaiseau, France). The filtrate was diluted 3 times with a 0.2 M lithium citrate buffer (pH 2.2) and the AAs were quantified by a cation exchange chromatography on a Biochrom30 automatic AA analyzer (Biochrom Ltd., Cambridge, G. B.). The AA bioaccessibility was calculated by two different equations as follows:

$$\text{overall AA bioaccessibility}\,(\%) = 100\,\text{x}\,\frac{[\text{AA(t)}\,] - [\text{AA}\,(\text{secretions})]}{[\text{AA(total)}\,]\,\text{x S}} \quad (3)$$

$$\text{net AA bioaccessibility}(\%) = 100\,\text{x}\,\frac{([\text{AA(t)}\,] - [\text{AA}\,(\text{secretions})\,])\,\text{x}\,1/\text{S} - [\text{AA(t0)}\,]}{[\text{AA(total)}\,] - [\text{AA(t0)}]} \quad (4)$$

where the term [AA(t)] is the concentration of free AAs after t min of digestion, and [AA(total)] is the total amount of each AA obtained following total acid hydrolysis of the undigested infant foods in 6 N HCl at 110 °C for 24 h. The total content of sulfur-containing AA (cysteine and methionine) were measured as methionine sulphone and cysteic acid after a performic oxidation treatment. S is the estimated ratio of infant food within the digesta (expressed as L of food/L of digesta) determined from Elashoff's equation and the secretion flows observed within the dynamic digestion system. The term [AA(secretions)] is the concentration of free AAs arising from bile and pancreatin (mg/L of intestinal digesta). Concentrations of free glycine and proline fell under the detection limit and tryptophan was not quantified.

### 2.7. Lipids analysis

#### 2.7.1. Lipids extraction

Lipids were extracted from samples of digesta (0.4 mL) following the method described above (section 2.2) after a preliminary acidification with 160 μL of 0.1 M HCl. For the analysis of free fatty acids (FFAs), C11:0 and C20:1n9c (0.5 mg/mL) were added as internal standards (160 μL). FFAs in the samples were isolated from the Folch extract (1 mL) using a solid-phase extraction column ($NH_2$, 3 mL/500 mg, Macherey-Nagel) after elution of lipid class by 10 mL of hexane:isopropanol (3:2, v:v) and the collection of the FFAs with 3.5 mL of diethyl ether, 2% (v/v) formic acid. Total fatty acids (TFAs, C6:0 to C24:0) were extracted from the undigested samples (100 μL) by direct transmethylation with 1 mL of sodium methylate 0.5% (w/v) in the presence of internal standard (20 μL of Glyceryl tri-C13, 5 mg/mL) at 50 °C for 10 min. Both FFAs and TFAs were methylated with BF3 (14% v/v in methanol) at 70 C for 15 min and the resulting fatty acid methyl esters were extracted with hexane after centrifugation (5000 *g* for 5 min).

#### 2.7.2. Determination of the degree of lipid hydrolysis (DLH) by thin layer chromatography

The quantification of the lipid classes was performed by thin layer chromatography (TLC). Briefly, lipid extracts were separated on a silica plate utilizing a solvent mixture of hexane/diethyl ether/acetic acid (70/30/2, v/v/v), then stained using a copper sulphate/orthophosphoric acid solution and heated for 15 min at 150 °C. Plates were then scanned using an Image Scanner III (GE Healthcare) at 300 dpi resolution, and the densitometric analysis on bands was performed using Image Quant TL™ (GE Healthcare). To obtain a quantitative evaluation of each lipid class, three calibration curves were generated from the area values obtained by a series of dilutions of triacylglycerols (TAGs), diacylglycerols (DAGs) and FFAs across the range 0.25–5 mg. The data were converted into moles by using the average molar masses calculated from the fatty acid composition of HM (TAG: 832; DAG: 585; FFA: 265). The DLH during digestion was expressed as the percentage level of FFAs over the total acyl chains from residual glycerides ([TAGs] and [DAG]) and FFAs quantified at a given time, according to the following equation:

$$\mathrm{DLH}\,(\%) = \frac{100\,\mathrm{x}\,[\mathrm{FFAs}]}{3^{*}[\mathrm{TAGs}] + 2^{*}[\mathrm{DAGs}] + [\mathrm{FFAs}]} \quad (5)$$

#### 2.7.3. The fatty acids profile of undigested DMF and BMF and quantification of FFAs released during digestion by gas chromatography

Standard calibration curves were generated to enable the quantification of fatty acids present. Analysis by GC/GC-TOFMS was performed by means of a LECO Pegasus BT 4D GCxGC/TOFMS instrument (Leco Corp., St. Josef, MI, USA) equipped with a LECO dual stage quad jet thermal modulator. The GC part of this instrument was an Agilent 7890 gas chromatograph (Agilent Technologies, Palo Alto, CA), equipped with a split/splitless injector. The first dimension column was a 30 m DB-FATWAX-UI (Agilent Technologies, Santa Clara, CA) with a diameter of 0.25 mm and a film thickness of 0.25 μm, and the second dimension chromatographic columns was a 2 m Rxi-17Sil MS (Restek Corp., Bellefonte, PA) with a diameter of 0.25 mm and a film thickness of 0.25 μm. High-purity helium (99.99%) was used as the carrier gas with a flow rate of 1.4 mL/min. 1 μL of sample was injected in split mode (ratio 30) at 250 °C. The temperature program for metabolites analysis was as follows: the initial temperature was 65 °C, then ramped 10 °C/min up to 130 °C, 7 °C/min to 240 °C and then held at this value for 15 min. The second column was maintained at + 5 °C relative to the GC oven temperature of the first column. Electron impact ionization was applied (70 eV). The ion source temperature was set at 250 °C, the mass range was 40 to 400 *m/z* with an extraction frequency of 32 kHz. The acquisition rates was 200 spectra/s. The modulation periods for the program were set at 2.5 s for the start of the run to 299.99 s, then 2 s from 299.99 to 953.94 s and finally 3 s from 953.94 until the end of the analysis. The modulator temperature offset was set at + 15 °C relative to the second column oven temperature, whilst the transfer line was set at 280 °C. The chromatograms were acquired in TIC (total ion current) mode. Peaks with a signal-to-noise value lower than 500.0 were rejected. The software, ChromaTOF (version 5.31) was used for raw data processing. Mass spectral assignment was performed by matching with NIST MS Search 2.3 libraries and standards. The relative content of each FFA were expressed as mass %.

### 2.8. Statistical analyses

Data analyses were performed using R software (version 4.1.3).

#### 2.8.1. Analysis of variance (ANOVA)

In the case of undigested food samples, statistical analysis was performed using one-way ANOVA with the supplement type as the factor. For the analytical data arising from samples taken during the digestion, a two-way ANOVA was fitted using a mixed linear model for repeated measurements (nlme package) applied separately on data relating to gastric and intestinal sampling. Time and the type of food supplement were set as the fixed factors whilst the digestion replicates were set as random factors. Residual normality and variance homogeneity were tested for each variable using a Shapiro-Wilks and Levene's tests, respectively. Where the threshold of statistical significance was reached ($p$ value $< 0.05$), pairwise multiple comparison of the means was carried out using Tukey's test (lsmeans package).

#### 2.8.2. Principal component analysis (PCA)

For each of the two foods and for each digestion time (G0, G30 and G90 for gastric phase and I30, I90 and I180 for the intestinal phase), relating variables were log10-transformed [log10 (abundance + 1)]. PCA was carried out using the FactoMineR package and graphics were developed using the ggplot2 package. The variables considered were: (i) characteristics relating to particle size distribution (mode value for particle diameter, D[4,3]-diameter and specific surface of particles), (ii) % of protein bands intensity with respect to G0 (LDS-PAGE), (iii) AA bioaccessibility, (iv) hydrolysis of TAGs and FFAs and DAG liberation (TLC), (v) the relative percentage of FFAs (GCxGC/TOFMS). Any missing value for free AAs at G30 and G90 were set as G0, due to the absence of aminopeptidases in the gastric compartment. In the case of missing values for the FFAs and lipolysis products at time G30, these were estimated as the average of values noted for G0 and G90.

## 3. Results and discussion

Fortifying HM with extra-nutrients is a common strategy in NICUs, in order to cover special nutritional requirements for the premature baby. This study set out to answer the question if two supplements differing in protein source and form (extensively hydrolysed bovine whey proteins vs. whole donkey milk proteins) may differently affect the HM-fortified digestion. In this study, it was demonstrated that, despite differences in the protein and lipid composition of the undigested HM fortified either with the bovine or the donkey milk derived products, the two fortified HM samples reached at the end of the intestinal phase a similar net degree of proteolysis and lipolysis. However, the two infant foods did differ in other ways: in the microstructure of the gastric digesta, in the bioaccessibility of the AAs and in the release of FFAs.

### 3.1. Nutritional composition of fortifiers and fortified HMs

As previously reported (Coscia et al., 2018), the chemical composition of FPD and FPB differed both in term of protein origin, structure and protein and lipid profiles. The nitrogen content of the FPD was predominantly in the form of whole milk protein nitrogen (Supplementary data Table 2), with solely 10.05 ± 0.25% (w/w) of NPN out of total N,

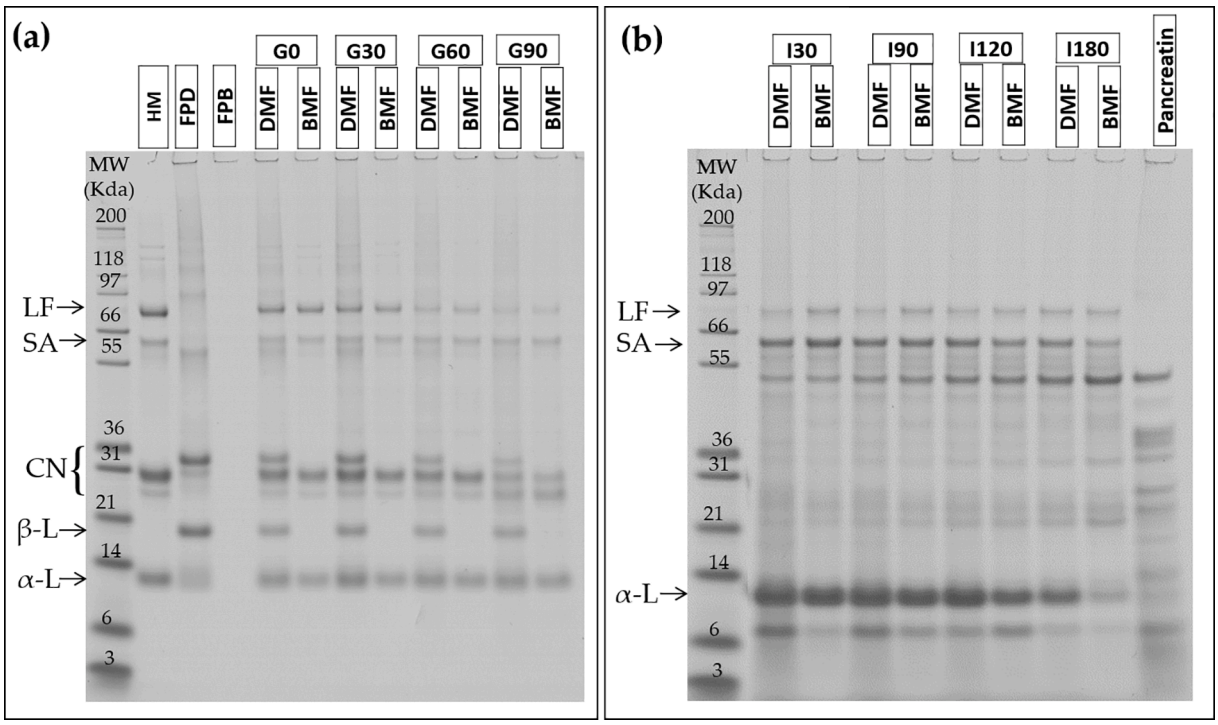



**Fig. 1.** Protein profiles for human milk (HM), donkey milk fortifier (FPD), bovine milk fortifier (FPB), donkey (DMF) and bovine (BMF) milk fortified HM. Digestion profiles of the DMF and BMF during gastric (a) and intestinal (b) digestion at different times. G0-G90: gastric digestion at time 0–90 min. I30-I180: intestinal digestion at time 30–180 min. Proteins were loaded in the gels allowing for the dilution of the gastric and intestinal juices at the sampling time. Molecular weight standards (MW). Lactoferrin (LF), serum albumin (SA), caseins (CN), β-lactoglobulin (β-L) and α-lactalbumin (α-L) were taken from previous papers.

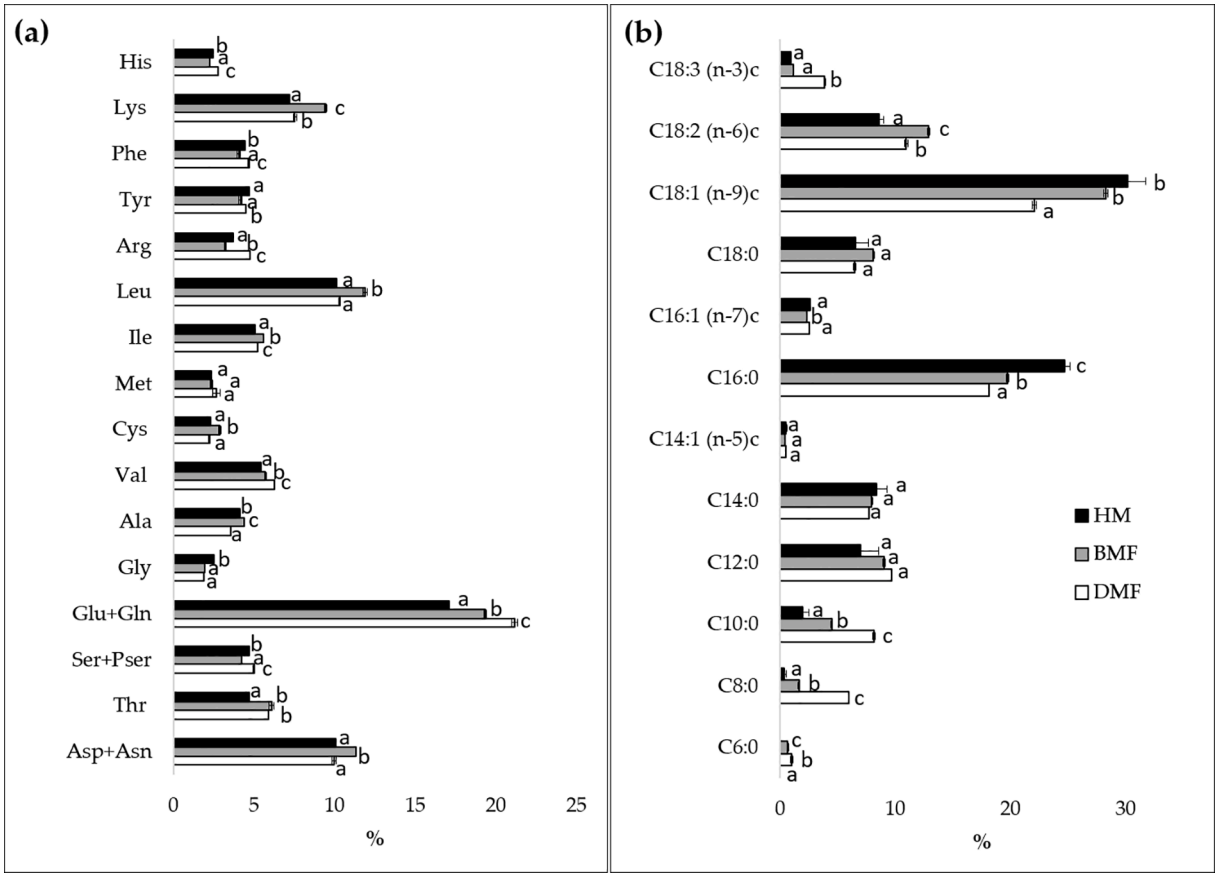



**Fig. 2.** Amino acid (a) and fatty acid (b) profiles of undigested donkey (DMF) and bovine (BMF) fortified human milk compared to the human milk (HM). Data represented as means ± standard deviations (N = 3). Tukey's post hoc test was performed in each case, and letters indicate the differences between samples.

vs. 98.53 ± 0.58% (w/w) for FPB, with no intact protein observed by LDS-PAGE electrophoresis (Fig. 1). The combined samples of collected HM contained 1.23% (w/v) of crude protein. After fortification, the crude protein content reached 2.16% (w/v) in both infant feeds, although the true protein content of BMF was significantly lower than that of DMF (1.23 vs 2.06% w/v). Accordingly, the two infant foods strongly differ in their content of specific free AAs before digestion (Supplementary data Table 3). Nevertheless, the total AA profile only slightly varied between the two fortified foods and was similar to that of unfortified human milk (Fig. 2a). The higher content of free lysine and leucine in the BMF was in accordance with the presence of whey proteins among FPB ingredients. The major proteins in HM (lactoferrin, serum albumin, casein and α-lactalbumin) were revealed in both infant foods by electrophoresis (Fig. 1). In the case of the DMF, β-lactoglobulin and an additional band of casein were also detected.

Preterm HM contained 2.37% (w/v) of fat and after fortification, the BMF contained slightly less fat than the DMF (2.41 vs 2.6% w/v). The fatty acid profile was dominated by oleic (C18:1n-9), palmitic (C16:0), linoleic (C18:2n-6), stearic (C18:0), myristic (C14:0) and lauric (C12:0) acids in both fortified and unfortified HM (Fig. 2b). The DMF had 3.6 times more caprylic (C8:0) and 1,8 times more capric (C10:0) acid than BMF. The difference in the composition of polyunsaturated fatty acids (PUFA) composition led to a significantly lower ω6/ω3 PUFA ratio for the DMF (2.78) in comparison to both the BMF (9.12) and unfortified HM (9.89).

### 3.2. Structural changes during gastric and intestinal digestion

Fig. 3 illustrates the particle size distribution for the two infant foods during the simulated gastric and intestinal digestion. Prior to digestion (G0), the fortified infant foods strongly differed in terms of their particle size characteristics (D[4,3], specific surface area and mode diameter) (Fig. 3a). DMF was characterized by the presence of large particles, bigger than those detected in BMF (mode diameter 69.3 μm vs 9.3 μm). Confocal images (Fig. 3b) highlighted that the particles in the DMF were formulated by aggregated proteins that shielded the embedded fat globules (Fig. 3b, yellow arrow). The FPD used in the study was obtained by freeze-drying of pasteurized concentrated donkey milk (Coscia et al., 2018). Protein insolubility, as consequences of freeze-drying, has already been reported for infant formulas, due to changes in fat-protein interactions. Mechanisms include the unfolding of whey proteins followed by aggregation with caseins (Baldwin, 2010), and, in a fat-containing system, the formation of protein-bridged fat globule clusters (Singh & Ye, 2010).

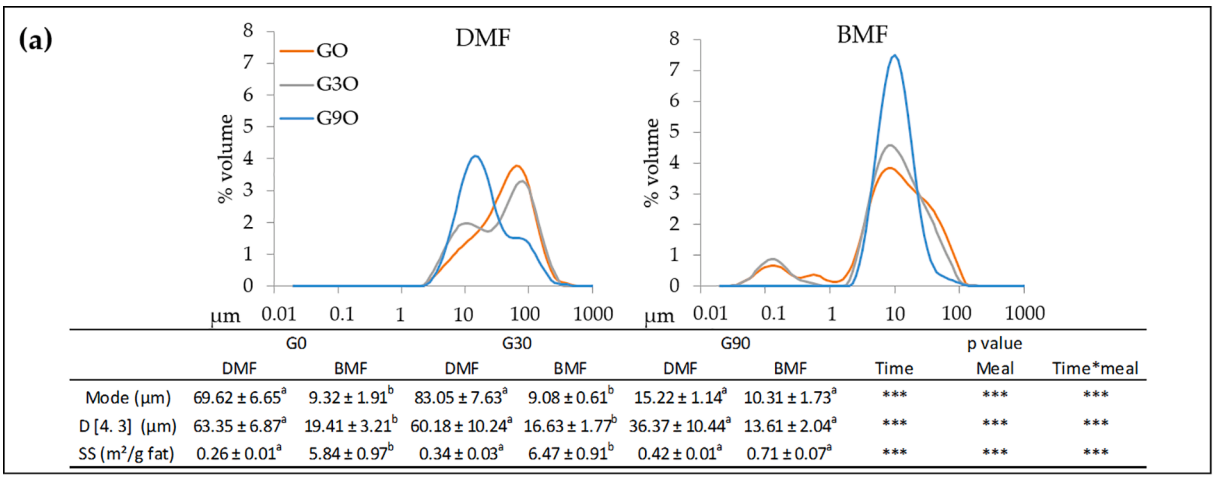
After 30 min of gastric digestion, the particle characteristics of the two infant foods remained similar to that observed for each at G0, whilst a decreased particle size was observed at the end of gastric phase in the case of the DMF only. For BMF, the particle size analysis did not reveal any major changes during the gastric digestion, other than a change from a bimodal to a unimodal distribution at G90. At the end of the gastric phase (G90), the differences between the two infant foods were attenuated (mode diameter 15.2 vs 10.3 μm), although some particles with a diameter above 100 μm were still present in the DMF; larger protein particles were also detected in the BMF (Fig. 3b). Lipolysis products were observed within fat globules in the case of the BMF (Fig. 3b, white arrows). The corresponding particle size during the digestion of this food was similar to that observed for the *in vitro* digestion of Holder pasteurized HM (de Oliveira et al., 2016; Nebbia et al., 2020). However, the difference in particle size found before and during gastric digestion of the two prepared infant foods did not result in any difference in the *in vivo* gastric emptying time, as also observed in a sub-cohort of reported clinical trials (Cresi et al., 2020). Finally, in the intestinal phase, both infant foods presented a similar bimodal profile in terms of the mode diameter and D[4,3] with an increased proportion of smaller particles (~0.1 μm) as digestion progressed (Fig. 3c).

### 3.3. Proteolysis during gastric and intestinal digestion

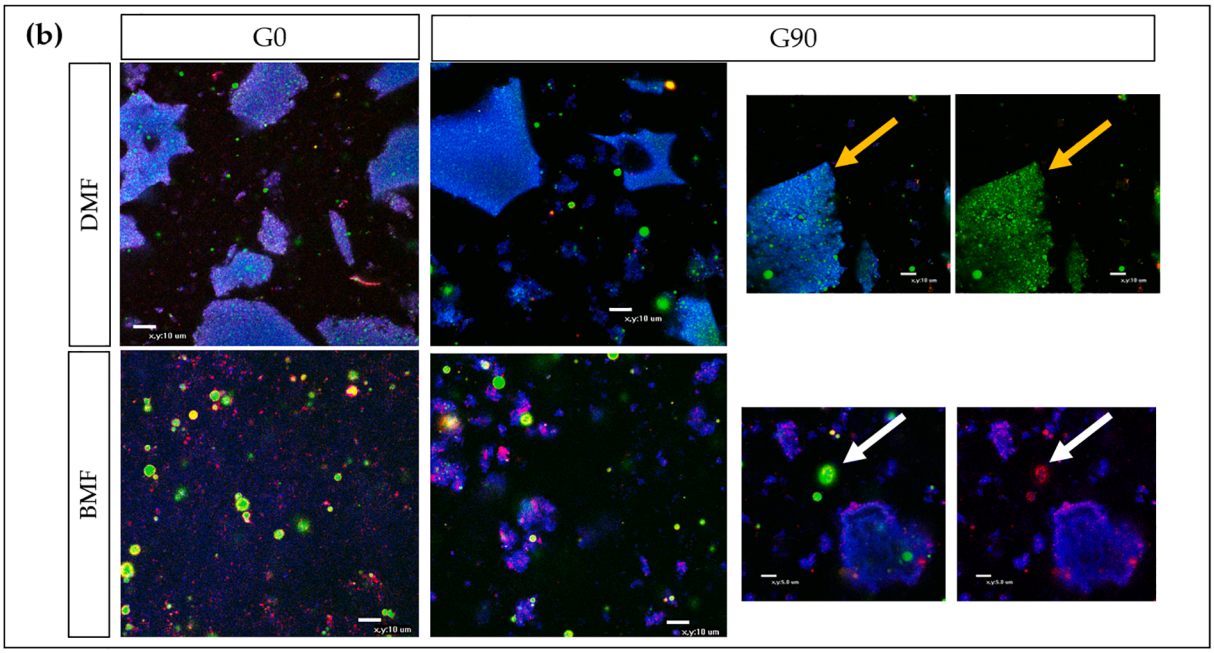
Although the two added supplements greatly differed in terms of the type of nitrogen source involved, a similar trend of the digestion of the specific proteins in HM was found between the two infant foods (Fig. 1 and Supplementary data Fig. 1). In particular, lactoferrin was digested mainly in the gastric phase for both infant foods, but intact lactoferrin was still visible at the end of the intestinal phase. Caseins were hydrolyzed during the gastric phase and were undetectable as the intact proteins in the intestinal phase. Serum albumin and α-lactalbumin were mostly digested in the intestinal phase. Similar results have already been reported for unfortified HM following a Holder pasteurization using the same *in vitro* digestion system at the preterm conditions (de Oliveira et al., 2016; Nebbia et al., 2020). The protein profile for the DMF was characterized by the extra presence of casein, that was quickly digested in the gastric phase, and of β-lactoglobulin, undigested in the gastric phase: both proteins were virtually undetectable in the intestinal phase, as reported previously in donkey milk after *in vitro* digestion experiments (Tidona et al., 2014). These results suggested that fortification, irrespectively of the type of protein source, do not affect the digestion of individual HM proteins. Likewise, small differences were reported by other authors on the release of the HM peptides during the infant digestion of unfortified HM and those fortified with bovine milk derived supplements (Beverly et al., 2019; Cattaneo et al., 2020; Demers-Mathieu et al., 2018; Nielsen et al., 2018; Pica et al., 2021).

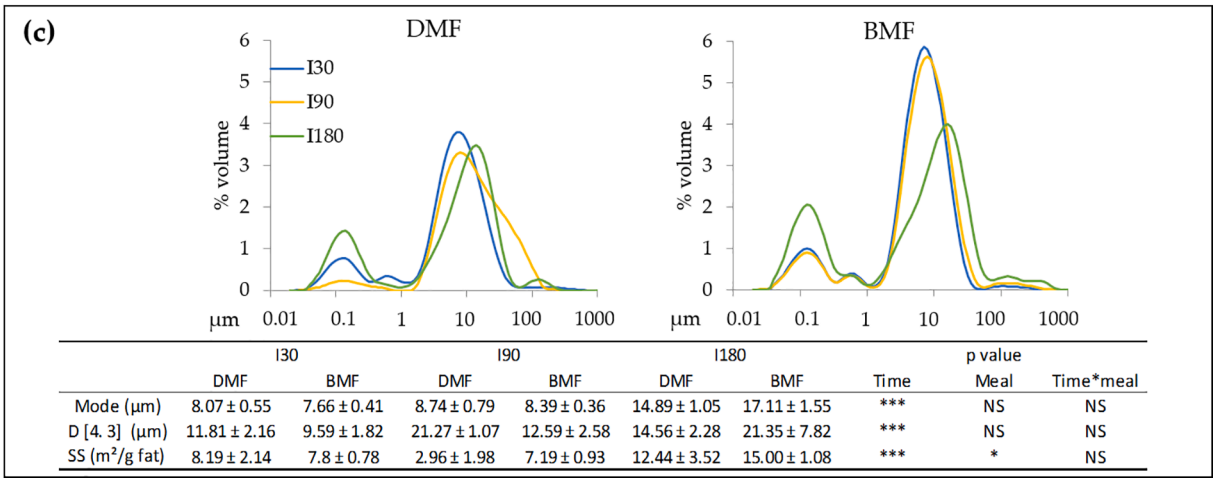
The DPH before digestion (Fig. 4a) was significantly higher in the BMF (30.1 ± 2.4% DPH), than for the DMF (11.5 ± 1.4% DPH). The difference in DPH was maintained during gastric digestion but was then greatly diminished in the intestinal phase by the time I180 (51.12 ± 5.6% DPH for the BMF vs. 42.6 ± 0.5% DPH for the DMF). When the net DPH was considered (Fig. 4b), the level of proteolysis emerges as not significantly different between the two prepared infant foods, except at I30, when the DMF formulation was significantly higher. Feeding proteins under different molecular forms has been shown to affect the postprandial metabolic fate of nitrogen in adults, with a greater AA oxidation when the kinetics of their absorption was faster (Deglaire et al., 2009). However, the clinical trial conducted with infants by our group has shown that this same factor did not affect their overall nutritional efficiency, as demonstrated by the comparable gain of weight and length observed at the end of the intervention period for babies fed with DMF and BMF (Bertino et al., 2019). Nevertheless, feeding infants with intact and minimally processed proteins may represent a better strategy for providing important bioactive compounds (Arslanoglu et al., 2006, 2019; Picaud et al., 2016).

Fig. 4 reports the overall bioaccessibility of the AAs at G0, and that released during intestinal digestion (Panel c, d and e), as well as the net bioaccessibility of the AAs at each digestion time, for each type of fortified HM (Panel f, g and h). Despite the difference in the total bioaccessibility of the AAs in the undigested fortified HM samples (2.3 ± 0.3% for DMF vs 16.9 ± 0.2% for BMF), the difference between the two was substantially reduced by the end of the intestinal phase (17.6 ± 1.8% for DMF vs 24.8 ± 0.3% for BMF), due to a greater release of free AAs during the digestion of DMF, such as indicated by the net bioaccessibility of the AAs (Fig. 4f-h and in Supplementary data Table 4). Each AA displayed a different release pattern in the two foods (Supplementary data Table 5). The free AA profile of undigested DMF and of unfortified HM was dominated by glutamine + glutamic acid (>57%) whilst, in the case of undigested BMF, the free AA profile was dominated by lysine (19.3%), leucine (17.8%) and glutamine + glutamic acids (15%) (Supplementary data Fig. 2). Throughout all of the intestinal digestion stage, the free AA profile of the DMF was dominated by arginine (15.5%), glutamine + glutamic acid (15.3%), lysine (14.1%) and leucine (13.6%), whilst in the case of the BMF samples, the free AA profile was dominated by the same free AAs as detected before digestion and in similar proportions. The free AAs profile in the DMF was similar of what is already found for the unfortified HM following a Holder




| | G0 | | G30 | | G90 | | p value | | |
|---|---|---|---|---|---|---|---|---|---|
| | DMF | BMF | DMF | BMF | DMF | BMF | Time | Meal | Time*meal |
| Mode (µm) | 69.62 ± 6.65$^{a}$ | 9.32 ± 1.91$^{b}$ | 83.05 ± 7.63$^{a}$ | 9.08 ± 0.61$^{b}$ | 15.22 ± 1.14$^{a}$ | 10.31 ± 1.73$^{a}$ | *** | *** | *** |
| D [4. 3] (µm) | 63.35 ± 6.87$^{a}$ | 19.41 ± 3.21$^{b}$ | 60.18 ± 10.24$^{a}$ | 16.63 ± 1.77$^{b}$ | 36.37 ± 10.44$^{a}$ | 13.61 ± 2.04$^{a}$ | *** | *** | *** |
| SS (m²/g fat) | 0.26 ± 0.01$^{a}$ | 5.84 ± 0.97$^{b}$ | 0.34 ± 0.03$^{a}$ | 6.47 ± 0.91$^{b}$ | 0.42 ± 0.01$^{a}$ | 0.71 ± 0.07$^{a}$ | *** | *** | *** |




| | I30 | | I90 | | I180 | | p value | | |
|---|---|---|---|---|---|---|---|---|---|
| | DMF | BMF | DMF | BMF | DMF | BMF | Time | Meal | Time*meal |
| Mode (µm) | 8.07 ± 0.55 | 7.66 ± 0.41 | 8.74 ± 0.79 | 8.39 ± 0.36 | 14.89 ± 1.05 | 17.11 ± 1.55 | *** | NS | NS |
| D [4. 3] (µm) | 11.81 ± 2.16 | 9.59 ± 1.82 | 21.27 ± 1.07 | 12.59 ± 2.58 | 14.56 ± 2.28 | 21.35 ± 7.82 | *** | NS | NS |
| SS (m²/g fat) | 8.19 ± 2.14 | 7.8 ± 0.78 | 2.96 ± 1.98 | 7.19 ± 0.93 | 12.44 ± 3.52 | 15.00 ± 1.08 | *** | * | NS |

**Fig. 3.** Particle size distribution and characteristics (described as: mode, D[4,3]-diameter, SS - specific surface) of donkey (DMF) and bovine (BMF) fortified HM samples during gastric (G0-G30-G90: 0–90 min) and intestinal (I30-I90-I180: 30–180 min) digestion. Particle size distribution (given as % volume) for gastric samples (a). Confocal laser scanning microscopy images (40x, zoom 1 and zoom 6) of undigested and digested samples after 90 min of gastric digestion (b). Proteins are stained in blue (FastGreen®), apolar lipids in green (Lipidtox®) and polar lipids in red (Rhodamine-PE®). Arrows indicate specific detail in the images. (c) Particle size distribution (given as % volume) for intestinal samples. Data represented as means ± standard deviations (N = 3). $p < 0.001$ (***); $p < 0.01$ (**); $p > 0.05$ (NS). Tukey's post hoc test was performed in each case and letters indicate the differences between samples when the interaction factor was significantly different.

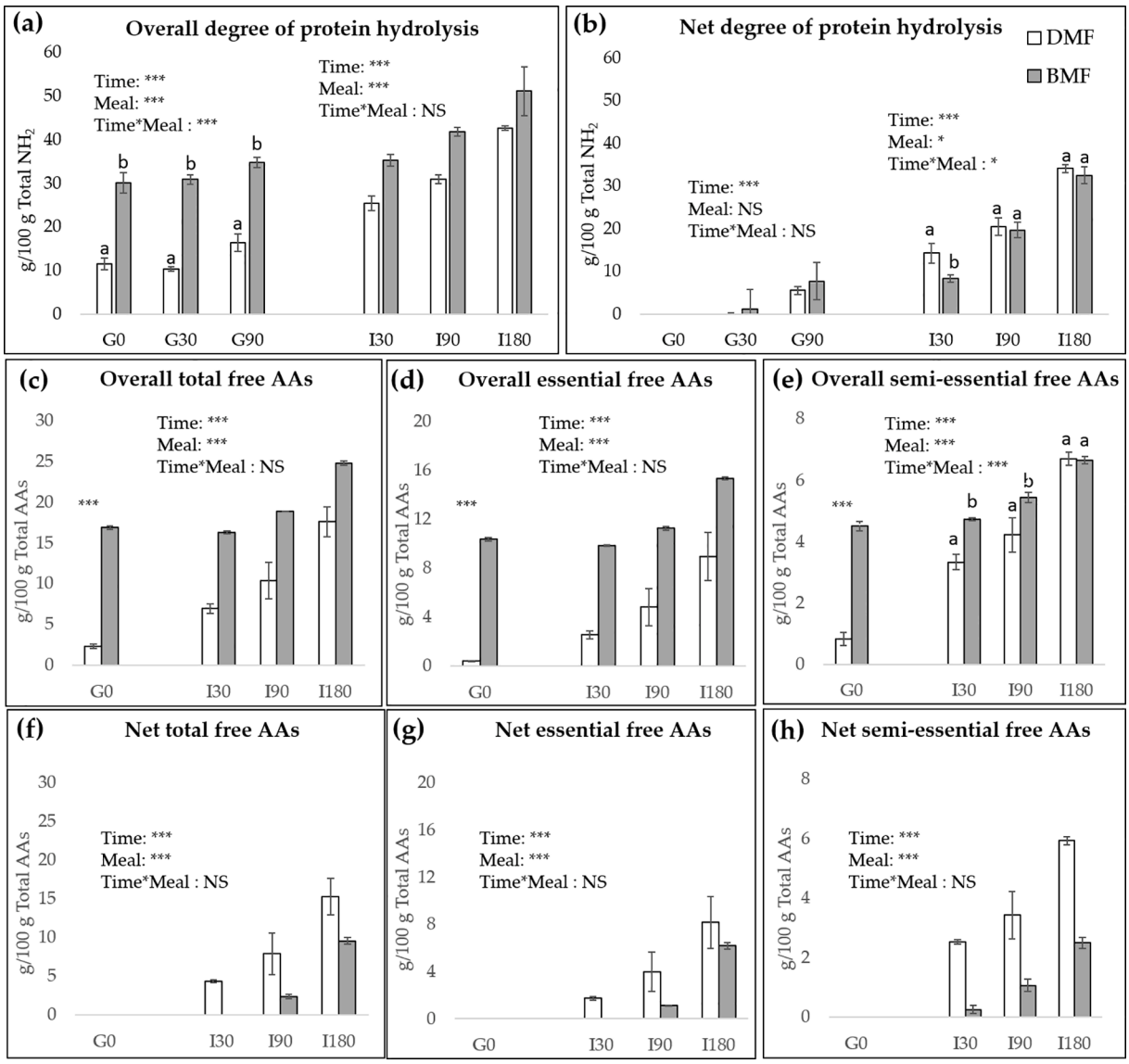


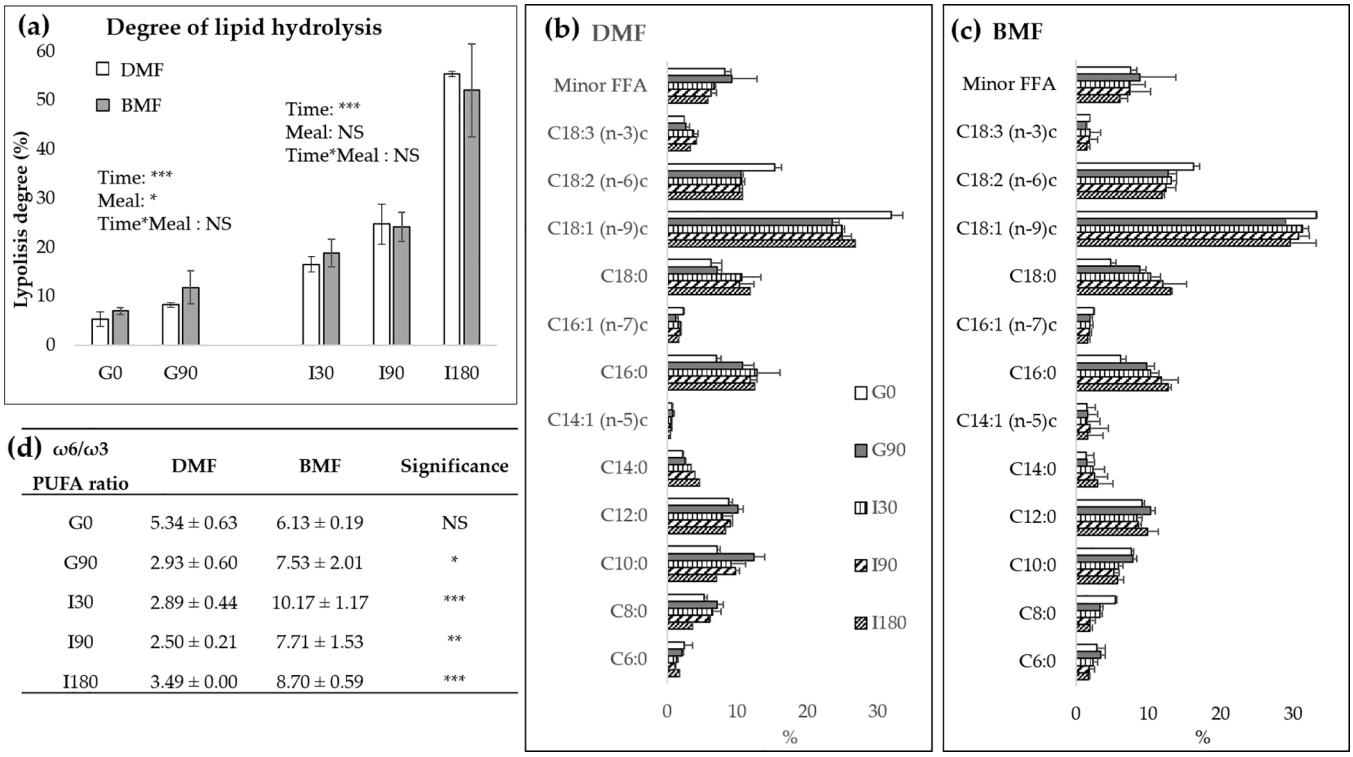

**Fig. 4.** Proteolysis of donkey (DMF) and bovine (BMF) fortified HM samples during gastric (G0-G30-G90: 0–90 min) and intestinal (I30-I90-I180: 30–180 min) digestion. The overall (a) and net (b) degree of protein hydrolysis. Overall (c-d-e) and net (f-g-h) bioaccessibility of total, essential and semi-essential AAs. $p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*); $p > 0.1$ (NS). Tukey's post hoc test was performed in each case and letters indicate the differences between samples when the interaction factor was significantly different.

pasteurization in our previously study (Nebbia et al., 2020) during all of the subsequent intestinal digestion. In any case, because of food fortification the amount of free AAs detected in the DMF was higher than the unfortified HM. The changes in the urinary metabolome of the premature babies fed with different fortified foods led to a significantly higher level of the excretion of leucine and lysine (for those fed with the BMF) than for those fed with a DMF (Giribaldi et al., 2020), which is in accordance with the observed predominance of these two AAs in the profile of the digested BMF.

### 3.4. Lipolysis during gastric and intestinal digestion

In this study, a pre-lipolysis (10%) was observed that was in accordance with figures reported in other studies (de Oliveira et al., 2016; Nebbia et al., 2020). A small yet significantly higher, difference in DLH during the gastric digestion was found in the case of the BMF. During intestinal digestion, lipolysis increased from ~ 20% to ~ 55% at the end of intestinal digestion (Fig. 5a), and was not differently affected by the two fortifiers. Overall, considering the negligible amount of lipids (+0.28 g/100 mL in the DMF, +0.04/100 mL in the BMF), fortification is not expected to substantially modify lipolysis in HM. Accordingly, when considering the relative proportion of the different lipid classes present during digestion, a decrease in TAG content and a concomitant increase in FFAs, in particular during the intestinal phase, with a similar profile was observed for both BMF and DMF (Supplementary data Fig. 3).

Fig. 5b and c show the profile of specific fatty acids in the DMF and BMF, reported as total esterified fatty acids and as fatty acids released during *in vitro* digestion. The short chain FAs (C6:0 to C10:0) were found to be more readily released during the gastric digestion phase, as they are located predominantly on external positions (*sn*-1,3) of the glycerol backbone, corresponding to the regioselectivity of the gastric lipase (*sn*-3) (Bourlieu & Michalski, 2015). The most abundant products of lipolysis were C18:1 (n-9)c and C18:2 (n-6)c, which was the case for both infant foods although they were more abundant in BMF. Conversely, C8:0, C10:0 and C18:3 (n-3) were present in higher amounts in the DMF, since they are naturally present in donkey milk (Gastaldi, 2010). The high proportion of ω3 PUFA in the DMF (in particular C18:3 (n-3)) resulted in a significantly lower ω6/ω3 free PUFA ratio in the DMF




(d) ω6/ω3 PUFA ratio

| | DMF | BMF | Significance |
|---|---|---|---|
| G0 | 5.34 ± 0.63 | 6.13 ± 0.19 | NS |
| G90 | 2.93 ± 0.60 | 7.53 ± 2.01 | * |
| I30 | 2.89 ± 0.44 | 10.17 ± 1.17 | *** |
| I90 | 2.50 ± 0.21 | 7.71 ± 1.53 | ** |
| I180 | 3.49 ± 0.00 | 8.70 ± 0.59 | *** |

**Fig. 5.** Degree of lipolysis of donkey (DMF) and bovine (BMF) fortified human milk during gastric (G0-G90: 0–90 min) and intestinal (I30-I90-I180: 30–180 min) digestion. Degree of lipolysis determined by means of densitometry over the thin layer chromatography (a). Profiles of free fatty acids released from DMF (b) and BMF (c) during digestion. ω6/ω3 free PUFA ratio in DMF and BMF during digestion (d). Fatty acids were determined by means of GC/GC–MS/MS analysis. Data represented as means ± standard deviations (N = 3). $p < 0.001$ (***); $p < 0.01$ (**); $p > 0.05$ (NS).

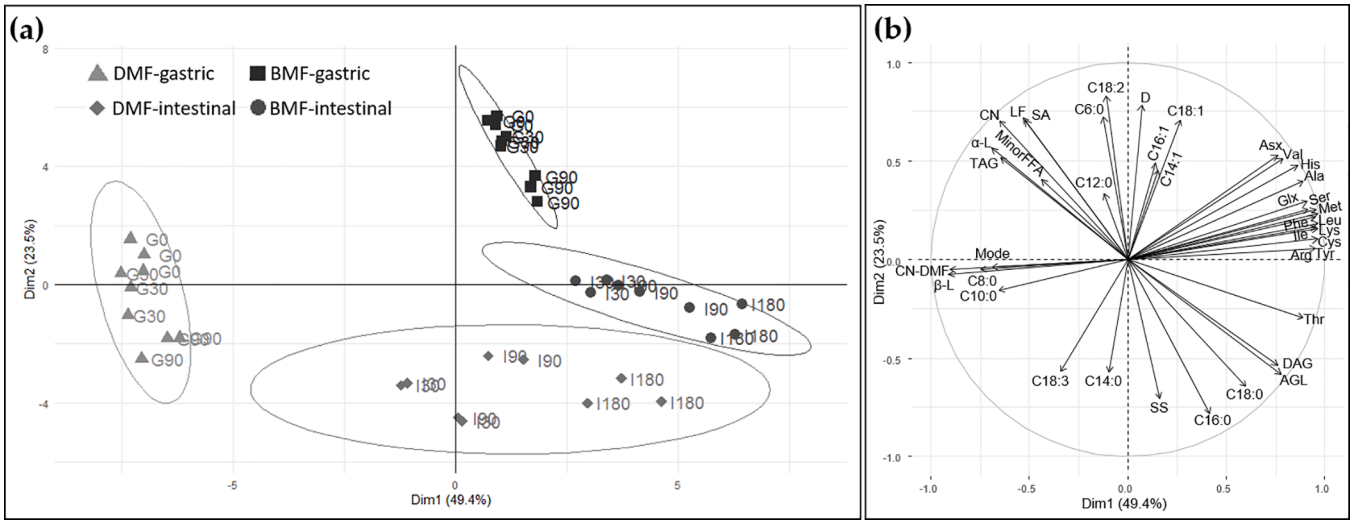



**Fig. 6.** Projection of the individual (a) and variables (b) on the first two dimensions (Dim1 and Dim2) of the principal component analysis (PCA) of donkey (DMF) and bovine (BMF) supplements during gastric (G0-G30-G90: 0–90 min) and intestinal (I30-I90-I180: 30–180 min) digestion. Changes in the particle size distribution: Mode, D[4,3]-diameter (D), specific surface (SS). Protein digestion: Lactoferrin (LF), serum albumin (SA), casein (CN), casein detected in DMF (CN-DMF), β-lactoglobulin (β-L) and α-lactalbumin (α-L). Free amino acids: asparagine + aspartic acid (Asx), thronine (Thr), serine (Ser), glutamin + glutamic acid (Glx), alanine (Ala), valine (Val), cysteine (Cys), metionine (Met), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), lysine (Lys), arginine (Arg) and histidine (His). Degree of lipolysis: triglycerides (TAG), free fatty acids (FFA) and diglycerides (DAG). Relative % of fatty acids released detected by GC/GC–MS/MS: C8:0 to C18:3 (n3)c and the minor free fatty acids (Minor FFA).

(value < 3.49) than found in the BMF samples (value > 7.53), during both the gastric and intestinal digestion phases, whereas the free PUFA ratio was the same in the two infant foods before digestion (5.7) (Fig. 5d). A low ω6/ω3 PUFA ratio may lead to important physiological effects, such as a decrease in the production of compounds causing inflammation generated from arachidonic acid, resulting in improved inflammatory conditions and protection against oxidative stress (Di Pasquale, 2009).

### 3.5. Principal component analysis

In order to explore the possible correlations between the two types of food fortification, the digestion time, and the different analyses (products of proteolysis, lipolysis, as well as particle size characteristics), a PCA was carried out (Fig. 6). The PCA allowed to separate the two digestion phases (gastric and intestinal) but also the two fortifications (DMF and BMF) (Fig. 6a). The fortified HM samples grouped separately during the gastric phase, mostly depending on the higher bioaccessibility of free AAs present, and on a lower concentration of C18:1 (n-9)c and C18:2 (n-6)c fatty acids in the BMF(Fig. 6b). During the gastric digestion, the DMF was associated with the formation of larger particles, with a higher concentration of C8:0, C10:0 and C18:3 (n-3)c, and with milk proteins (β-lactoglobulin and a different casein isoforms) that relate specifically to donkey's milk. Due to the high level of protein and lipid hydrolysis occurring in the intestinal phase, and with the increase of free AAs and fatty acids, and together with the decrease of TAG, particle size diameter and of the concentration of the main HM intact proteins (lactoferrin, casein, α-lactalbumin, and serum albumin), both intestinal samples grouped separately with respect to the gastric phase, and both correlated positively with the first axis. Always in the intestinal phase, samples were separated in the graph of individuals (Fig. 5a), but the differences were considerably less evident than in the gastric phase, revealing a closer profile of hydrolysis products than at the end of the gastric phase.

In order to achieve more information on the overall effect of fortifying HM, data arising from the use of DMF and BMF were compared with previously published values on HM following Holder pasteurization (Nebbia et al., 2020), leading to a further PCA (Supplementary data Fig. 6). The time of digestion was positively correlated with the first dimension, with the overall DPH and DLH, and negatively correlated with particle size distribution. The DLH was similar in all samples whereas unfortified HM led to a lower DPH than with the BMF, whereas the DMF displayed an intermediate behaviour. Of note is that, irrespectively of the starting point, by the end of intestinal digestion the differences in the digesta were generally reduced, although in the case of the BMF, it was still characterized by higher DPH when compared to DMF. In other hand, fortification did not affect the degree of lipolysis but can have an impact on the overall release of free peptides and AAs.

## 4. Conclusion

The use of an *in vitro* dynamic model to simulate infant digestion revealed the different impacts of an experimental donkey milk-based fortifier when compared to that a commercial bovine milk-based fortifier on the digestion of proteins and lipids in HM. DMF reached at the end of digestion a similar net degree of proteolysis and lipolysis to BMF and could represent a better source of proteins and lipids with a low ω6/ω3 free PUFA ratio, the latter being an important nutritional parameter for the protection of the infant from oxidative stress. Nevertheless, further peptidomic analysis will be necessary to provide an insight into the specific features that distinguish the two fortified infant foods studied in terms of the bioactive peptides release and in depth study of the digestion of the donkey proteins.

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## CRediT authorship contribution statement

**Stefano Nebbia:** Investigation, Methodology, Data curation, Writing – original draft. **Amélie Deglaire:** Investigation, Data curation, Supervision, Project administration, Writing – review & editing. **Olivia Ménard:** Investigation, Data curation, Supervision, Methodology, Writing – review & editing. **Gwénaële Henry:** Investigation, Data curation, Methodology, Writing – review & editing. **Elettra Barberis:** Investigation, Methodology, Writing – review & editing. **Marcello Manfredi:** Investigation, Methodology, Writing – review & editing. **Enrico Bertino:** Resources, Methodology, Writing – review & editing. **Alessandra Coscia:** Resources, Methodology, Writing – review & editing. **Didier Dupont:** Conceptualization, Data curation, Funding acquisition, Writing – review & editing. **Marzia Giribaldi:** Conceptualization, Data curation, Writing – review & editing. **Laura Cavallarin:** Conceptualization, Data curation, Supervision, Project administration, Funding acquisition, Writing – original draft.

## Declaration of Competing Interest

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

## Data availability

Data will be made available on request.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.133579.

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