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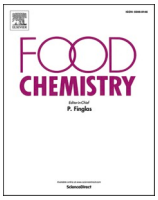

Protein-bound and free glycation compounds in human milk: A comparative study with minimally processed infant formula and pasteurized bovine milk

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ABSTRACT

The role of the Maillard reaction and the accumulation of non-enzymatic glycation compounds in human milk have been scarcely considered. In this study, we investigated the proteins most susceptible to glycation, the identity of the corresponding modified residues and the quantitative relationship between protein-bound and free glycation compounds in raw human milk and, for comparison, in minimally processed infant formula and pasteurized bovine milk. In human milk, total protein-bound lysine modifications were up to 10% of the counterparts in infant formula, while *Nε*-carboxymethyllysine reached up to 27% of the concentration in the other two products. We demonstrated that the concentration of free pyrraline and methylglyoxal-hydroimidazolone were of the same order of magnitude in the three milk types. Our results delineate how the occurrence of some glycation compounds in human milk can be an unavoidable part of the breastfeeding and not an exclusive attribute of infant formulas and pasteurized bovine milk.

1. Introduction

The divergence between human milk (HM), as a major example of unprocessed foods, and infant formulas, a part of processed or ultra-processed dairy products, represents an intriguing and critical challenge in the field of food chemistry and infant nutrition (Pischetsrieder & Henle, 2012). The World Health Organization (WHO) guidelines encourage breastfeeding, as it represents the golden standard for infant nutrition and correlates with beneficial aspects for both the mother and the child; in this context, exclusive breastfeeding is recommended up to 6 months of age (Koletzko et al., 2019). However, when breastfeeding is not possible, infant formulas become the best alternative. Infant formulas are produced by the extensive processing of bovine, goat and soy milk and nutritional ingredients; operations such as homogenization, thermal treatment and spray drying can introduce some unavoidable consequences in terms of modifications of proteins, carbohydrates and lipids (Lund, Bechshøft, Ray, & Lund, 2022). Indeed, infant formulas are included among the family of ultra-processed foods, in which the main drawback is represented by the possibility that an array of chemical modifications alters the digestibility of milk proteins and the subsequent bioavailability of amino acids (Arena et al., 2010; Renzone, Arena, & Scaloni, 2015).

During infant formula processing, reactions on milk proteins are mainly driven by the Maillard reaction, with non-enzymatic glycation and oxidation processes mostly mediated by reducing monosaccharides, oligosaccharides and their degradation products, and to a lesser but consistent extent, by lipids and their degradation products (Delgado-Andrade & Fogliano, 2018). Because of mother's nutrition and metabolism, chemical changes on proteins and amino acids can possibly occur also in HM; mother's dietary components can be transferred

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through the milk to the infant and become physiological effector compounds impacting on newborn's health (Garcia-Mantrana & Collado, 2016). Independently of the kind of feeding, the chemical nature of the side chain, the occurrence as protein-bound or free form, as well as the corresponding bioaccessibility and bioavailability, are the major drivers for the definition of any nutritional outcome of amino acid derivatives in infant's health (Cordova et al., 2020; Hellwig & Henle, 2014). A diet rich in modified components impacts the composition of gut microbiota wherever modified peptides are not digested or absorbed (van der Lugt et al., 2020). Moreover, poorly bioavailable modified milk proteins have been often associated with carbon limitation, in which protein catabolism and resulting amino acids become the primary source of energy (Chow et al., 2014). Poorly bioavailable proteins boost the amount of the corresponding non-canonical species that reach the colon (Lassak, Aveta, Vougioukas, & Hellwig, 2023); therefore, the quantification of the real intake of glycation and oxidation compounds from HM or infant formula is a prerequisite to define their impact on the infants' gut microbiota metabolism in the short and long term. Solid evidence has demonstrated that glycation compounds, such as *N*ε-fructosyllysine, *N*ε-carboxymethyllysine (CML), pyrraline, and other oxidation compounds can be metabolized by the microbiota, with the formation of short chain fatty acids (SCFA) from the Amadori compound and carboxymethylated biogenic amines and other organic acids from CML (Bui, Troise, Fogliano, & De Vos, 2019; Hellwig et al., 2015). Formula-fed infants can efficiently degrade *N*ε-fructosyllysine because of their microbiota (Bui et al., 2020), while Amadori compounds and secondary bile acids are mainly present in the feces of formula fed infants (Sillner et al., 2021).

Previous studies have fully detailed the occurrence of glycated amino acids, peptides and proteins in raw bovine milk and various dairy products, including infant formulas (Hegele, Buetler, & Delatour, 2008; Henle, 2005; Meltretter, Wüst, & Pischetsrieder, 2013; Meltretter, Wüst, & Pischetsrieder, 2014; Milkovska-Stamenova & Hoffmann, 2016a; Milkovska-Stamenova & Hoffmann, 2016b; Milkovska-Stamenova & Hoffmann, 2019). This is not the case of HM, where the scientific literature consisted only in very few preliminary reports on the concentration of protein-bound lysine modification products (Dittrich et al., 2006; Sebeková et al., 2008; Marousez et al., 2022). In HM, a systematic investigation on non-enzymatically glycated and oxidized lysine and arginine derivatives in protein bound and free form was not accomplished so far and a quantitative relationship between both forms has not yet been satisfactorily established. To address this lack of knowledge and to assign compounds deriving from mother's diet putatively transferred to the infant, we used three mass spectrometry-based techniques that evaluated the protein modification targets, the identity of the modified residues and the concentration of arginine and lysine glycation and oxidation products both in protein-bound and free forms in HM. Furthermore, a straightforward comparison of glycation and oxidation compounds in HM with minimally processed infant formula and pasteurized bovine milk contributed to the definition of a cause-effect relationship between amino acid and protein modifications in milk and the overall potential intake of the above-mentioned products in breastfed and formula-fed infants.

2. Materials and methods

2.1. Chemicals

Acetonitrile, water, and formic acid were of mass spectrometry grade and were obtained from Merck (Darmstadt, Germany). Analytical standards *N*ε-fructosyllysine, fructosylarginine hydrochloride >80% were purchased from TRC (Toronto, Canada), while CML, *N*ε-carboxyethyllysine (CEL), pyrraline, methylglyoxal-hydroimidazolone (MG-H1) were obtained from IRIS-biotech (Marktredwitz, Germany). Prolidase (208 U/mg protein), pepsin (3555 U/mg protein), and leucine aminopeptidase (19 U/mg protein), *N*ε-acetyllysine, lysine and arginine were obtained from Merck-Sigma-Aldrich (Darmstadt, Germany). All the other chemicals were of analytical grade and were purchased from Merck-Sigma-Aldrich, unless otherwise stated.

2.2. Human milk, and pasteurized bovine milk and minimally processed infant formula

Fresh mature HM samples were provided by healthy mothers ($n = 5$) with healthy full-term and exclusively breast-fed infants from MAMI cohort (PMID: 31053102, García-Mantrana et al., 2019). Mother participants were familiarized with the purpose and nature of the study; preventive informed written consent was obtained that was approved by the Ethics Committees of the Hospital Clínico Universitario de Valencia, Spain, and the Ethical Committee of the CSIC, Spain. Fresh HM samples were collected at lactation stages in the range 3–7 months, rapidly homogenized by mixing, then stored at −20 °C until freeze-drying. In parallel, commercial pasteurized bovine milk (PM) samples were purchased at a local supermarket (Valencia, Spain). Powdered minimally processed infant formula (MPIF) samples were obtained from a MPIF prototype (66 kcal/100 mL), containing proteins (1.3 g/100 mL), lipids (3.4 g/100 mL) and carbohydrates (7.2 g/100 mL) of which the latter included a scGOS/lcFOS prebiotic mixture, consisting of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (9:1, 0.8 g/100 mL), which were manufactured per good manufacturing practices (ISO 22000). During the production of this prototype product, heat treatment was reduced to a minimum, while still adhering to requirements per EU legislation (15 s at 72 °C). All HM, MPIF and BM samples were freeze dried in different aliquots and stored at −20 °C. Samples were reconstituted in sterile water (1.2:10 *w/v*), aliquoted and directly subjected to three analytical approaches, as reported below. Specifically, (i) milk proteins were precipitated and subjected to proteomics to assign the main molecular modification targets and the identity of the corresponding modified residues (providing qualitative information on protein-bound derivatives). In parallel, a milk fractionation based on molecular mass was accomplished to obtain (ii) a retentate fraction that was subjected to extensive enzymatic hydrolysis and hydrophilic interaction liquid chromatography (HILIC) high-resolution tandem mass spectrometry to quantify protein-bound lysine and arginine modification products (providing quantitative information on protein-bound derivatives) and, (iii) a permeate fraction that was directly subjected to HILIC high-resolution tandem mass spectrometry to quantify free lysine and arginine modification products (providing qualitative and quantitative information on free derivatives). In all cases, comparative experiments were performed on human milk, minimally processed infant formula and pasteurized bovine milk.

2.3. Proteomics

Proteins from milk samples (100 μL) were precipitated by the addition of cold acetone (1:6 *v*/v), pelleted by centrifugation at 8000x *g*, for 10 min, at 4 °C, and dried in SpeedVac centrifugal evaporator (Thermo Fisher Scientific, Bremen, Germany). Recovered proteins were redissolved in 100 μL of 100 mM tetraethylammonium bicarbonate (TEAB) and quantified by bicinchoninic acid-based assay (Thermo Fisher Scientific). Proteins (100 μg) were reduced with 5 μL of 200 mM tris(2-carboxyethylphosphine), at 55 °C, for 60 min, alkylated by adding 5 μL of 375 mM iodoacetamide, for 30 min, in the dark, at room temperature, precipitated by addition of 6 vol of cold acetone, pelleted by centrifugation at 8000x *g*, for 10 min, at 4 °C, and dried by centrifugal evaporation. Recovered alkylated proteins (90 μg) were digested with freshly prepared trypsin (quantitative ratio of trypsin to protein 1:50) in 100 mM TEAB, at 37 °C, overnight. Protein digests were directly analyzed in duplicate according to a shotgun approach with a nanoLC-hybrid quadrupole-Orbitrap consisting of an UltiMate 3000 HPLC RSLC nano-system (Thermo Fisher Scientific) coupled to a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific) through an Easy-Spray ion nano-source, and mounting an Easy-Spray column packed

with PepMap RSLC C18 resin (150 mm × 75 μm ID; 2 μm particle size; 100 Å pore size, Thermo Fisher Scientific) and thermostated at 40 °C. After loading onto the column, peptides were eluted with a gradient of solvent B (19.92/80/0.08 water/acetonitrile/formic acid *v/v/v*) in solvent A (99.9/0.1 water/formic acid v/v), at a flow rate of 300 nL/min. The following gradient of solvent B (minutes/%B) was used: (0/3), (35/40), (40/80), (44/80), with 21 min for equilibration. The mass spectrometer operated in data-dependent mode, using a full scan (range *m/z* 375–1500, nominal resolution of 70,000), followed by MS/MS scans of the 8 most abundant ions. MS/MS spectra were acquired in a dynamic *m/z* scan range, using a nominal resolution of 17,500, a normalized collision energy of 28%, an automatic gain control target of 50,000, and a maximum ion target of 110 ms. A dynamic exclusion value of 10 s was used.

For protein and peptide assignment, raw mass data files were analyzed with Proteome Discoverer v. 2.4 software (Thermo Fisher Scientific), running Mascot software, v. 2.4.2 (Matrix Science, London, UK). Criteria for database searching were the following: *Bos taurus* (134,396 sequences) and *Homo sapiens* (204,942 sequences) database (UniProtKB) depending on the milk nature, carboxyamidomethylation at cysteine as fixed modification, and glycation (glucose/galactose) at lysine, lactosylation at lysine, carboxymethylation at lysine and arginine, oxidation at methionine, pyroglutamate formation at glutamine, and phosphorylation at serine, threonine and tyrosine as variable modifications. Peptide mass tolerance was set to ±10 ppm, and the fragment mass tolerance to ±0.05 Da. Proteome Discoverer peptide candidates were considered confidently identified only when the following criteria were satisfied: i) protein and peptide False Discovery Rate (FDR) confidence: high; ii) peptide Mascot score > 30; iii) Peptide Spectrum Matches (PSMs): >2. For amino acid numbering, assigned protein sequences from UniProtKB included the signal portion.

For semi-quantitative analysis, data were analyzed using in the processing step "minora feature detector" node, setting PSM high for component verification. "Feature map" node was used for chromatographic alignment with selection of maximum peptide retention time shift of 10 min and a mass tolerance of 10 ppm. Peptide precursor ion quantification was performed using the corresponding intensity; normalization was obtained using the total sum of the abundance values for all peptides. ANOVA test was used to assess statistical confidence.

2.4. Protein enzymatic hydrolysis and isolation of protein-bound modified amino acids

Milk samples (100 μL) and control samples (BSA 10 mg/mL) were loaded onto Amicon 10 kDa centrifugal filter units (Merck-Millipore, Darmstadt, Germany) and centrifuged at 18,000 xg, for 30 min, at 4 °C. Retentate samples underwent enzymatic hydrolysis, while permeates were freeze-dried then stored for analysis of free markers. Enzymatic hydrolysis was set up as previously reported (Thornalley & Rabbani, 2014), with minor modifications. Freeze dried retentates with protein fraction >10 kDa were suspended in 50 μL of water and mixed with 10 μL of 100 mM HCl, 5 μL of a 20 mM HCl solution containing pepsin (2 mg/mL), and 5 μL of a 20 mM HCl solution containing thymol (2 mg/mL), and finally incubated at 37 °C for 24 h. Samples were then mixed with 12.5 μL of 100 mM potassium phosphate buffer (pH 7.4), 5 μL of 260 mM potassium hydroxide, 5 μL of a 10 mM potassium phosphate solution containing pronase E (2 mg/mL), pH 7.4, and 5 μL of a solution containing penicillin (1000 units/mL) and streptomycin (1 mg/mL), and incubated again at 37 °C, for 24 h. The last step included the addition of 5 μL of a 10 mM potassium phosphate buffer solution containing aminopeptidase (2 mg/mL), pH 7.4 and 5 μL of a 10 mM potassium phosphate buffer solution containing prolidase (2 mg/mL), pH 7.4. Upon the incubation at 37 °C for 48 h, the enzymatic hydrolysis was stopped by the addition of acetonitrile, and samples were centrifuged at 18,000 xg, for 10 min, at 4 °C, and transferred to glass vials for HILIC high-resolution tandem mass spectrometry.

2.5. Isolation of free markers

Permeate samples were freeze dried then dissolved in 50 μL acetonitrile/water (1:1, *v/v*), centrifuged at 18,000 x *g*, for 10 min, at 4 °C; resulting acetonitrile/water supernatants were analyzed by HILIC high-resolution tandem mass spectrometry, as detailed below.

2.6. HILIC high-resolution tandem mass spectrometry

For protein-bound and free modified lysine and arginine derivatives obtained from permeate and retentate fractions, LC-MS/MS data were separately acquired using an Exploris 120 quadrupole Orbitrap high-resolution mass spectrometer interfaced to a Vanquish Core liquid chromatographic system (Thermo Fisher Scientific, Bremen, Germany). Compounds listed in Supplementary Table S1 (namely: lysine, *N*ε-fructosyllysine, *N*ε-lactulosyllyine, CML, CEL, *N*ε-formyllysine, *N*ε-acetyllysine *N*ε-lactoyllysine, pyrraline, arginine, fructosylarginine, 3-deoxyglucosone-hydroimidazolone, 3-DG-H and MG-H1) were separated through a zwitterionic sulfobetaine column (Atlantis Premier BEH, *Z*-HILIC, 100 × 2.1, 1.7 μm, Waters, Milford, MA) thermostated at 35 °C, which was eluted with 0.1% formic acid in acetonitrile (solvent A) and 0.1% formic acid in water (solvent B), at a flow rate of 0.2 mL/min. The following gradient of solvent B (minutes/%B) was used: (0/5), (1.5/5), (10/50), (13/50). For positive ion mode, H-ESI interface parameters were as follows: static spray voltage 3.3 kV, ion transfer tube and vaporizer temperature were both at 280 °C; sheath gas flow and auxiliary gas flow were 30 and 15 arbitrary units, respectively. Upon a preliminary screening in full scan mode in the *m/z* range 50–600 with the analyzer resolution set at 60,000 (FWHM at *m/z* 200), analytes were identified in product ion scan mode screening the precursor ions according to an *in-house* mass list generated in Trace Finder (v. 5.1, Thermo Fisher Scientific, Waltham, MA). For product ion scan, normalized collision energy was set according to Supplementary Table S1, while the quadrupole and Orbitrap resolution values were set at 1 and 15,000 (FWHM at *m/z* 200), respectively. Profile data were collected using Xcalibur 4.5 (Thermo Fisher Scientific, Waltham, MA) and fragmentation spectra were recorded by using Free Style software (v. 1.8, Thermo Fisher Scientific, Waltham, MA). EASY-IC with fluoranthene in positive ion mode (*m/z* 202.0777 $[M]^+$) was used to improve mass accuracy in both full scan and product ion targeted MS^2 scan mode. Analytical performance robustness, sensitivity, reproducibility, repeatability, linearity, accuracy, carry over and matrix effects were evaluated by following a procedure previously reported (Troise, Fiore, Wiltafsky, & Fogliano, 2015) using the analytical standards reported in Supplementary Table S1.

2.7. Statistical analysis

Concentration of amino acid derivatives was achieved through standard addition technique and was reported as ng/mg of protein for protein-bound markers and as ng/mL for free markers. Each sample was analyzed from two independent replicates and injected twice to monitor reproducibility over the running batch. Statistical analysis and Tukey test were performed in GraphPad Prism (v. 6.01, GraphPad software, San Diego, CA).

3. Results

3.1. Proteomics compares modifications of proteins in human milk, minimally processed infant formula and pasteurized bovine milk

Proteomics was the starting investigation for evaluating the differences in the modifications among HM, MPIF and PM. As putative targets of variable protein modification, we included reaction products of lactose and hexoses (glucose/galactose) with the ε-amino group of lysine, leading to the formation of *N*ε-lactulosyllysine and *N*ε-

hexosyllysine, along with the corresponding carboxymethylated derivative, as well as the glycation and carboxymethylation adducts at the guanidino side chain of arginine. Other variable modifications considered during *in-silico* research of modified peptides included products deriving from oxidation at methionine, and phosphorylation at serine, threonine and tyrosine. Although the latter modifications highly affected peptide profiles, corresponding results were not of interest in this study and will be not described below. Supplementary Table S2 outlines an overview of the 2,616 assigned peptides corresponding to 599 proteins identified in all above-reported milk typologies, including a total of 144 modified peptides that underwent chemical modifications as result of the Maillard reaction and its associated oxidation pathways. Considering the sum of lactosylated and hexosylated residues, the percentage number of Amadori compounds at lysine residues varied among the three milk types. While for infant formula and pasteurized milk the ratio between the number of glycated peptides and that of total peptides was 14.4% and 13.7%, respectively, in the case of human milk samples this parameter was about ten times lower and ranged from 1.4 to 2.3%. These findings were in good agreement with previous observations on raw and heated bovine milks (Milkovska-Stamenova & Hoffmann, 2016a; Milkovska-Stamenova & Hoffmann, 2016b; Milkovska-Stamenova & Hoffmann, 2019; Renzone et al., 2015), and definitively demonstrated the occurrence of non-enzymatic modification products also in human milk.

In HM, we identified 33 modified peptides that were associated with human proteins, namely αS1-casein (αS1-CN), β-casein (β-CN), lactotransferrin (LTF), α-lactalbumin (LAC), polymeric immunoglobulin receptor (PIGR) and serum albumin (ALB) (Table 1, Supplementary Table S2). Among that, 17, 4 and 12 modified peptides included *N*ε-lactulosyl-, *N*ε-hexosyl- and *N*ε-carboxymethyllysine, respectively. Two peptides occurred both as lactosylated and hexosylated species at the same lysine, while three ones exhibited both lactosylated and carboxymethylated adducts at the same amino acid (Table 1). At present, no information is available to discriminate whether the observed carboxymethylated peptides may derive from the oxidative fragmentation of the corresponding protein Amadori/Heyns adducts or the direct modification of proteins by reactive sugar-derived oxidation products glyoxal, glycolaldehyde and glyoxylic acid. Among the modified peptides reported above, 31 molecules were associated with human proteins (Table 1, Supplementary Table S2). For example, Fig. 1 shows two of them, whose fragmentation spectrum proved their nature as human LTF peptide (279–292) carboxymethylated at Lys283 (panel A) and human LAC peptide (78–88) bearing carboxymethylation Lys81 (panel B). As expected, the intensity values of the parent signals demonstrated their occurrence only in HM. The remaining two modified peptides identified in HM were associated with ALB peptides (161–168) and (438–452) bearing *N*ε-lactosylation at Lys161 and Lys438, respectively (Table 1, Supplementary Table S2). The identical sequence of human and bovine albumin at these portions hampered exclusively assigning these peptides to one of these mammalian protein homologues. Only the latter peptide was also detected in MPIF and PM samples. The detection of peptides that might have both human and bovine origin in HM prompted us to search mass spectrometric data obtained for human milk samples against the bovine database (data not shown). This was done because previous studies based on optimized prefractionation techniques and stable isotope-labeled standards already demonstrated the presence in HM of exogenous peptides from bovine αS1-CN, αS2-CN, β-CN, κ-CN and β-lactoglobulin (LGB) that arose from the mother's diet (Zhu, Garrigues, Van den Toorn, Stahl, & Heck, 2019). No additional lactosylated, hexosylated and carboxymethylated peptides having bovine origin were

Table 1
Peptides bearing glyco-oxidative modifications deriving from proteins present in human milk (HM) samples. Protein description, protein accession, peptide sequence, modifications (including modified amino acid), similarity with the bovine counterpart (in red are reported differing amino acids), number of PSMs, number of missed cleavages, theoretical $[M + H]^+$ value [Da], and Mascot score are reported. Additional data are reported in Supplementary Table S2.

Protein Description	Protein Accession	Peptide Sequence	Modifications	Bovine Sequence	#PSMs	# Missed cleavages	Theor. $[M+H]^+$ [Da]	Mascot Score
Albumin	P02768	ADLAKYICENQDSISSK	1xHex [K5]	ADLAKYICDNQDTISSK	4	1	2103.97506	67
Albumin	P02768	AEFAEVSKLVTDLTK	1xHex [K8]	AEFVEVTKLVTDLTK	4	1	1812.9477	36
Albumin	P02768	FKDLGEENFK	1xHex(2) [K2]	FKDLGEEHFK	2	1	1550.71083	31
Albumin	P02768	FKDLGEENFK	1xHex [K2]	FKDLGEEHFK	2	1	1388.658	42
Albumin	P02768	KQTALVELVK	1xHex [K1]	KQTALVELLK	19	1	1290.75151	36
Albumin	P02768	KQTALVELVK	1xHex(2) [K1]	KQTALVELLK	9	1	1452.80433	36
Albumin	P02768	KVPQVSTPTLVEVSR	1xHex(2) [K1]	KVPQVSTPTLVEVSR	6	1	1964.04339	77
Albumin	P02768	KYLYEIAR	1xHex(2) [K1]	KYLYEIAR	3	1	1379.69405	34
Albumin	P02768	LAKTYETTLEK	1xHex(2) [K3]	LAKEYEATLEECCAK	2	1	1620.81021	38
Albumin	P02768	TCVADESAENCDKSLHTLFGDK	1xHex(2) [K13]	TCVADESHAGCEKSLHTLFGDE	2	1	2821.20263	32
Albumin	P02768	YKAAFTECCQAADK	1xHex(2) [K2]	YNGVFQECCQAADK	2	1	1986.8307	80
Alpha-lactalbumin	A0A080YV01	ALCTEKLEQWLCEK	1xHex(2) [K6]	ALCSEKLDQWLCEK	12	1	2131.97736	37
Alpha-lactalbumin	A0A080YV01	ALCTEKLEQWLCEK	1xCarboxymethyl [K6]	ALCSEKLDQWLCEK	2	1	1865.8772	58
Alpha-lactalbumin	A0A080YV01	ALCTEKLEQWLCEKL	1xHex(2) [K6]	ALCSEKLDQWLCEKL	7	2	2245.06143	35
Alpha-lactalbumin	A0A080YV01	GIDYWLAHK	1xCarboxymethyl [K9]	GINYWLAHK	16	0	1160.57348	47
Alpha-lactalbumin	A0A080YV01	ILDIKGIDYWLAHK	1xHex(2) [K5]	ILDKVGINYWLAHK	5	1	2009.04775	32
Alpha-lactalbumin	A0A080YV01	ILDIKGIDYWLAHK	1xCarboxymethyl [K5]	ILDKVGINYWLAHK	4	1	1742.94758	61
Alpha-lactalbumin	A0A080YV01	LWCKSSQVPQSR	1xCarboxymethyl [K4]	IWCKDDQNPHSSNICNISCDK	8	1	1533.74784	40
Alpha-lactalbumin	A0A080YV01	NICDISCDKFLDDDITDDIMCAK	1xCarboxymethyl [K9]	NICDISCDKFLDDDLTDDIMCVK	12	1	2835.18276	83
Alpha-lactalbumin	A0A080YV01	NICDISCDKFLDDDITDDIMCAK	1xHex(2) [K9]	NICDISCDKFLDDDLTDDIMCVK	8	1	3101.28293	80
Alpha-lactalbumin	A0A080YV01	NICDISCDKFLDDDITDDIMCAK	1xCarboxymethyl [K9]; 1xOxidation [M20]	NICDISCDKFLDDDLTDDIMCVK	7	1	2851.17767	34
Alpha-lactalbumin	A0A080YV01	QFTKCELSQLLK	1xHex(2) [K4]	QLTKCEVFRELK	3	1	1818.90412	32
Alpha-S1-casein	P47710	EKQTDEIKDTR	1xHex(2) [K2]	sequence identity <30%	3	2	1686.7916	43
Beta-casein	P05814	AKDTVYTK	1xCarboxymethyl [K2]	sequence identity <30%	3	1	983.5044	34
Beta-casein	P05814	VMPVLKSPTIPFFDPQIPK	1xHex(2) [K6]	EMPFPKYPVEPFTER	5	1	2478.30879	41
Lactotransferrin	B3VMW0	DLLFKDSAIGFSR	1xCarboxymethyl [K5]	sequence identity <30%	5	1	1526.78493	60
Lactotransferrin	B3VMW0	KCSTSPLLEACEFLR	1xHex(2) [K1]	KCSTSPLLEACAFLTR	3	1	2134.98826	55
Lactotransferrin	B3VMW0	KSEEEVAAR	1xCarboxymethyl [K1]	ETAEEVKAR	3	1	1076.52184	34
Lactotransferrin	B3VMW0	KSEEEVAAR	1xHex(2) [K1]	ETAEEVKAR	2	1	1342.62201	34
Lactotransferrin	B3VMW0	NGSDCPDKFCLFQSETK	1xCarboxymethyl [K8]	NGKNCPDKFCLFKSETK	9	1	2090.87938	68
Lactotransferrin	B3VMW0	SPKFQLFGSPSGQK	1xHex(2) [K3]	SRSFQLFGSPPGQK	2	1	1831.896	44
Lactotransferrin	B3VMW0	SVNGKEDAIWNLLR	1xCarboxymethyl [K5]	SVDGKEDLIWKLLS	14	1	1672.86531	65
Polymeric immunoglobulin receptor	P01833	NADLQVLKPEPELVYEDLR	1xCarboxymethyl [K8]	NIDLQVLEPEPELVYGDLR	3	0	2299.18162	77

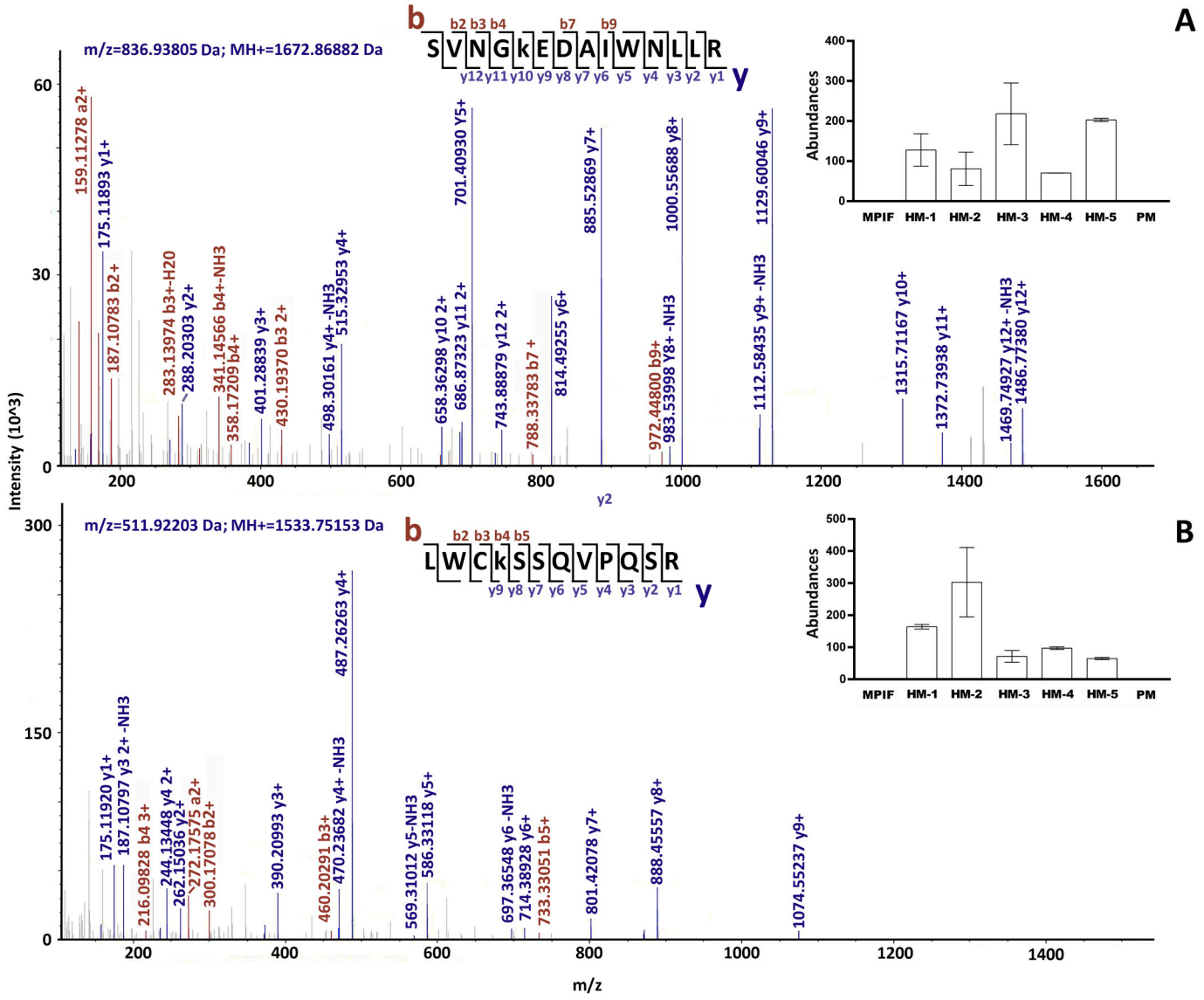

Fig. 1. High resolution tandem MS spectrum in positive ion mode and quantitative information (inset) of two carboxymethylated peptides exclusively fragmented in human milk (HM) and not in minimally processed infant formula (MPIF) and pasteurized bovine milk (PM). Panel A. Lactotransferrin peptide (279–292) bearing carboxymethylation at Lys283. Panel B. α-Lactalbumin peptide (78–88) bearing carboxymethylation at Lys81. The b series is outlined in red, while the y series is labeled in blue; the lowercase letter indicates the modified amino acid. Peptide semi-quantitative information derived from the evaluation of the peptide precursor ion intensity values measured in HM, MPIF and PM samples. ANOVA test was used to assess data statistical confidence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

detected. This may be attributed to the real absence of modified peptides from bovine proteins in the analyzed HM samples as well as to the technical power of the shotgun procedure that we used. At present, we cannot exclude that lactosylated, hexosylated and carboxymethylated peptides from bovine proteins that are ingested by mothers can migrate from their plasma to their milk and become part of newborns' diet. The original detection of lactosylated, hexosylated and carboxymethylated peptides in HM samples provided relevant information on the possibility that non-enzymatic glycation and oxidation reactions can also occur at human proteins and be part of physiological processes linked to milk secretion in the human mammary gland.

In MPIF and PM samples, we identified 110 modified peptides belonging to ten bovine proteins, namely αS1-CN, αS2-casein (αS2-CN), β-CN, κ-casein (κ-CN), LTF, LAC, ALB, LGB, glycosylation-dependent cell adhesion molecule 1 (GLYCAM) and butyrophilin (BUT) (Supplementary Table S2). Among that, 33, 30 and 47 modified peptides included *N*ε-lactulosyl-, *N*ε-hexosyl- and *N*ε-carboxymethyllysine, respectively. Most of these proteins were already identified as modified species in previous studies on various commercial dairy products (Arena et al., 2010; Meltretter et al., 2013, 2014; Milkovska-Stamenova & Hoffmann, 2016a; Milkovska-Stamenova & Hoffmann, 2016b; Renzone et al., 2015). In some cases, their oxidized and phosphorylated forms were also detected. Seventeen peptides occurred in their concomitant lactosylated, hexosylated and carboxymethylated arrangement at the same lysine; 8 ones were present both as hexosylated and carboxymethylated species at the same amino acid, while only 1 exhibited both the lactosylated and carboxymethylated adducts at the same residue (Supplementary Table S2). Hundred and eight modified peptides assigned in MPIF and PM were distinctive for bovine proteins since they differed in the amino acid sequence with respect to the human homologues (Supplementary Table S3). Among the two remaining ones having sequence identity with respect to human counterparts, only one was also identified in HM (Table 1). Fig. 2 shows two exemplificative tandem mass spectra of modified bovine peptides uniquely fragmented in MPIF and

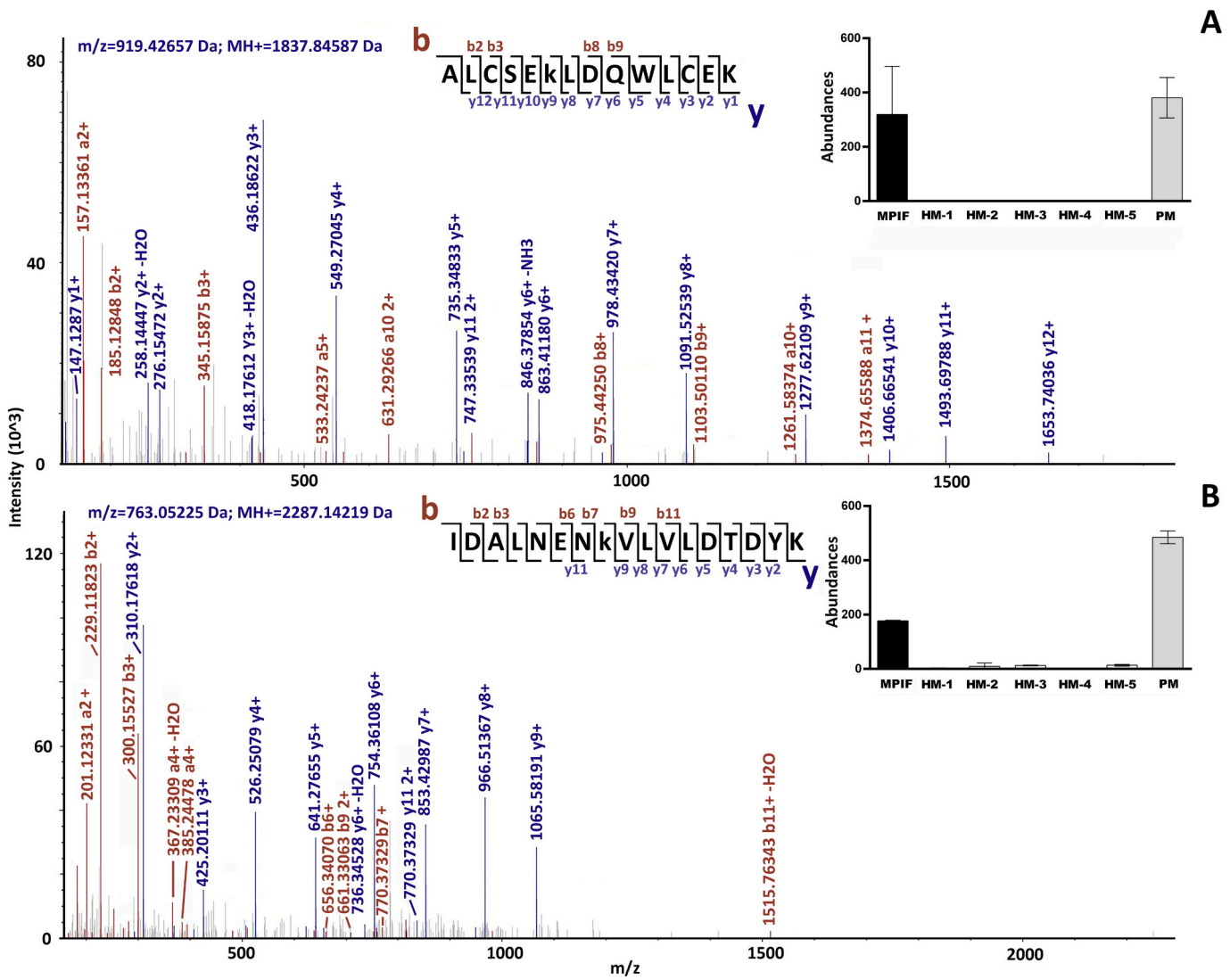

Fig. 2. High resolution tandem MS spectrum in positive ion mode and quantitative information (inset) of two modified peptides exclusively fragmented in minimally processed infant formula (MPIF) and pasteurized bovine milk (PM) and not in human milk (HM). Panel A. α-Lactalbumin peptide (128–141) bearing carboxymethylation at Lys133. Panel B. β-Lactoglobulin peptide (100–116) bearing lactosylation at Lys107. The b series is outlined in red, while the y series is labeled in blue; the lowercase letter indicates the modified amino acid. Peptide semi-quantitative information derived from the evaluation of the peptide precursor ion intensity values measured in HM, MPIF and PM samples. ANOVA test was used to assess data statistical confidence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

PM, which were practically absent in HM samples based on the measured intensity values of the corresponding parent signals. Fig. 2A shows the tandem mass spectrum and the abundance in the different milk samples of the carboxymethylated LAC peptide (128–141) bearing carboxymethylation at Lys133, while Fig. 2B reports the corresponding results for the LGB peptide (100–116) lactosylated at Lys107. These modified peptides were already identified in pasteurized and infant formula samples (Arena et al., 2010; Arena, Renzone, Novi, & Scaloni, 2011). The latter lactosylated peptide was used as a marker of heat treatment in thermally treated dairy products, such as heated milks, coffee cream and powdered milk (Arena, Salzano, Renzone, D'Ambrosio, & Scaloni, 2014; Meltretter et al., 2014).

3.2. Quantitation of protein-bound glycation and oxidation compounds in human milk, minimally processed infant formula and pasteurized bovine milk

The identification of food processing markers is the prerequisite for the collective classification of products into raw, processed, and ultra-processed goods as well as for the definition of a quantitative cause-effect relationship between diet and health (Göncüoğlu Taş, Kocadağlı, & Gökmen, 2022). Straightforward analytical procedures based on liquid chromatography high resolution tandem mass spectrometry have been efficiently used to depict the chemical nature of modified amino acids in dairy products (Akıllıoğlu & Lund, 2022; Yan, Hemmler, & Schmitt-Kopplin, 2024). Accordingly, the combination of compound fractionation based on molecular mass, enzymatic hydrolysis and targeted mass spectrometry provided the analytical paradigm to quantitatively monitor the occurrence of protein-bound and free modified arginine and lysine residues in the above-reported milk types. Exhaustive enzymatic hydrolysis of proteins avoided the formation of chemical artifacts due to hard conditions typical of acid hydrolysis and minimized the loss of pyrraline and MGH1 (Troise, 2018).

Supplementary Table S1 summarizes the key analytical performances upon method optimization; each compound was screened in targeted tandem MS mode by using a mass tolerance within ±3 ppm. The response was by far below acceptance limit, providing a mass error below 1 ppm for both protein-bound and free modified amino acids,

with the sole exception of 3-DG-H showing a mass error of 1.89 ppm. Along with mass spectrometry results, HILIC separated similar glycated compounds with highly polar moieties. Supplementary Fig. S1 shows the tandem mass spectra and the HRMS full scan peak of two glycated amino acids having the highest response: *N*ε-fructosyllysine (retention time, 9.52 min) and *N*ε-lactulosyllysine (retention time, 9.82 min). Without their optimized chromatographic separation, fragmentation of the lactose moiety in *N*ε-lactulosyllysine can lead to the overestimation of the compounds with *m/z* 309.1654 through *in source* formation of *N*ε-glycosyllysine. This drawback is illustrated in Supplementary Fig. S1A, which reports the fragmentation spectrum of *N*ε-fructosyllysine showing three consecutive losses of water (*m/z* 291.1547, 273.1444 and 255.1344), the loss of hexose (*m/z* 147.1128), and the two most intense ions corresponding to pipecolic acid and α-amino-ε-caprolactam ions at *m/z* 84.0808 and 130.0861, respectively. Supplementary Fig. S1B shows the MS/MS spectrum of *N*ε-lactulosyllysine (*m/z* 471.2183); along with the diagnostic losses of two molecules of water from a primary alcohol (*m/z* 453.2080 and 435.1972), this spectrum showed the two neutral losses indicative of lysine medication [M-hexose-hexose]$^+$ and [M-hexose]$^+$ leading to lysine (*m/z* 147.1128) and fructosyllysine (*m/z* 309.1656), respectively. The enlarged view of the latter fragmentation spectrum (Supplementary Fig. S1C) highlights a clear overlap of signals with respect to counterparts from *N*ε-fructosyllysine.

The use of HILIC was also helpful for the separation and quantification of the structural isomers *N*ε-lactoyllysine and CEL ([M + H]$^+$ at *m/z* 219.1339), having common intense fragment signals at *m/z* 84.0808 and 130.0861 related with pipecolic acid and α-amino-ε-caprolactam ions, respectively (Supplementary Table S1). Their optimized chromatographic resolution as well as the selective formation in the case of *N*ε-lactoyllysine of the characteristic ion at *m/z* 173.1285 indicative of the molecular formula $C_8H_{17}N_2O_3$ [M-e]$^+$ guaranteed the accurate quantification of both analytes (Henning & Glomb, 2016).

When HM, MPIF and PM samples were analyzed for protein-bound glycation and oxidation modifications, lysine was identified as the preferred amino acid target, in agreement with results from proteomic experiments (Figs. 3 and 4). Fig. 3 reports the concentration of lysine and lysine glycation and oxidation products in different milk types as referred to the total amount of proteins. The concentration of lysine in HM samples ranged from 24,488 to 33,245 ng per mg of protein, while the average concentration in PM and MPIF samples was 17,129 and 41,007 ng/mg of protein, respectively. In HM, the sum of the Amadori products *N*ε-lactulosyllysine and *N*ε-fructosyllysine, the intermediate glycation compounds CML and CEL, and the amide-AGEs *N*ε-formyllysine, *N*ε-lactoyllysine and pyrraline ranged from 394 to 809 ng/mg of protein. Based on these findings, the average value of total glycated and oxidized lysine ingested by infants with a HM-based diet was about 593 ng/mg of protein. When the above-reported values were compared to the amount of lysine present in HM samples, the corresponding percentage of glycated and oxidized lysine compounds ranged from 1.5 to 2.4% of non-modified lysine. By evaluating the same parameters in MPIF and PM, the average percentage of glycated and oxidized lysine derivatives in these samples was 20.0% to 19.1% of non-modified lysine, respectively. Total protein-bound modified lysine in HM accounted for 7.3% and 18.0% of the counterparts measured in MPIF and PM, respectively. Amadori compounds *N*ε-fructosyllysine and *N*ε-lactulosyllysine in HM were 1.3% and 13.5% of their glycated counterparts in MPIF, respectively.

In MPIF, *N*ε-fructosyllysine and *N*ε-lactulosyllysine accounted for 74.4% and 23.2% of total modified lysine, respectively, proving that Maillard reaction early-stage modifications provided the highest contribution (97.6%) to the overall modified lysine residues in this milk type. Assuming a ratio furosine:*N*ε-lactulosyllysine 1:5 and a ratio furosine:*N*ε-fructosyllysine 1:3, according to the model system of α-hyppuryllysine (Krause, Knoll, & Henle, 2003), our quantitative results on the above-reported Amadori compounds were lower with respect to those derived from furosine concentration values measured in bovine milk-based conventional infant formulas (Gazi et al., 2022; Yu et al., 2021), but were in line with those reported in similar dairy products having a different whey to casein ratio (Xie, van der Fels-Klerx, van Leeuwen, & Fogliano, 2023) or functionalized with encapsulated ascorbic acid (Troise, Vitiello, Tsang, & Fiore, 2016). A quantitative predominance of modified amino acids deriving from the early stages of the Maillard reaction was also verified in the other milk samples here described, although the contribution of *N*ε-lactulosyllysine to total modified lysine residues was higher than that of *N*ε-fructosyllysine, being 43% and 11% in HM, and 82% and 16% in PM, respectively.

Focusing on glycation and oxidation products related to intermediate stages of the Maillard reaction, concentration of CML and CEL in MPIF (Fig. 3) were of the same order of magnitude of those determined in similar dairy products (Akıllıoğlu, Chatterton, & Lund, 2022), but lower than those reported for conventional infant formulas (Aalaei, Sjöholm, Rayner, Teixeira, & Tareke, 2019; Hegele et al., 2008). The latter finding was here related to the technological processes associated with the preparation of the different products. CML concentration in HM samples matched the average values previously reported in human milk (Marousez et al., 2022; Sebeková et al., 2008), while results on protein-bound CEL in HM were in line with data reported by Marousez et al. (2022). Based on these results, the calculated percentage ratio between CML concentration in HM and the same parameter in MPIF accounted for a value of 26.6%. In the case of CEL concentration, this percentage ratio decreased to 15.0%.

Regarding non-canonical lysine modifications, this study introduced original information on protein-bound pyrraline, and amide-AGEs as *N*ε-lactoyllysine and *N*ε-formyllysine in HM, MPIF and PM samples. Protein-bound pyrraline was not detected in HM, while its average concentration values were 49 and 4.8 ng/mg protein in MPIF and PM, respectively. Such concentrations paralleled those already reported on other infant formulas (Lund et al., 2022). Pyrraline can be formed upon Paal-Knorr condensation between lysine and 3-deoxyglucosone, and it was identified as one of discriminating products of the Maillard reaction, being only formed in foods and not as result of biochemical processes occurring *in vivo* (Förster, Kühne, & Henle, 2005). Novel data were also obtained for *N*ε-lactoyllysine and *N*ε-formyllysine concentrations in different milk types. Based on these results, the calculated percentage ratio between the average concentration of *N*ε-formyllysine in HM samples and the same parameter in MPIF accounted for a value of 49.4%. In the case of *N*ε-lactoyllysine, this percentage ratio was ten-fold higher in HM than in MPIF. To date, *N*ε-formyllysine and *N*ε-lactoyllysine as part of amide-AGEs family have been detected in plasma; specifically, *N*ε-lactoyllysine was identified as a marker of oxidative stress in liver tissues upon enzymatic hydrolysis (Baldensperger, Jost, Zipprich, & Glomb, 2018). Overall, our data confirmed previous reports pinpointing the key role of these abundant amide-AGEs, with *N*ε-formyllysine being the most relevant compound belonging to this family (Milkovska-Stamenova & Hoffmann, 2019).

Along with lysine modifications, we also provided a snapshot of possible glycation and oxidative modifications at arginine in proteins. The average concentration of non-modified arginine in HM, MPIF and PM was 25,883, 28,363 and 17,509 ng/mg of protein, respectively (Fig. 4). Two imidazole derivatives resulting from the modification of the guanidino side chain of arginine were detected, namely 3-DG-H and MG-H1, whose levels in HM, MPIF and PM were around three orders of magnitude below their precursor. Results demonstrated that 3-DG-H and MG-H1 concentration values in HM were close to the ones reported for MPIF, and higher than those of PM. Indeed, the percentage ratio between the average concentration of MG-H1 in HM and MPIF was about 99.0% (19.8 ng/mg of protein in HM *vs* 20.0 ng/mg of protein in MPIF), while in the case of 3-DG-H it was about 27.8%.

The arginine non-enzymatic modifications have historically received less attention than lysine or N-terminal ketoamide modifications; the relevance of methylglyoxal-derived arginine modifications is lower than thermal modifications mediated by lactose and other reducing sugars

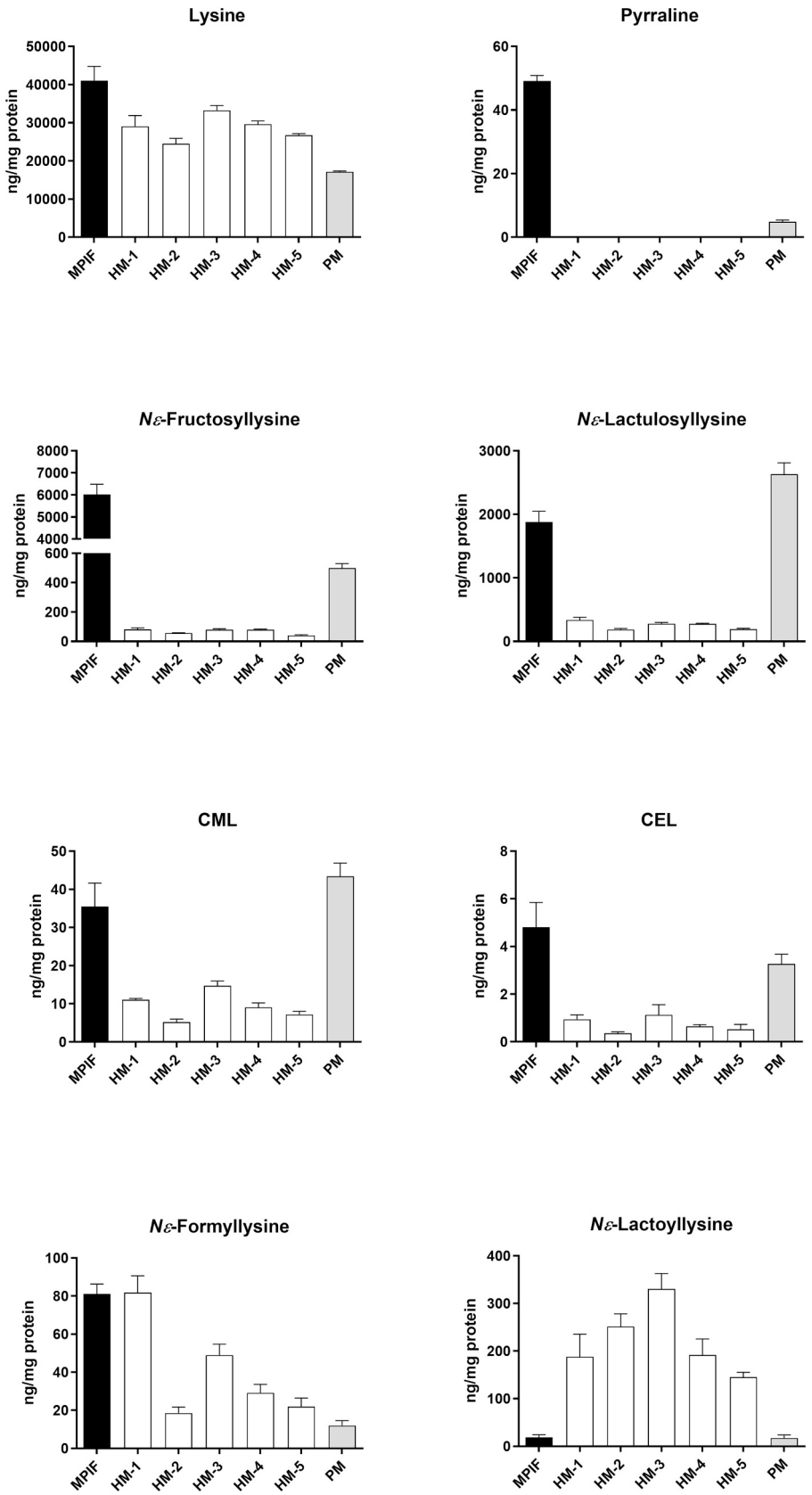

Fig. 3. Concentration of protein-bound lysine and glycated and oxidized lysine compounds measured in human milk (HM, white), minimally processed infant formula (MPIF, black) and pasteurized bovine milk (PM, grey). Results are reported as ng of compound/mg of protein ($n = 4$ for each milk sample). CML, *N*ε-carboxymethyllysine, CEL, *N*ε-carboxyethyllysine.

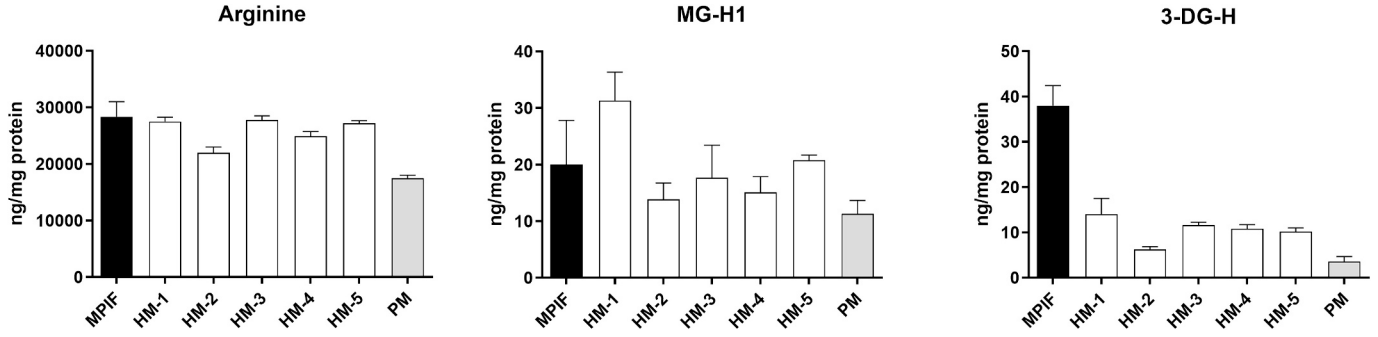

Fig. 4. Concentration of protein-bound arginine and glycated and oxidized arginine compounds measured in human milk (HM, white), minimally processed infant formula (MPIF, black) and pasteurized bovine milk (PM, grey). Results are reported as ng of compound/mg of protein (n = 4 for each milk sample). MG-H1, methylglyoxal-hydroimidazolone; 3-DG-H, 3-deoxyglucosone-hydroimidazolone.

that preferentially react with the amino group rather than the guanidino side chain (Meltretter et al., 2014). Besides Amadori compounds and amide-AGEs, we noticed that the concentration of MG-H1 in foods is often higher than that of other lysine modifications such as CML and CEL, which are in turn used as markers of thermal treatments (Scheijen et al., 2016). Our results in milk outlined the quantitative relevance of arginine modifications; both 3DG-H and MG-H1 concentration values were comparable to CML and CEL counterparts and in line with that measured in two recent surveys of milk-based products by Xie et al. (2023) and Akıllıoğlu and coworkers (Akıllıoğlu et al., 2022), which reported an average concentration of MG-H1 and glyoxal-hydroimidazolone (GH1) at the same level as CML and CEL. We here originally report the same trend also in HM, demonstrating that MG-H1 and 3-DG-H are in some cases formed at higher concentrations than in MPIF and a quantity of MG-H1 up to 38 ng/mg of protein can be part of the infants' diet independently of the kind of feeding.

3.3. Quantitation of free glycation and oxidation compounds in human milk, minimally processed infant formula and pasteurized bovine milk

Fig. 5 reports the concentration value of free lysine and its modification products. The two Amadori compounds *N*ε-lactulosyllysine and *N*ε-fructosyllysine varied among milk types, reaching the highest values in HM (333 ng/mL); *N*ε-lactulosyllysine was detectable only in two out of five HM samples (up to 44 ng/mL), while it was absent in MPIF and PM. CML was measured in all the samples, with concentration peaking in MPIF (63 ng/mL) and PM (61 ng/mL). In HM, the average CML concentration was 7 ng/mL, corresponding to about 11.6% of CML in MPIF.

When considering the advanced glycation end-products, we quantified two free amide-AGEs, *N*ε-formyllysine and *N*ε-lactoyllysine in HM, MPIF and PM. While *N*ε-lactoyllysine was only present in two out of five human milk samples up to 8 ng/mL, *N*ε-formyllysine exhibited the highest concentration among the free modified amino acids, reaching a value of 538 ng/mL. Considering all the compounds measured, total free glycated and oxidized lysine compounds observed in HM accounted for 87.0% of the counterparts measured in MPIF. Pyrraline in free form was generally detected in all milk types. However, in HM pyrraline showed an opposite trend with respect to what observed for its protein-bound form; free pyrraline accumulated up to 46 ng/mL, corresponding to an average concentration of 21 ng/mL, while in protein-bound form it was not detected. Such values accounted for 31.0% of the concentration measured in MPIF.

Fig. 6 shows the levels of free arginine and its modification products measured in HM, MPIF and PM. The average concentration of free arginine in HM was about 9,000 ng/mL, in line with MPIF, but below the concentration observed in PM. Only MG-H1 was detected in free form in HM and MPIF, and not in PM, while for 3-DG-H and G-H1 their response in all milk types was below the detection limit. Free MG-H1 concentration in HM was up to 30 ng/mL, a value close to the highest concentration reported for MPIF (31 ng/mL). Considering the average concentration in the five HM samples, we noticed that the MG-H1 concentration was 32% lower in HM than MPIF.

4. Discussion

The interconnection between diet, physiology and health makes human milk a dynamic fluid, in which protein, peptide and metabolite compositions are intensively studied because of their function as essential substrates for the healthy growth of infants (D'Alessandro, Scaloni, & Zolla, 2010; Dekker et al., 2020). Although various proteomic, peptidomic and metabolomic investigations have been accomplished on HM (Dingess, van den Toorn, Mank, Stahl, & Heck, 2019), detailed quantitative studies on protein and metabolite modifications supervised by the Maillard reaction and oxidation reactions are missing, differently from processed bovine milks (Lund & Ray, 2017).

Providing information on the concentration of protein-bound and free markers, we stress that a discrimination between protein-bound and free glycation compounds in relation to their ability to interact with AGEs receptors is mature (Zhao et al., 2019). In this view, CML is one of the key examples as, along with Amadori compounds, it is considered one of the most relevant products of the Maillard reaction in foods and *in vivo* (Tessier, Boulanger, & Howsam, 2021). Its pathophysiological implication was thoroughly investigated for both protein-bound and free forms revealing that, while free CML increased quickly in plasma after an oral intake of foods rich in this amino acid derivative, protein-bound CML remained stable and unaffected by the same diet exposure (Alamir et al., 2013). CML and CEL can be formed as result of the fragmentation of the Amadori compounds or from the reaction of lysine with 2,4-dioxo intermediates (Kasper & Schieberle, 2005). Conversely, the mechanistic role of glyoxal and methylglyoxal in CML and CEL formation in dairy products remains uncertain because of the presence of other reactive sites sensitive to α-dicarbonyls (Nguyen, Van Der Fels-Klerx, & Van Boekel, 2016). Furthermore, both intermediates CML and CEL can undergo further glycation, decarboxylation and oxidation at the alfa side, as described by Hellwig and coworkers (Hellwig, Nitschke, & Henle, 2022). Hence the role of free CML in plasma and the putative transfer through mammalian gland into human milk can be a target of future investigations. Besides CML and CEL, the concentration of free pyrraline appeared quite relevant and suggested the potential role of this compound as a biomarker of processed food intake in lactating mothers. For free compounds, such as Amadori compounds, CML, CEL and lysine amide AGEs, endogenous formation can derive from *in vivo* metabolism or from reactions at cellular level as outlined for citrullination, asymmetric dimethylation and glycation of L-arginine, hydroxylation and glycation of L-lysine part of post-translational modifications in human milk (Baskal et al., 2022). Conversely, host metabolic pathways can be excluded as a source of endogenous pyrraline and, accordingly, it can

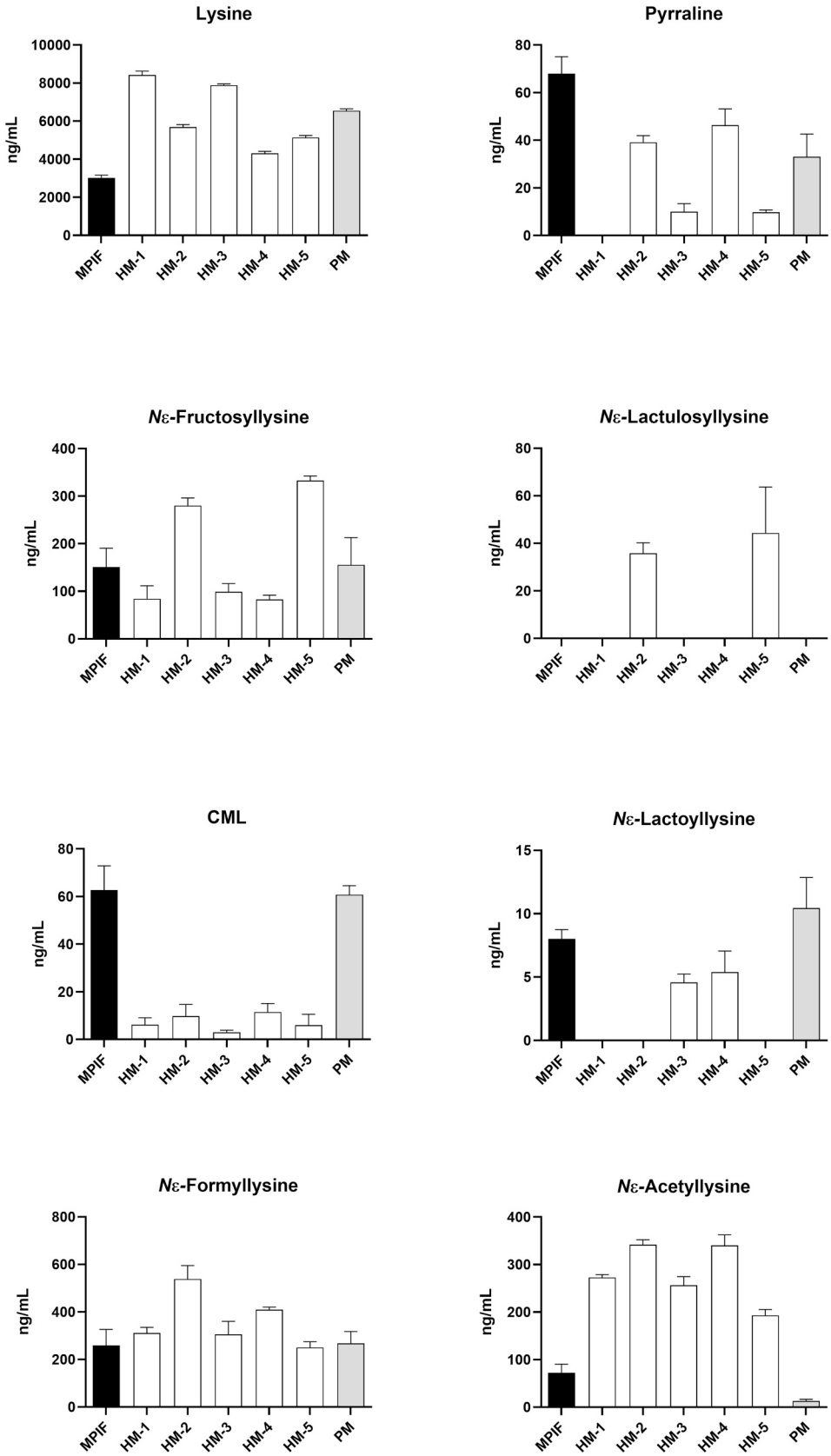

Fig. 5. Concentration of free lysine and lysine derivatives measured in human milk (HM, white), minimally processed infant formula (MPIF, black) and pasteurized bovine milk (PM, grey). Results are reported as ng of compound/ml of milk (n = 4 for each milk sample). CML, *N*ε-carboxymethyllysine.

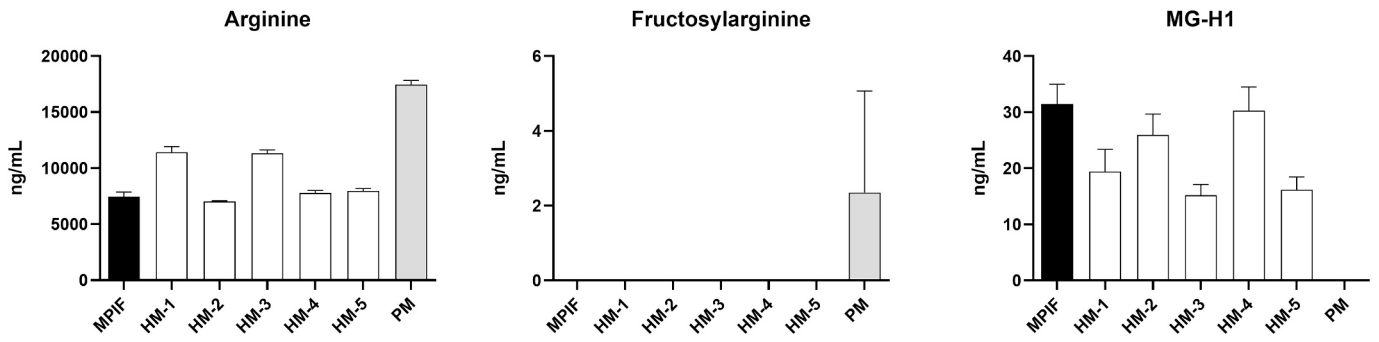

Fig. 6. Concentration of free arginine and glycated and oxidized arginine derivatives measured in human milk (HM, white), minimally processed infant formula (MPIF, black) and pasteurized bovine milk (PM, grey). Results are reported as ng of compound/ml of milk (n = 4 for each milk sample). MG-H1, methylglyoxal-hydroimidazolone.

derive only from foods present in mothers' diet. Indeed, overall differences between protein-bound and free pyrraline suggest that the lysine modifications can follow different biochemical pathways because of diet and nutritional status. Circulating free pyrraline can be found in plasma at a concentration up to 92 ng/mL (Hohmann et al., 2017), and pyrraline-containing dipeptides have been reported being transported by hPEPT1 in an electrogenic manner into intestinal cells (Geissler et al., 2010).

Arginine modifications can follow some of the pathways described for lysine modifications; we can hypothesize that plasma may funnel the accumulation of MG-H1 part of arginine modifications, in line to what is envisaged for pyrraline. The chemical nature of MG-H1 including the imidazole ring can fit with the hypothesis of passive diffusion that in any case deserves further dedicated studies for its role. Once present in plasma, MG-H1 (and in theory also pyrraline) is hypothesized to migrate into milk through the mammalian gland, in a similar fashion to other odor active molecules sharing some chemical features with this compound (Debong & Loos, 2020). Pioneering studies on vegetable products and HM outlined the infants' ability to detect diet-transmitted flavors in human milk (Spahn et al., 2019); in this context, molecular-oriented studies demonstrated that the extent of aroma transfer of a customary curry dish into milk is shaped by the interindividual variability, and it is related to the chemical nature of the odor active molecule (Debong et al., 2021). Along with aroma compounds, also non-volatile molecules, such as acrylamide, represent a valuable example of food neoformed compounds found in HM determining a potentially risk for infants' health (Mojska, Gielecińska, Winiarek, & Sawicki, 2021). Of note, other lifestyle molecules, such as medications and their metabolites, and industrial sources, such as plasticizers, cosmetics, and other personal care products, can be found in HM and in newborns' stools (Thomas et al., 2022).

The biological implications of protein-bound and free modified amino acids are connected to the kind of technological process behind infant formula production or to the mother's nutritional status. Indeed, the dichotomy between raw and ultra-processed foods toward health outcomes involves the entire food production chain, from primary production, through processing, packaging, and storage, to food delivery to the consumers (Knorr & Augustin, 2021). One of the most frequently proposed strategies to achieve an adequate consciousness for the choice of healthy and unhealthy foods is to categorize food products according to the set of processes affecting composition and structure of primary foods (Braesco et al., 2022). Indeed, the consumer approach toward HM and infant formulas involves a strict association of the latter with chemically modified, non-canonical amino acids and proteins, while HM is supposed to have the total absence of modified molecules. In the case of processed and ultra-processed foods, this approach can lead to an inaccurate categorization, when safety, composition and portion size issues are neglected (Petrus, do Amaral Sobral, Tadini, & Gonçalves, 2021), thus inappropriately determining a final negative perception by the consumers (Sánchez-Siles, Román, Fogliano, & Siegrist, 2022; Visioli et al., 2023). In this frame, we provided a general attempt to establish that food categorization necessitates a detailed molecular characterization at different levels (protein-bound *vs* free) of food ingredients, with accurate quantitation of glycation and oxidation compounds or any other bioactive molecules with potential health implications, even in human milk.

Indeed, the different chemical nature of protein-bound and free compounds can lead to diverse effects during their transit in the human body (Henning & Glomb, 2016), moving from their gastrointestinal digestion, through their intestinal absorption and *in vivo* circulation, down to renal clearance, accumulation in milk or, when unabsorbed, interaction with the microbiota (Förster et al., 2005; Hellwig, Matthes, Peto, Löbner, & Henle, 2014). This neat discrimination between protein-bound and free non-canonical amino acids becomes even more important in the case of infant nutrition; different distribution and more complex sources of non-canonical amino acids can have a direct influence on the infant gut microbiota, but an unequivocal cause-effect relationship between food glycation compounds and health effects in mothers and infants is still far to be established.

Our pilot study has some limitations related to its observational nature, the sample size and the corresponding technical power. The latter parameter may have somewhat hampered our ability to detect other modified peptides and free compounds in different milk types as well as significant observations related to the nutritional status of the mothers. In this context, large-scale prospective longitudinal multi-location studies are needed to tackle donor health status, dietary habits, environments, pollution and socio-economical differences. In HM profiles, the impact of maternal diet would influence the ratio between glycated compounds toward MPIF and PM. Finally, the milk processing and storage of MPIF would also be affected by environmental conditions and different food compositions in terms of reducing oligosaccharides or because of the addition of lipid-based ingredients. Despite all these limitations, we here have shown that non-canonical amino acids are ubiquitous even in human milk and their role can be relevant for newborn growth, regarding both the bioavailability of amino acids and peptides, both the optimal shaping of the gut microbiota. In a broader context, this molecular characterization of human milk provided relevant information on the priming role of this natural food in the newborn and the corresponding microbiome toward the development of a complete functionality for the metabolization of glycation compounds.

5. Conclusion

We introduced new bases for deciphering the effects of the Maillard reaction and potentially of other chemical modifications in breastfeeding. For the first time, we demonstrated that non-enzymatic glycation and oxidation products, part of post-translational modifications and non-canonical amino acids can be present in human milk, both in

protein-bound and in free forms. The same products can be found in infant formula and pasteurized milk of bovine origin albeit at higher amounts. In the case of Amadori compounds, there are large differences in the concentration between HM and MPIF, but solid evidence has demonstrated that microbial populations can efficiently use Amadori compounds to synthesize bioactive compounds as SCFA. For other intermediate and advanced glycation and oxidation products, such as CML, CEL and *N*ε-lactoyllysine, the concentration between minimally processed infant formula and human milk are similar. Furthermore, an overview of arginine modifications revealed that MG-H1 deserves attention independently of the kind of diet and feeding. Although one of the limitations of this study is the lack of food frequency questionnaires in mothers, we suggest here that free pyrraline can cross the mammary gland barrier and be present in human milk. Considering that pyrraline was not formed in protein-bound form, we can assume that it has an exclusive exogenous origin coming from the mothers' diet. These findings along with database-stored on pyrraline concentration in foods can create a striking connection between mothers' diet and infant intake of advanced glycation products, building a straight separation between endogenous and exogenous sources of glycation compounds.

CRediT authorship contribution statement

Sabrina De Pascale: Writing – review & editing, Validation, Methodology, Formal analysis. **Valentina Ciaravolo:** Writing – review & editing, Methodology, Formal analysis. **Mariela Mejia Monroy:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Joost W. Gouw:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Bernd Stahl:** Writing – review & editing, Supervision, Resources. **Christine Bäuerl:** Writing – review & editing, Resources, Investigation, Formal analysis, Data curation. **Maria Carmen Collado:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization. **Carlotta De Filippo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Andrea Scaloni:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition. **Antonio Dario Troise:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships, which may be considered as potential competing interests: Joost W. Gouw and Bernd Stahl reports a relationship with Danone Research & Innovation that includes: employment. 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.141265.

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