Short-term effects of dietary bovine milk on fatty acid composition of human milk: a preliminary multi-analytical study

Adele Cutignano[a], Francesco Siano[b], Raffaele Romano[c], Alessandra Aiello[c], Fabiana Pizzolongo[c], Roberto Berni Canani[d,e,f], Lorella Paparo[d,e,f], Rita Nocerino[d,e,f], Carmen Di Scala[d,e,f], Francesco Addeo[c], Gianluca Picariello[b,*]

[a] Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche (CNR), Via Campi Flegrei, 34 80078 Pozzuoli (Napoli), Italy

[b] Istituto di Scienze dell'Alimentazione, Consiglio Nazionale delle Ricerche (CNR), Via Roma 64, 83100 Avellino, Italy

[c] Dipartimento di Agraria - Università di Napoli "Federico II", Parco Gussone 80055 Portici (Napoli), Italy

[d] Dipartimento di Science Mediche Traslazionali e Laboratorio Europeo per lo Studio delle Malattie Indotte da Alimenti - Università degli Studi di Napoli Federico II, Via S. Pansini 5, 80131 Napoli, Italy

[e] ImmunoNutritionLab at CEINGE Biotechinogie Avanzate, Università degli Studi di Napoli Federico II, Via Comunale Margherita, 484-538, 80131 Napoli, Italy

[f] Task Force di Ateneo per gli Studi sul Microbioma - Università degli Studi di Napoli Federico II, Via S. Pansini 5, 80131 Napoli, Italy

*Corresponding author:

Gianluca Picariello, email: picariello@isa.cnr.it Tel. +39 0825 299521

ABSTRACT

The fatty acid (FA) composition of human milk (HM) from N = 9 Italian healthy donors following a free diet exhibited FA-dependent ranges of variability, as assessed by GC-FID. The possible short-term changes in the FA profile were monitored in the milk of lactating mothers (three) collected at five time points over a 6 h period, following an oral load (200 mL) of bovine milk. An array of techniques was exploited, including UHPLC-ESI-MS/MS of intact lipids and MALDI-TOF MS before and after chemical hydrogenation or bromination, in addition to MALDI-TOF MS analysis of FA after saponification, to monitor short-chain and odd-chain FA in HM as markers of bovine milk fat.

A single administration of bovine milk did not appreciably modify the lipid pattern, suggesting that the maternal diet could induce not detectable short-term changes on the lipid composition of HM. Diet-induced increase of butyric acid was also excluded by ^{13}C-NMR.

The functions that HM FA exert in infant physiology appear finely regulated through maternal metabolism.

Keywords: Human milk fat; fatty acids; maternal diet; butyric acid; GC-FID; UHPLC-QExactive MS/MS; MALDI-TOF mass spectrometry; ^{13}C-NMR

1. Introduction

Breast milk is the natural exogenous source of energy and key bioactive components for growth and development of neonates and infants. More than other nutrients, lipids in human milk (HM) supply energy and indispensable metabolites, such as the essential fatty acids (FA). Similar to the milk from other species, 96-98% of breast milk fat consists of a complex mixture of triacylglycerols (TAG), while the remainder comprises minor amounts of partial glycerides and free FA, as well as phospholipids, glycolipids and free or esterified cholesterol stemming from the milk fat globule membrane [1]. As a source of biosynthetic precursors and reflecting the physiological needs of the infants, HM contains a unique FA composition that is clearly distinct from the milk of ruminants and non-ruminant mammals. The fat content of mature term HM spans over a relatively large range (2.6-4.5 g/100 mL) due to a series of variability factors [2, 3]. Accordingly, FA composition undergoes inter- and intra-individual fluctuation [4].

FA of HM TAG can be derived from three sources: i) *de novo* synthesis in the mammary glands, ii) dietary lipids, and iii) mobilization of endogenous lipids stored in the adipose or hepatic tissues, which are correlated to dietary habits and are featured by a slow turnover [5, 6].

Medium-chain FA (C10-C14) arise almost exclusively from *de novo* synthesis, and they are incorporated directly into TAG without any further modification [7-9]. Long-chain FA (LCFA, > 16 carbons) are taken up from circulating lipids, while C16 FA are derived from both *de novo* synthesis and blood lipids. Overall, according to many lines of evidence, diet influences the FA composition of HM more than genetic factors [1, 10].

Diet-induced fluctuations of mono- (MUFA) and polyunsaturated fatty acids (PUFA), including oleic (C18:1*n-9*), essential linoleic (C18:2*n-6*), and α-linolenic (C18:3*n-3*) acids, have been established in detail [11]. Nearly 30% of the HM linoleic acid appears to derive directly from the maternal diet [12]. A specific increase in long chain PUFA arachidonic acid (AA, 20:4*n-6*), docosahexaenoic acid (DHA, 22:6*n-3*) and eicosapentaenoic acid (EPA, 20:5*n-3*) levels was observed in the milk of women who had regular access to seafood [3]. However, independent targeted studies carried out with stable labelled isotopes (i.e. ^{13}C-labelled DHA) gave contrasting results [13, 14], highlighting that the impact of diet on the LCFA profile of HM is still a matter of debate.

A few studies have considered the possible enrichment of dietary short-chain fatty acids (SCFA) in human milk TAG. SCFA are increasingly recognized as key physiological signalling metabolites, biosynthetic precursors or epigenetic modulators [15, 16, 17]. Unlike long-chain FA, dietary SCFA as well as MCFA directly enter blood vessels and then the portal circulation, after which they are rapidly oxidized in the liver and utilized for energy production or for metabolic processes.

Most of the butyrate and SCFA (e.g. acetate or propionate) produced by microflora in the colon is consumed at the local level, as it represents the main metabolic fuel for the colonic epithelial cells, while only a portion enters several metabolic routes [18]. The role of SCFA in the synthesis of endogenous lipids and their possible incorporation in breast milk TAG remains poorly investigated [19]. However, immediately after birth, before the development of a mature microbiota, endogenous production of SCFA in the infant gut is very low [20]. Considering its discrete concentration (up to 3.5 mg/100 mL), HM may represent the main source of butyrate and other SCFA during the first stages of life [17, 21]. Despite its physiological importance, the origin (endogenous or exogenous), form (free or esterified) and fluctuations of butyrate in HM still await precise assessment. In a very recent study,

SCFA and MCFA have been quantified in HM samples, after comparing several methods of FA derivatization and analysis [22]. Another study has demonstrated that the regular maternal intake of dairy products and beef can affect the level of HM branched-chain fatty acids (BCFA), which may also include OCFA [23].

However, direct excretion of free butyrate in HM or incorporation in TAG, following an oral administration of butyrate-containing food (*e.g.* bovine milk) has not been monitored to date.

Ruminants' milk fat contains 2-5% (w/w) of butyrate, which constitutes nearly one-third of the palmitate on a molar basis [24], while OCFA account for 1.5-2-5% (w/w) overall [25]. Thus, the intake of one cup of bovine milk (200 mL) supplying 0.25-0.35 g and 0.1-0.2 g of butyrate and OCFA, respectively, exposes several human tissues to millimolar levels of these FA [26], which might be of physiological relevance especially for infants.

In the present work, we analysed mature breast milk from N = 9 Italian healthy donors following a Mediterranean diet including also milk and dairy products, to assess the possible fluctuation of SCFA or OCFA. Furthermore, we profiled the milk of lactating mothers (N=3), collected at five time points over a 6 h period, after drinking a cup of bovine milk to monitor the possible short-term changes in the FA patterns. A variety of chromatographic and mass spectrometry-based analytical methods were exploited to detect free and esterified butyric acid along with other SCFA and OCFA, as markers of bovine milk fat in HM.

2. Experimental

2.1. Sampling

Mature HM samples (~10 mL) were obtained from lactating healthy donors (N=9) through a breast pump equipped with plastic disposable pump sets. HM samples were obtained in the 2-3 h interval after the mothers had fed their babies. Mothers were between two weeks and three months of lactation after term delivery. Donors had no dietary restrictions and they were asked to have a varied omnivore diet of around 1800-2000 kcal/day, which also included bovine milk and dairy products. Mothers ate cereals, legumes, meat, eggs, fish and fruit according to a "Mediterranean diet" and they were not exposed to any other dietary intervention. The breast milk of three different mothers (approximately 5 mL) was sampled over a 6 h period at time intervals (1, 2, 3, 4, and 6 h) following a 200 mL oral load of pasteurized bovine milk, obtained from the local market. In this case, the lactating mothers complied with an isocaloric varied diet as above that was completely free of cow's milk and dairy for at least 1 week. Control milk samples (baselines) were collected before the mothers were given cow's milk. The dose of 200 mL was chosen because it corresponds to an average amount of cow's milk eaten in the context of a realistic diet. The fat content of cow's milk was 3.6 g/100 mL according to the label. The GC-FID profile of bovine milk FA as methyl ester derivatives and the MALDI-TOF MS profile of TAG (see below for abbreviations and methods) of cow's milk is displayed in **Supplementary material, Fig. S1**. The relative FA amount is reported in **Supplementary material, Table S1**.

Milk was expressed into plastic sterile tubes, mixed with a serine-protease inhibitor (Pefabloc®, Sigma, St. Louis, MI, USA, 1 mM final concentration), and immediately frozen at -20 °C to prevent undesired hydrolysis.

A skilled operator supervised the administration of the bovine milk, the sample collection, and the code labelling. Prior to collecting the milk samples, mothers provided informed consent.

The milk was thawed in an ice bath, and 5 mL aliquots were centrifuged (3,000 x *g*, 15 min, 4 °C). The floating fat layer was hardened at -20 °C for 5 min, withdrawn with a spatula and collected in clean glass vials. This procedure was repeated twice and fat layers were pooled. Lipids from an aliquot of bovine milk were fractionated by centrifugation with the same procedure above to assess the FA composition.

2.2. GC-FID analysis

The base-catalyzed *trans*-esterification guarantees the most effective conversion of both free and TAG-esterified FA, especially of SCFA and MCFA [22]. Thus, FA composition of HM was obtained by gas chromatography (GC) after derivatization to fatty acid methyl esters (FAME) using 2N potassium hydroxide in methanol [27]. The gas chromatograph was an Agilent Technologies 6850 Series II instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with a programmed temperature vaporizer (PTV), a flame ionization detector (FID), and a fused silica capillary column, 100 m, 0.25 mm i.d.; 0.20 mm film thickness (Supelco Bellefonte, PA). The oven was set at an initial temperature of 100 °C for 5 min. The temperature was increased at a rate of 3 °C/min to 165 °C, held for 10 min, then increased to 260 °C with a second 3 °C/min ramp, and finally held for 28 min. The PTV was operated at 50 °C for 0.1 min, increased at 400 °C/min to 260 °C and held constant at 260 °C for 5 min. The split ratio selected was 1:30. Helium was used as a carrier gas with a linear velocity of 20 cm/s. The FID was operated with 10:1 ratio of air:hydrogen at 260 °C. Fatty acid methyl esters (FAME) were assigned and quantified using the Supelco 37 Component FAME MIX (Supelco Bellefonte, PA) as external standard. FA concentration was calculated through response factors to convert peak areas into weight percentages. To monitor possible free FA in HM , a 100 µL aliquot of two unfractionated milk samples

was subjected to direct trans-methylation and analysed under the same GC conditions, except that the split ratio was 1:5. Samples were analysed in triplicate, and the relative FA abundances were expressed as the mean + standard deviation.

To determine the FA composition of bovine milk, a 10 mg aliquot of the lipid fraction was *trans*-methylated and analysed by GC-FID of FAME using the same workflow used for HM.

2.3. TAG hydrogenation and bromination

Unfractionated HM lipids (10 mg) were hydrogenated in 1 mL absolute $CHCl_3$ in the presence of a catalytic amount of platinum under 1 bar H_2 pressure (2 h at room temperature). The resulting solution was centrifuged (4500 x *g,* 15 min, 4 °C), and the supernatant was used for subsequent analysis.

Bromination was carried out by dropwise addition of a solution of bromine in $CHCl_3$ to fat (10 mg) dissolved in $CHCl_3$ (1 mL) until the solution turned reddish. After discoloration by shacking with 1 mL of 10% aqueous sodium thiosulfate, which indicated the reduction of the excess bromine, the aqueous layer was discarded and the organic layer was used for subsequent analysis.

2.4. Saponification of HM lipids

For mass spectrometry (MS) analysis of FA, the fat samples were saponified according to the procedure of Hlongwane et al. [28] with minor modifications. Briefly, 50 mg of fat was suspended in 10 mL of NaOH in methanol (6%, w/v) and refluxed for 30 min under continuous stirring. Then, the mixture was transferred into a clean tube containing ~2 g of crushed ice. The resulting gelatinous soap was vacuum-filtered, air dried and stored at -20 °C until use.

2.5. Matrix assisted laser desorption/ionization - time of flight (MALDI-TOF MS) mass spectrometry (MS)

The HM fat was melted at 37 °C in a N_2 atmosphere for 5 min. Prior to MALDI-TOF MS analysis, 5 µL of a 1 mg/mL $CHCl_3/CH_3OH$ (2:1, v/v) solution of the total lipid extract was diluted in 1 mL of $CHCl_3$ and vigorously vortexed with 1 mL of 0.5 M aqueous sodium acetate in order to promote the ionization of TAG exclusively as Na^+ adducts and to minimize fragmentation by depleting traces of acidic residues [29]. After separation of the biphasic system, the organic layer was used for the analysis. The same procedure was used for analysing hydrogenated and brominated TAG as well as bovine milk fat.

MALDI-TOF mass spectra were acquired on a Voyager-DE PRO (PerSeptive Biosystems, Framingham, MA, USA), as previously described. Analyses were performed in the reflector positive ion mode using 10 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 50% aqueous acetonitrile (v/v) containing 5 mM sodium acetate or meso-tetrakis(pentafluorophenyl)porphyrin (F20TPP, Sigma Aldrich), 10 mg/mL in $CHCl_3$, as the matrix for TAG or FA, respectively. The spectra were acquired in the *m/z* ranges of 400-1500 or 100-450 for the analysis of TAG or FA, respectively. At least 400 laser shots were averaged for each spectrum, acquired in several random points of the sample wells. External mass calibration was performed with separate acquisitions of standard TAG or FA (obtained from Sigma-Aldrich) approximately each six samples analyzed. Analyses were carried out in triplicate to verify repeatability. The mass spectra were elaborated and compared using the Data Explorer 4.0 software (PerSeptive Biosystems).

2.6. UHPLC-QExactive MS/MS analysis

Chromatographic separation of HM lipid extracts was achieved on an Infinity 1290 UHPLC System (Agilent Technologies, Santa Clara, CA, USA), equipped with a Kinetex Biphenyl 2.6 µm, 150 x 2.1 mm column, (Phenomenex, Castel Maggiore, Bologna, Italy) at 28 °C according to Cutignano et al. [30]. Briefly, the elution programme consisted of a gradient of A: water and B: MeOH, ranging from 40% B to 80% B in 2 min, then to 100% B in 13 min, holding at 100% B for 7 min. The flow rate was 0.3 mL/min.

HM fat from 1 mL aliquots of the samples collected at different time intervals (before, 2 and 4 h after bovine milk assumption) was dissolved in $CHCl_3/CH_3OH$ (2:1) and vortexed twice with 1 mL deionized water. The organic layer was dried under a N_2 stream and re-suspended in 1 mL methanol:isopropanol (9:1) for LC-MS processing. The injection volume was 10 µL, and the autosampler was maintained at 10 °C.

LC-MS/MS analyses were carried out on a QExactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with a HESI source. Source parameters were as follows: spray voltage positive polarity 3.2 kV, negative polarity 3.0 kV, capillary temperature 320 °C, S-lens RF level 55, auxiliary gas temperature 350 °C, sheath gas and auxiliary gas flow rates 60 and 35 arbitrary units, respectively.

Full MS scans were acquired over the range of 200-1800 with a mass resolution of 70000. The target value (AGC) was 1e6, and the maximum allowed accumulation time (IT) was 100 ms. For the ddMS2 analyses, a Top10 method was used. The ten most intense peaks were selected for fragmentation with a stepped normalized energy of 25-28-35 and 20-40% in positive and negative ionization mode, respectively. AGC was 2e5 with IT 75 ms and 17500 mass resolution. Calibration and performance check tests in both positive and negative ion modes were routinely carried out using commercial

calibration mix standards (Thermo Scientific). For lipid identification, LC-MS/MS data were processed by Xcalibur 3.0.63 and LipidSearch 4.1.16 packages (Thermo Scientific).

All results are average values of triplicate measurements.

2.7. ^{13}C NMR

Monodimensional ^{13}C nuclear magnetic resonance (NMR) spectra of HM fat collected before and after administration of bovine milk were acquired in $CDCl_3$ on an AVANCE-III HD-400 spectrometer (Bruker, Hamburg, Germany) equipped with a CryoProbe™ Prodigy fitted with a gradient along the Z-axis, at a probe temperature of 27 °C (300 K). The chemical shifts were expressed in parts per million (ppm) and referenced to the solvent signal at 77.0 ppm. Each sample (approximately 10 mg) was acquired with number of scans NS=15000 with a delay D1=4 sec. Bruker TopSpin software was used for data processing. All spectra were subjected to exponential phase multiplication before Fourier transformation. Manual phase and baseline corrections were applied.

2.8. Statistical analysis

All analyses were performed in triplicate and the measured values were averaged. The inter-individual variability of FA (N=9 HM samples) was assessed by determining a % relative standard deviation (%RSD = (SD / $\bar{x}$)*100, where SD is the standard deviation and $\bar{x}$ the mean of FA abundance), as an index of data dispersion. Because of the low number of samples, the statistical significance of the variability of FA levels in HM sampled at varying time intervals compared to the control (before administration of bovine milk) was assessed using the nonparametric Kruskal-Wallis and Wilcoxon paired-samples tests. Significance levels were established at $p \leq 0.05$. The analyses were performed

using the XLSTAT software suite (Addinsoft Inc. New York, USA). MALDI-TOF MS spectra were baseline corrected, and the datasets were exported in Microsoft Excel vers. 2013; the signal intensities were averaged among three technical replicates, and the mass tolerance for signal assignment was ± 0.1 Da. Signals from different samples were compared in terms of both signal intensity and peak area. LC-MS spectra acquired with the QExactive platform were processed by LipidSearch software, and the response signals (peak areas) were averaged over three technical replicates; the mass tolerance for precursor ion and product ion identification was set at ± 10 ppm.

3. Results and discussion

3.1. GC-FID analysis of HM FA

In a meta-analysis, German & Dillard [1] observed that the FA of HM span over wide ranges of composition in response to long-term maternal dietary habits.

In our analysis, the levels of FA determined by GC-FID in the milk of a geographically homogeneous set of N=9 donors following independent diets were affected by a FA-dependent degree of fluctuation (**Table 1**). The variability of individual FA over the samples was estimated through the %RDS (also known as coefficient of variation), which is ordinarily used as an index of the data dispersion.

The most abundant FA, i.e. oleic acid (C18:1*n-9*c), ranged between 27.17 and 35.58%, %RSD = 8.44, in line with many values published in the literature. Such figures of variability are compatible with the 30% diet-induced relative variation reported for C18 FA [12]. Essential C18:2*n-6* (linoleic acid) varied within comparable percentages (%RSD=10.10), although its levels in absolute terms fell within a narrower range (7.83-10.63%). In all the samples, butyrate was detected in trace amounts, just above the limit of detection (LOD=0.01%). Therefore, although %RSD appeared relatively high (43.96%), in

absolute terms the inter-individual variability of butyrate implies slight deviations from the mean value (0.02%). Odd chain C15:0 and C17:0 saturated FA occurred in detectable amounts, varying in the 0.26-0.49% and 0.25-0.56% ranges, respectively, in agreement with recent data [31]. Interestingly, long-chain PUFA, namely AA, DHA and EPA, exhibited rather constant levels affected by low inter-individual variability within the sample set analysed in this study. For instance, %RDS of DHA was 5.93%, with a mean abundance of 0.33%, which is in line with the indicative abundance described by other authors [13]. However, the margins of long chain PUFA fluctuation in HM are still undefined [12].

To monitor possible short-term food-induced changes of the FA profiles, we assessed the FA in breast milk samples collected from three independent donors at several time points after an oral load of bovine milk. GC-FID-based determinations of FA in the time range of 0-6 h are reported for the three samples in **Table 2**. In this case, intra-individual variability of main FA was non-significant ($p>0.05$), indicating that they did not appreciably vary compared to the baseline control (milk collected before drinking bovine milk) in response to the administration of bovine milk within the 6h of monitoring. In particular, the most abundant MCFA and LCFA of bovine milk (C14-C18) were not transferred into breast milk at appreciable amount. The $p<0.05$ occasionally recorded for the very low-abundance FA has to be considered a consequence of their low fluctuations in absolute terms, therefore may not reflect a real bovine milk-induced variation trend.

Furthermore, the levels of butyrate and OCFA (i.e. C15:0 and C17:0) did not change upon administration of bovine milk. Slight fluctuations of the amount of C15 and C17 FA observed among lactating women of different geographical origin have been attributed to the habitual consumption of ruminants' milk and meat or dairy [31]. A very slight increase was observed only in one case for C10

FA after administration of bovine milk (donor 3, Table 2). Remarkably, the average amount of butyrate determined for the three lactating donors was comparable with those of the nine donors having a free diet, regardless of the avoidance or assumption of bovine milk.

In agreement with very recent determinations [32], the occurrence of significant levels of non-esterified butyric acid in HM after skimming was excluded by direct *trans*-methylation of unfractionated aliquots of HM and GC-FID analysis, since the FA compositions determined with or without previous separation of fat from HM samples did not differ from each other (not shown). The volatile nature should not be considered a main confounding factor for the determination of butyric acid because the pKa is 4.82, thereby indicating that it is almost completely dissociated and dissolved in milk (pH 6.8-7.4). Alternatively, small amounts of free butyric acid may remain associated with the lipid fraction and hence, undergo *trans*-esterification before GC analysis [17, 22].

The low number of lactating mothers enrolled is a weakness of this study, which does not allow to infer statistically robust conclusions. On the other hand, these findings appear in line with a bulk of literature data, since the amounts of butyrate and SCFA reported so far are not different than those here reported, regardless of the mothers' diet. In a recent meta-analysis that included 55 studies carried out worldwide on 4374 term and 1017 preterm HM samples it has been emphasized that butyrate has almost never been computed due to its very low amount [33].

3.2. MALDI-TOF MS analysis of HM fat

The HM fat samples were analysed by MALDI-TOF MS without any previous fractionation step. We were not able to find any previous attempt of MALDI MS-based characterization of human milk fat in the literature. The use of MALDI MS for the analysis of complex lipid mixtures is still largely

underestimated, despite its relative simplicity and elevated informative level [34]. MALDI MS enables access to information that cannot be obtained with other techniques and boasts a series of analytical advantages for the characterization of a lipidome, including minimal sample handling, no required derivatization, speed of analysis, specificity, sensitivity and large dynamic range, and relative ease in the assignment of individual components. In the classical GC analysis of FA, the information about the native TAG is lost. Similar to the shotgun lipidomic strategies, MALDI-MS analysis enables lipids to be analysed while preserving their intact molecular structures.

Typical spectra of HM fat samples obtained from a single donor before (**Fig. 1A**) and 2 h after (**Fig. 1B**) an oral load of bovine milk are compared in **Fig. 1**. Although MALDI MS suffers from a certain intrinsic signal variability, the spectra were practically overlapping, with coefficients of variation for the relative intensity of TAG signals, calculated as the %RSD for five replicates, lower than 10% in all cases. This finding indicated the practical equivalence between the two lipid samples.

Compared to typical ruminant milk fat (Figure S1B)[35], HM fat exhibits a lower molecular complexity and a narrower TAG distribution due to the substantial lack of SCFA and low levels of MCFA, which in turn determine the lack (or very low abundance) of TAG with carbon number < 36. As expected, the MALDI spectra of TAG from HM fat are dominated by the C52 cluster [36], reflecting the relatively high contribution of palmitic acid (C16:0), which is known to region-specifically occupy the *sn*-2 position of HM TAG [10, 37]. The intensity of C48-C36 TAG clusters progressively decreased with decreasing carbon number. C54 TAG also occurred at significantly high intensity, especially as a consequence of the relative abundance of oleic and linoleic acids. The molecular multiplicity within each TAG cluster is the result of the presence of FA with varying degrees of unsaturation. It must be emphasized that isobaric TAG and FA compositions of single TAG species cannot be assigned with a

simple MALDI MS analysis. However, this task has been accomplished through an LC-MS/MS approach on HM fat samples, as described below.

The analysis of intact acylglycerols also allows detecting possible diacylglycerols (DAG), whose presence cannot be assessed using the classical GC analysis of *trans*-esterified FA. In fact, the spectra in **Fig. 1** show the presence of minor signals of DAG, which can result from a partial endogenous lipolysis or, alternatively, from incomplete biosynthesis. The intensity of DAG signals did not change in the monitored time interval after the bovine milk oral load compared to the baseline control.

3.3. MALDI-TOF MS analysis of hydrogenated and brominated HM TAG

The signal dispersion due to variable degrees of unsaturation of FA might render the lowest-abundance TAG clusters minimally detectable in a complex lipid mixture. To enhance the detection of very low/very high carbon numbered as well as odd numbered TAG, the samples of HM fat were analysed by MALDI-TOF MS after catalytic hydrogenation. In this way, exclusively saturated TAG species were detected, and the assignment of the cluster was simplified. The exemplary spectrum of **Fig. 2A** shows the clear presence of even- and odd-carbon number saturated TAG in the ranges of C36-58 and C43-C53, respectively. A part of the natural fluctuations of the C56-58 and odd-carbon numbered TAG signals detected by MALDI, which are hardly visualized by other techniques, were detected in all of the samples analysed, and they did not exhibit significant inter- or intra-individual variability, also indicating that they constitutively occur in HM fat.

We were interested in distinguishing possible completely saturated TAG in HM, which normally escape detection because they occur at relatively low abundance and fall in the isotopic cluster of preceding monounsaturated counterparts. For this purpose, an aliquot of TAG was brominated by the

addition of bromine to double bonds. This reaction is very fast, does not require particular skills and can be easily controlled until completeness, as the early excess of bromine is visually detected by the reddish colour of molecular bromine [35]. The unsaturated components shifted by 160 units/double bond, while saturated species were not affected by bromination.

A representative MALDI-TOF MS spectrum of brominated HM TAG is shown in **Fig. 2B**. Saturated TAG practically fitted a Gaussian distribution centred on C44, which dominated the spectrum. Except for slight fluctuations of signal intensities, the pattern was common to all of the analysed samples, suggesting that it could be a general trend of HM. In particular, no significant modifications could be appreciated in the HM samples collected before and after the oral load of bovine milk.

The spectral quality of brominated TAG was lower in terms of the signal to noise ratio when compared to the hydrogenated species due to the relatively low abundance of saturated TAG in the native fat.

In the high molecular weight region of the spectrum, the signals of brominated monounsaturated TAG appeared. In general, the latter produce low intensity signals due to extensive fragmentation of the brominated TAG, as demonstrated by metastable ions (**Fig. 2B**), and due to signal dispersion resulting from the natural occurrence of two stable Br isotopes with almost-equal distribution, namely, ^{79}Br (51%) and ^{81}Br (49%). On the other hand, the brominated species can be easily distinguished because of the characteristic isotopic pattern (magnified view in **Fig. 2A**). Signals of brominated TAG arising from PUFA were not detected at all in the higher molecular weight spectral range (not shown).

3.4. MALDI-TOF MS analysis of HM FA

The use of MALDI MS for the analysis of small molecules is infrequent due to matrix interference, which crowds the low-mass range with very intense signals that suppress those of the analytes occurring at lower abundance. Ayorinde et al. [38] developed a MALDI MS-based method to determine the FA compositions of oils and fats using F20TPP (molecular weight 974) as the matrix. F20TPP produces practically no ions < 500 Da, thus preventing matrix interference in the molecular weight range of FA. These researchers also demonstrated that the MALDI-TOF MS analysis provides reliable quantitative results under controlled conditions, consistent with the GC-based determination, but with the additional advantage of superior sensitivity in selected cases, allowing FA detection that is not observed with GC [28]. In spite of its undisputed advantages, this method has been applied only in a handful of research papers over almost 20 years. According to these authors, we used F20TPP as the matrix to analyse saponified HM fat by MALDI-TOF MS.

A typical spectrum of HM FA is shown in **Supplementary Material, Fig. S2**. In the MALDI TOF MS positive ion mode and in the presence of Na^+ as a dopant, FA are detected as sodiated sodium carboxylates $[RCOONa + Na]^+$ and can be identified by matching with their expected molecular weights. While the most abundant FA were clearly detected (**Fig. S2**), low-intensity signals were detected for myristic and lauric acids (*m/z* 245.2 and 217.2, respectively), in line with their relative abundances.

No signal for FA shorter than C10:0 was detected (considering a signal to noise ratio of 3:1). Thus, C4-C8 FA were not detected in any of the samples analysed. Among the OCFA, C15:0 (*m/z* 287.2) and C17:0 (*m/z* 315.2) were clearly detected. The very slight inter-sample fluctuations of signal intensity recorded for both most and least abundant signals appeared to result from technique-dependent stochastic variability, rather than as the consequences of actual diet-induced modifications, as

confirmed by the analysis of replicate samples. Especially in the case of HM samples collected at time intervals, variability was very low and no statistically significant modification could be assessed by MALDI MS.

3.5. UHPLC-QExactive MS/MS analysis of HM TAG

Liquid chromatography joined with MS analysis offers the advantage of resolving and spreading the totality of lipids included in a complex extract over the chromatographic run time. In particular, in the UHPLC-MS method here adopted [30], TAG were detected as sodium adducts and were identified by their high-resolution masses. FA composition of each TAG species was assessed by MS/MS data based on diglyceride product ions and FA neutral losses obtained in Data Dependent Scan Analysis. The interpretive analysis was supported by LipidSearch software, which allowed the identification of 60 different TAG species in the C34-C60 range (**Fig. 3**). Assignment of individual TAG, including the TAG 12:0/12:0/12:0 is substantially in agreement with recent MS-based characterizations [39]. A unique species containing C8 was detected, i.e. TAG 16:0/8:0/18:1 (C42:1, M-Na^+ = *m/z* 743.6144), which was also observed by MALDI-TOF MS (Fig. 1); butyrate containing TAG, which could occur at trace abundance levels as recently demonstrated with supercritical CO_2 LC-MS [40], were not detected in any of the samples. Except for the TAG56:4 group (TAG 18:0/18:1/20:3 and 18:1/18:1/20:2), no isobaric species were recorded. On average, TAG52 and TAG54 were the richest groups, dominated by C16:0/C18:1 FA. TAG-containing PUFA, such as ARA, EPA or DHA, were also clearly detected, mainly in TAG56-60 homologous series. However, no significant variation was observed in the FA composition of individual MCFA- and LCFA-containing TAG before and after bovine milk consumption.

3.6. ^{13}C NMR of HM lipids

NMR spectroscopy has been widely used in metabolomics of milk from different species, including HM. Most frequently, ^{1}H-NMR analysis of HM has been carried out to measure low-molecular weight polar compounds, such as sugars, amino acids and nucleotides, with the aim of linking the metabolite profile to nutritional and quality aspects [41].

Very recently, Prentice et al. [17] related HM butyrate, determined by ^{1}H-NMR, to energy metabolism and anthropometric parameters in breastfed newborns. Slight differences in butyrate – which occurs at trace levels – have been found among HM samples from mothers of different geographic origin by ^{1}H NMR-based metabolomic analysis [42].

With specific concern for milk fat, high-resolution ^{13}C NMR is a powerful method to identify the different classes of glycerol lipids and to determine the positional distribution of saturated and unsaturated FA based on chemical shift differences of glycerol-backbone and carbonyl signals [43]. To the best of our knowledge, ^{13}C NMR has not been applied to specifically investigate HM fat, except in a recent comparison between HM and infant formulas [44].

A typical ^{13}C NMR spectrum of HM recorded in $CDCl_3$ is shown in **Fig. 4**. The two insets display enlargements of the carbonyl and glycerol-backbone regions. Carbon signals belonging to differently substituted glycerol carbons allow distinguishing 1-MAG (monoacylglycerols), 2-MAG, 1,2-DAG (diacylglycerols), 1,3-DAG and TAG species in HM. In fact, the two prominent signals at 62.10 and 68.87 ppm correspond to *sn*-1/3 and *sn*-2 glycerol carbons of TAG, respectively. Both 1,2- and 1,3-DAG were detectable. 1,3-DAG was characterized by two glycerol signals at 68.41 ppm (C-2) and 65.04 ppm (C-1/3). On the other side, 1,2-DAG showed three distinct signals for carbons at the *sn*-1 (62.00 ppm), *sn*-2 (72.11 ppm) and *sn*-3 (61.56 ppm) positions. Minor MAGs were also detected, and the corresponding carbons were annotated by comparison with data from the literature [45, 46]. Signals

observed in the carbonyl region confirmed the presence of all of the different classes of glycerol-lipids indicated above. Furthermore, slight differences in chemical shifts of the *sn*-1,3 (173.30/173.26 ppm) and *sn*-2 carbonyl signals (172.89/172.84 ppm) can be measured and correspond to saturated (SFA)/unsaturated (UFA) FA, respectively, occurring at these regioisomeric positions. The diagnostic carbonyl resonances of butyric acids esterified at either the *sn*-1,3 (173.13 ppm) or *sn*-2 positions (172.65) of glycerol [47] were absent in HM samples either before (**Fig. 5A**) or 2 h (**Fig. 5B**) and 4 h after (**Fig. 5C**) intake of bovine milk, as confirmed by comparison with the ^{13}C NMR spectrum of tributyrin standard (**Fig. 5D**).

Trace levels of butyric acid that have been detected in HM by other studies and observed by GC herein escaped detection by ^{13}C NMR, most likely due to suppression effects and to the limited dynamic range of the technique.

4. Conclusions

In agreement with previous determinations performed on samples from geographically homogeneous donors, HM LCFA were affected by higher inter-individual variability compared to MCFA and SCFA [10, 42]. A single administration of bovine milk, did not induce striking short-term fluctuations of the FA acid profile of HM. Minor fluctuations of low abundant FA might be induced by medium- or long-term dietary changes [13, 31], although evidence of direct correlations between diet and FA composition of HM is still scarce [48].

Because of the essentially endogenous origin, MCFA and SCFA do not significantly vary in HM. Butyrate that occurs at very low amounts in HM is not an exception. The butyrate levels determined in the HM samples analysed in this study fall in the 0-3.5 mg/100 mL range recently defined by GC-MS

[17]. In addition to the intrinsic individual production, slightly different levels of butyrate and SCFA could be the result of varying metabolism of oligosaccharides by human microbiome. However, it is difficult to establish clear correlations between such small fluctuations and genetic or environmental factors, microbiome contribution or the influence of extraction and analytical methods.

The findings of this study suggest that the consumption of bovine milk does not appreciably increase the levels of SCFA and other FA in HM while bovine milk-specific OCFA are not incorporated in HM TAG at all.

This evidence emphasizes that the influence of diet on HM composition is limited to specific FA components. The maternal metabolism appears to finely regulate the balance of FA in HM, thus supporting the concept that HM lipids are physiologically relevant for infants, as their role extends well beyond the mere nutritional function. Nevertheless, the outcomes of this study are preliminary and should be confirmed by enrolling a higher number of lactating donors and extending HM monitoring to longer time. The dietary and technological practical interest (e.g. development of infant formulas or food supplements containing precursors of SCFA) promises further investigation.

Compliance with Ethical Standards

Conflicts of interest

The authors have declared no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors. Prior to collecting milk samples, mothers were asked to sign written informed consent.

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Legends to Figures

Fig. 1 MALDI-TOF analysis of HM fat samples obtained from a single donor before (**A**) and 2 h after (**B**) an oral load of bovine milk. Mass values are indicated in panel A, while TAG clusters are assigned in the panel B.

Fig. 2 MALDI-TOF MS analysis of hydrogenated (A) and brominated (B) human milk fat. The analysis of hydrogenated samples allowed a straightforward assignment of the TAG families, also including those with low/high or odd carbon numbers. The analysis of brominated TAG enabled the selective detection of native saturated TAG in HM fat.

Fig. 3 UHPLC-ESI-MS/MS-based comparison of HM TAG before, after 2 h and after 4h the maternal uptake of 200 mL of bovine milk.

Fig. 4 Typical ^{13}C NMR spectrum of HM recorded in $CDCl_3$. The two insets display enlargements of the carbonyl and glycerol-backbone regions. 1-MAG (monoacylglycerols), 2-MAG, 1,2-DAG (diacylglycerols), 1,3-DAG and TAG species were clearly distinguished.

Fig. 5 ^{13}C NMR monitoring of the diagnostic carbonyl resonances of butyric acids. The α-carbonyl resonances of butyrate at *sn*-1,3 (173.13 ppm) or *sn*-2 glycerol positions (172.65 ppm) were not detected in HM samples either before (A) or 2 h (B) and 4 h after (C) intake of bovine milk. The resonances were confirmed by comparison with the ^{13}C NMR spectrum of standard tributyrin (D).