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ScienceDirect

Journal of Nutritional Biochemistry 126 (2024) 109580

Journal of Nutritional Biochemistry

RESEARCH PAPER

Lipids and lipid signaling molecules in human milk and infant formula, a chemical characterization of relevant biochemical components

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Received 7 November 2023; received in revised form 8 January 2024; accepted 8 January 2024

Abstract

Breastfeeding is the gold standard in infant nutrition and continuous researches aim to optimize infant formula composition as the best alternative available. Human milk lipid content provides more than 50% of energy requirements for infants together with essential vitamins, polyunsaturated fatty acids, and other bioactive components. While fatty acids and vitamins human milk content has been extensively studied and, when needed those have been added to infant formulas, less is known about polyunsaturated fatty acids functional derivatives and other bioactive components. Here we describe the comparison of lipid compositions in breast milk from 22 healthy volunteers breastfeeding mothers and the six most common infant formula devoting particular attention to two families of signaling lipids, endocannabinoids, and eicosanoids. The main differences between breast milk and formulas lie in a variety of saturated fatty and unsaturated fatty acids, in the total amount (45–95% less in infant formula) and a variety of endocannabinoids and eicosanoids (2-AG, 5(s)HETE, 15(S)-HETE and 14,15-EET).

Keywords: Human milk; Infant formulae; Lipids; Endocannabinoids; Breastfeeding; Eicosanoids; Fatty acids.

1. Introduction

Breastfeeding is the best way to feed healthy infants, as human milk (HM) is the natural source of nutrition [1]. HM provides numerous benefits, including psychological, ecological, economic, and nutritional advantages. Breastfeeding has been shown protective properties against childhood infections, malocclusions, overweight and diabetes [1] and may reduce the risk of allergic disorders such as asthma and allergic rhinitis [2]. Moreover, mothers who breastfeed have a reduced long-term risk of cardiovascular disease [3], diabetes [4], breast [5], and ovarian cancer [6]. Unfortunately, HM is not always available, and infant formulas (IFs) are widely regarded as the best alternative to HM. IFs are enriched with various amounts of vitamins, minerals, amminoacids, nucleic factors, essential fatty acids, and other relevant factors. Breast milk, on the other hand, is the sum of the mother's nutritional choices, starting from the preconceptional era. During the first 6 months of an infant's life, providing optimal nutrition is crucial as the consequences of inadequate nutrition can be very serious. Nevertheless, infant formula (IF) is often used as a substitute for breast milk. It contains various vitamins, minerals, amino acids, nucleic factors, essential fatty acids, and other relevant nutrients suggested by current knowledge.

Lipid fraction is the most variable constituent of HM, and the lipid content and composition are influenced by various factors, such as lactation period, diet, and individual differences [7]. Moreover, fats are important components in HM, as they are not only rich in energy, but also contain essential polyunsaturated fatty acids (PUFAs), fat-soluble vitamins, and hormones that are necessary for infants' growth. HM fat, a complex mixture of lipids, accounts for 3–5% by weight but provides more than 50% of energy requirements for infants [7,8]. About 98% of human milk fats [9] consists of more than 200 fatty acids (FAs) [7], 400 triacylglycerols (TAGs) [10] and also several complex lipids, such as glycerophospholipids, sphingolipids, and sterols. In addition, beyond the energetic and structural aspects, HM contains important functional lipids as eicosanoids and endocannabinoids (ECs) with fundamental signaling functions. In the last decade, increasing literature strongly suggests ECs and eicosanoids role in a plethora of physiological processes and organism homeostasis [11,12]. Atypical levels of eicosanoids and/or ECs, for example, have been as-

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https://doi.org/10.1016/j.jnutbio.2024.109580

sociated to pathological processes [13–16] or energy homeostasis deregulation [17]. The involvement of ECs and eicosanoids in health and disease is evidenced by the promotion of the endocannabinoid system as a therapeutic target, as well as by numerous studies exploring the possibility of regulating ECs and eicosanoids local or systemic levels to promote a return to physiological conditions [18–22]. Besides the documented involvement of ECs and eicosanoids in adulthood physiologic and pathological processes, recent evidence also suggests a role in childhood growth and development processes. Arachidonic acid (ARA) is the most predominant long-chain PUFAs in human milk and is essential for infant development [23]. Moreover, the ω-3 PUFA Docosahexaenoic acid (DHA) is vital for the development of structure and function of the brain in fetuses and infants, besides its well documented role in the maintenance of a healthy brain in adulthood [24]. Maternal DHA and ARA ~~are~~ accumulate rapidly within the cerebral cortex during the last trimester of pregnancy and postnatal 18 months [25]. In humans, ARA and DHA can be synthetized starting from the essential PUFAs linoleic acid (LA, ω-6) and α-linolenic acid (ALA, ω-3), respectively. However, there is a clear experimental evidence demonstrating that the conversion of ALA to longer-chain FAs is insufficient to ensure adequate tissue levels; therefore, those are modulated also by the dietary intake of the mothers [26]. Eicosanoids, as prostaglandin, prostacyclin and thromboxane, are highly active compounds synthesized from 20-carbon PUFAs, such as ARA, by enzymes cyclooxygenase, lipoxygenase, or cytochrome P450. The same enzymes also synthetize maresin, neuroprotectin, and resolvins (Rvs) from DHA. ARA and DHA metabolites perform a pivotal role in numerous physiological systems and pathological processes, including inflammation regulation. The levels of ARA and DHA metabolites in breast milk are influenced by the equilibrium between ω-6 and ω-3 PUFAs in the mother's diet, owing to their common enzyme system [27]. Current data, regarding eicosanoids, in HM mainly focused on the ARA-derived prostaglandins PGE2 and PGF2 [26]. The gastrointestinal tract contains prostaglandin receptors [28], and prostaglandins have various functional effects in the stomach and intestines [29]; however, the functional significance of eicosanoids in HM has not been fully understood yet. Only few studies, with a quite low number of samples, also reported DHA and EPA-derived eicosanoids in HM [30] with 18R-RvE1 and RvD1 similar concentrations in milk provided at different times across the first month of lactation and higher concentration of 18R- RvE1 in HM from women with mastitis. However, careful measurements of eicosanoids have to be performed due to the very low concentrations and stability of these molecules, indeed their levels in different studies vary widely, primarily due to methodological differences [28]. ARA and DHA are also precursors of ECs [26]. ECs regulate appetite and food intake [31] by activating cannabinoid receptor 1 (CB1) in the central nervous system [32]. CB1 is activated by two different EC, arachidonoylethanolamide (anandamide, AEA) and arachidonoyl glycerol (2-AG), both derived from ARA. As concern infant feeding behavior, 2-AG has been demonstrated to play a role in establishing the suckling response [33]. Evidence in mouse pups suggest that CB1 activation by 2-AG is needed to establish the suckling response by activating the oral-motor musculature behavior needed for milk suckling [33,34]. Different ECs functions in human milk as initiation of infant suckling [34], stimulation of infant appetite and a feeling of well-being have been suggested and their role in infant brain and neuronal development has been also proposed [35,36]. Moreover, recent literature explores the involvement of ECs in gut microbiota homeostasis and composition showing a correlation between ECs plasma levels (AEA, related acylethanolamides and 2-AG) and specific faecal bacterial genera implicated in maintaining gut barrier in young adults (18-25 years) [37] and suggesting an interplay between Oleylethanolamide (OEA) and the intestinal microorganisms in intestinal homeostasis [17]. The role of early nutrition in the development of the microbiome and of the immune system, affecting lifelong health, has been recently reviewed by Ames and co-workers [38], demonstrating the impact of nutritional sources on co- development of the gut microbiome, antigen tolerance, and immunity. Moreover, eicosanoids immunologic properties and their physiological and/or pathological roles in infants has been reported [39–41] and the presence of eicosanoids derived from ARA, DHA and EPA has been poorly studied devoting particular attention to resolvines families [30,42]. In this *scenario*, fatty acids and their derivatives, as ECs and eicosanoids derived from milk feeding, could play a pivotal role in infant gut and immune system development and response.

HM is considered to be a reference or the gold standard for the development of HM substitutes [7,8,43–45]. There are three major classes of IFs: cow-milk based formula, soy-based formula, and specialized formula. Bovine milk is the basis for most IFs. Because cow's milk contains higher levels of fat, minerals and protein than human breast milk, diluted skimmed cow's milk and a blend of vegetable oils are usually used to have a lipid composition similar to that of breast milk [46]. As in animals, bioactive lipids derived from fatty acids represent a small fraction of the total lipid content in plants, and their acyl composition generally reflects the acyl groups found in total lipids [47,48].Thus, cow's milk formulas should have a bioactive lipid content more similar to that of breast milk than soy-based formulas. Studying and understanding the lipid biology of HM could lead to better dietary advice for nursing mothers, as well as further opportunities to improve the composition of IFs. To propose a nutritionally adequate substitute for human milk, it is essential to consider not only the energy content but also the functional components. Therefore, promoting breast milk composition as a priority area of research can help improve infant formula and make it a real alternative to breastfeeding. Certainly, the in-depth characterization of the lipid compositions of HM in terms of fatty acids, ECs and eicosanoids can contribute to the achievement of this goal.

2. Materials and Methods

2.1. Chemicals

The reference materials N-arachidonoylethanolamide (AEA), N-linolenoylethanolamide (LNEA), N-linoleoylethanolamide (LEA), N-oleoylethanolamide (OEA), N-palmitoylethanolammide (PEA), N-stearoylethanolamide (SEA), and N-stearoylethanolamide-d4 (SEA-d4) were synthesized and completely characterized in our laboratories as previously described [49–51]. The reference materials N-docosahexaenoylethanolamide (DHEA), N-eicosapentaenoylethanolamide (EPEA), N-arachidonoyldopamine (ADA), N-oleoyldopamine (ODA), N-arachidonoylglycine (AGly), N-oleoylglycine (OGly), N-palmitoylglicine (PalGly), N-arachidonoylserine (ASer), N-arachidonoylserotonine (A5HT), N-oleoylserotonine (O5HT), N-palmitoylserotonine (Pal5HT), 2-arachidonoylglycerylether (2AGE), 2-arachidonoyglycerol (2AG), N-arachidonoyl-3-hydroxy-γ-aminobutyric acid (AGABA), arachidonic acid (AA), eicosapentaenoic acid (EPA), thromboxane-B2 (TXB2), prostaglandin-F2α (PGF2α), 6α-keto-prostaglandin-F1α (6α-keto-PGF1α), prostaglandin-E2 (PGE2), prostaglandin-D2 (PGD2), leukotriene-B4 (LTB4), 5-hydroxyeicosatetraenoic acid (5(S)-HETE), 15-hydroxyeicosatetraenoic acid (15(S)-HETE), (±)14(15)-epoxyeicosatrienoic acid (14,15-EET) and internal standards N-arachidonoylethanolamide-*d*8 (AEA-*d*8), N-oleoylethanolamide-*d*2 (OEA-*d*2), N-palmitoylethanolammide-*d*5 (PEA-*d*5), N-docosahexaenoylethanolamide-*d*4 (DHEA-

*d*4), N-eicosapentaenoylethanolamide-*d*4 (EPEA-*d*4), N-arachidonoyldopamine-*d*8 (ADA- *d*8), N-arachidonoylglycine-*d*8 (AGly-*d*8), N-arachidonoylserine-*d*8 (AS-*d*8), N-oleoylserotonine-*d*17 (O5HT-*d*17), 2-arachidonoyglycerol-*d*8 (2AG-*d*8), eicosapentaenoic acid-*d*5 (EPA-*d*5), thromboxane-B2-*d*4 (TXB2-*d*4), prostaglandin-F2α-*d*4 (PGF2α-*d*4), leukotriene-B4-*d*4 (LTB4-*d*4) were purchased from Cayman Chemical (Ann Arbor, USA). Fatty acid methyl esters (FAMEs) standards (Supelco 37 Component FAME Mix certified reference material) and all other reagents and solvents was purchased from Sigma–Aldrich (Milan, Italy).

2.2. Milk samples

Milk samples (HM) were obtained from 22 healthy volunteers Caucasian breastfeeding mothers at 4–8 weeks postpartum (25–40 years old), which gave informed consent to offer their biological samples for research intent. For this pilot study, volunteer mothers collected milk with an electric breast pump, following common home procedures. An aliquot of approximately 10 mL of breast milk was transferred into a sterile test tube and immediately home frozen. With this collection procedure we consider to have a good mix of fore and hind milk. Six common and most frequently used commercially available liquid cow's milk-based infant formulae for babies up to 6 months of age have been purchased at common supermarket. Each IF has been purchased in three different supermarkets and analyzed. The lipid sources and nutritional values reported on the labels of IFs are reported in Table 1.

2.3. Fatty acid analysis

The fatty acid profile of HMs and IFs was determined by gas chromatography using Hewlett-Packard 6890 gas chromatograph with flame ionisation detector as described elsewhere [52,53]. Briefly, lipids were extracted with chloroform/methanol (2:1, v/v) containing 0.2% butylated hydroxytoluene. Fatty acid methyl esters (FAMEs) were prepared by incubation with 140 g/l boron trifluoride in methanol at 90°C for 90 min, extracted with hexane and analyzed by capillary gas chromatography. Pure 37 components of the FAME Mix certified reference material were used for building the calibration curves and their retention times were used as a reference to identify the FAMEs obtained from the milk samples, heptadecanoic acid was used as the internal standard. Were identified and quantified the following fatty acids: caprylic acid (CAP, C8:0), caproid acid (CPC, C10:0), lauric acid (LAU, C12:0), myristic acid (MY, C14:0), palmitic acid (PAL, C16:0), palmitoleic acid (PALOL, C16:1), stearic acid (STA, C18:0), oleic acid (OLA, C18:1), linoleic acid (LA, C18:2, ω-6), alpha-linolenic acid (ALA, C18:3, ω-3), di-homo-gamma-linolenic acid (DHLA, C20:3, ω-6), arachidonic acid (ARA, C20:4, ω-6), timnodonic acid (eicosapentaenoic acid, EPA, C20:5, ω-3), cervonic acid (Docosahexaenoic acid, DHA, C22:6, ω-3). The amount of each considered fatty acid was calculated as μg/mL of milk and expressed as a percentage of the total fatty acid concentration. All the samples were analyzed in triplicate.

2.4. Endocannabinoids and eicosanoids analysis

A targeted HPLC-MS/MS analysis has been performed on milk samples to evaluate ECs and eicosanoids listed in chemicals paragraph, applying the analytical methods described before [54] and validated for the milk matrices. Sensitivity, specificity, precision, accuracy, recovery and matrix effect of extraction and quantification procedures have been assessed in compliance to the US Food and Drug Administration (FDA) guidelines for bioanalytical methods' validation. Surrogate analyte-free matrix (i.e., water or appropriate buffer) are usually used for the preparation of calibration (CS) and quality control (QC) samples for validation of endogenous compounds to overcome the lack of analyte-free matrix [55]. To avoid the interference of endogenous analytes, linearity, slope, recovery, and the influence of matrix effect were obtained by spiking milk with labelled internal standards (ISs). Pooled milk for CS and QC used for validation experiments were prepared combining 10 different samples. CS contain all compounds in the range of 0-5 ng/mL and 0-250 ng/ml for 2-AG, QC were prepared at low, intermediate and high concentration levels. Limit of detection and limit of quantification evaluation was performed on PBS [55]. The calibration curves showed good linearity with $R^2 > 0.991$ over the 0.1–2.5 ng/mL concentration range for all the compounds, and CS prepared in milk spiking ISs were parallel to that prepared in PBS that

Table 1
Lipid sources and nutritional values reported on the labels of infant formulae.

Product	Lipid sources	Lipids (g/100 mL)
IF1	Skim cow milk, palm kernel and palm oils, rapeseed oil, sunflower oil, microalgae oil (*Crypthecodinium cohnii*), *Mortiella Alpina* oil Emulsifiers: mono- and di-acylglycerols of fatty acids.	Total: 3.2 SFA: 1.3 MUFA: 1.3 PUFA: 0.6 LA: 0.330 ALA: 0.055 ARA: 0.0072 DHA: 0.0144
IF2	Skim cow milk, structured vegetable oil (Betapol: structured triglycerides), rapeseed oil, sunflower oil, *Mortierella alpina* oil, high oleic sunflower oil, high DHA fish oil Emulsifiers: mono- and di-acylglycerols of fatty acids.	Total: 3.4 SFA: 1.2 MUFA: 1.3 PUFA: 0.5 LA: 0.402 ALA: 0.042 ARA: 0.018 DHA: 0.018
IF3	Cow milk, high oleic sunflower oil, rapeseed oil, sunflower oil, coconut oil, whole milk Emulsifiers: soy lecithin.	Total : 3.6 SFA: 1.1 MUFA: 1.9 PUFA: 0.6 LA: 0.54 ALA: 0.0648
IF4	Skim cow milk, palm oil, coconut oil, rapeseed oil, sunflower oil, fish oil, *Mortierella alpina* oil Emulsifiers: mono- and di-acylglycerols of fatty acids, soy lecithin.	Total: 3.4 SFA: 1.5 MUFA: 1.4 PUFA: 0.5 LA: 0.418 ALA: 0.078 ARA: 0.00648 DHA: 0.00644 EPA: 0.0015
IF5	Skim cow milk, palm oil, coconut oil, rapeseed oil, sunflower oil, fish oil, *Mortierella alpina* oil Emulsifiers: mono- and di-acylglycerols of fatty acids.	Total: 3.2 SFA: 1.5 MUFA: 1.2 PUFA: 0.5 LA: 0.427 ALA: 0.081 ARA: 0.011 DHA: 0.01 EPA: 0.0022
IF6	Skim cow milk, coconut oil, rapeseed oil, sunflower oil, fish oil, *Mortierella alpina* oil Emulsifiers: mono- and di-acylglycerols of fatty acids, soy lecithin.	Total: 3.6 SFA: 0.9 LA: 0,555 ALA: 0,046 DHA: 0,018

can be used for samples quantifications. For the extraction and purification procedure, briefly, after centrifugation, 10 min at 350 g at 4°C degrees, ISs and 1 mL of ice-cold ACN were added to 500 μL of milk. After additional 10 min of centrifugation at 350 g at 4°C degrees the supernatant was transferred into glass test tubes and extracted with 4 mL of dichloromethane/isopropanol (8:2; v/v). After centrifugation at 350 g for 10 min, the organic layer was separated and dried under a stream of nitrogen. The dried residue was reconstituted with 60 μL methanol and a 3 μL were analysed. HPLC-MS/MS analyses have been performed in triplicate on basic extracts on a 1,290 Infinity UHPLC system (Agilent Technologies), coupled to a Q Trap 5,500 triple quadrupole linear ion trap mass spectrometer (Sciex) [56], equipped with an electrospray source operated in positive mode. Quantifications have been performed using deuterated internal standards calibration curves. All the analyses have been performed in triplicate.

2.5. Data presentation and statistical analysis

Data are presented as mean ± SD. Fatty acid profile data were analyzed using a randomized block design, with six treatments and three blocks. Each of the six IFs corresponded to a treatment, while HMs was used as a benchmark. Initially, lipid profile data were analyzed using ANOVA. When the differences were significant ($p<.05$) the Tukey test was performed at a 5% probability, using the StatistiXL software (version 1.5; StatistiXL, Western Australia). ECs and eicosanoids quantifications are expressed as ng/mL and presented as mean ± SD. One-way ANOVA tests were performed using Prisma GraphPad Prism 6.0c (GraphPad Software, La Jolla, CA, USA) [57] to assess statistical significance of groups differences. All mean values and standard deviations obtained are available in supplementary materials Table 1S for acids and table 2S for eicosanoids and endocannabinoids.

3. Results and discussion

Lipids play a critical role in infant nutrition. The compositional and biological effects of HM lipids have received considerable interest regarding their modulating effects on growth, metabolism and functions of the cardiovascular, membrane composition and function, immune and nervous systems [58]. Several advances have been made in IF composition so that formula can better mimic the nutritional functions of HM. Fatty acids and TAGs are a major source of energy provided with HM to the infant [59,60], but HM fat (HMF) also provide essential nutrients such as PUFAs and lipid soluble vitamins. For this purpose, a continue understanding and characterization of HMF has driven current lipid innovations in IF, with the overall goal of meeting the nutritional needs of formula-fed infants [61]. HMF substitutes are modified lipids whose fatty acid composition and distribution have been altered deliberately in order to mimic the fatty acid composition and distribution of HMF. So far, several HMF substitutes have been developed and commercialized successfully to satisfy customer needs, such as energy supplements, sources of essential fatty acids, and nutritional supplements in IF [61].

EC and EC-like compounds are present in breast milk [62–64]. EC-like compounds, referred to metabolites with entourage effects [65], can support the activity and physiological responses of the EC system by interacting with AEA and 2-AG, their enzymes or receptors. These EC-like compounds [66] include ethanolamide such as PEA, OEA, docosahexaenoyl ethanolamide (DHEA), eicosapentaenoyl ethanolamide (EPEA), eicosenoyl ethanolamide (EEA), and glycerol derivatives such as palmitoyl glycerol (PG), oleoyl glycerol (OG), docosahexaenoyl glycerol (DHG), eicosapenaenoyl glycerol (EPG), eicosaenoyl glycerol (EG);and long chain PUFA precursors: ARA, DHA, and EPA.

Aiming to obtain a detailed characterization of HM in respect to IF, and to explore the quite unexplored field of ECs and eicosanoids profile of HM this pilot study investigated the lipid composition, in term of fatty acids, ECs and eicosanoids, of 22 HM from healthy breastfeeding mother volunteers and 6 common and most frequently used commercially available IFs. The fatty acid profile of HMs and IFs was determined by gas chromatography while a targeted HPLC/MS-MS analysis has been performed to quantify the ECs and eicosanoids profile (20 ECs and 9 eicosanoids) as reported before [54]. In general, intra group IFs lipids compositions were much more different than HM, probably due to different lipid sources used for formulations, for this reason the comparisons reported and discussed below will consider the mean values obtained from the 22 HM and each IF. The lipid sources and nutritional values reported on the labels of IFs are reported in Table 1.

The total fatty acid concentration in IF is quite similar to HM. However, in comparison whit HMs, IFs had different percentages of capric acid (C 10:0), myristic acid (C 14:0), palmitic acid (C 16:0), palmitoleic acid (C 16:1), stearic acid (C 18:0), linoleic acid (C 18:2 ω-6), α-linolenic acid (C 18:3 ω-3), arachidonic acid (ARA, C 20:4 ω-6), and eicosapentaenoic acid (EPA, C 20:5 ω-3) acids (Fig. 1). In accordance with the fatty acid composition of the lipid sources used for their preparation, the IFs containing palm oil and Betapol® showed a higher palmitic acid content, while the IFs containing coconut oil had a higher content of myristic acid.

The quantification of 29 signaling lipids, listed in the experimental part, belonging to twenty endocannabinoids and nine eicosanoids, was performed by HPLC-MS/MS. The very sensitive method validated in out laboratories allows to simultaneously extract and quantify all these molecules in a single sample of 0,5 mL of milk, thanks to a double extraction and analysis previously described [55]. Considering the particular characteristics of the PUFA derivatives and metabolites (i.e., instability, autooxidation), the mayor instability of DHA and EPA and the poor knowledge on eicosanoids derived from these two PUFAS we decided, firstly, to focus our attention on ARA derivatives. From the ECs, ECs-like and eicosanoids lipids analyzed, serotonin and dopamine derivatives, arachidonoyl glycine, the ethanol-amides of DHA and EPA, 2-arachidonoylglycerylether and *N*-arachidonoyl-3-hydroxy-γ-aminobutyric acid were not detectable or under the quantification limit of the method in all assessed samples. Considering this ECs and eicosanoids, it is interesting to note their very lower content in IF. Data were calculated as mg of ECs plus eicosanoids content per 100 g of milk. Comparisons between HM and IFs were performed expressing each EC or eicosanoid as percentage of the total ECs and eicosanoids content using for HM the mean of the 22 HMs evaluated compared to each different IFs. IFs content of these lipids was very variable, from 45 up to even 95% less than that of HM (data not showed). For this reason, lipid concentrations obtained have been transformed in percentage of the total content of these two family of molecules to perform comparisons. One way-ANOVA test has been performed to analyze HM vs IFs differences, their statistically significance and obtained results are reported in Fig. 2. Beyond the high variability between the different IFs assessed afore mentioned, there is not a unique trend in the percentage compositions. LEA, LNEA, OEA, PEA, SEA and PALGly are higher in all IFs than in HM. IF1 and IF6 display a much higher content of AEA and only IF6 of NOGly than HM. Only IF1 reports a 2-AG content comparable to HM while the other IFs are significantly lower. The three eicosanoids 5(s)HETE, 15(S)-HETE and 14,15-EET content instead are lower in a statistically significant manner in all IFs in respect to HM.

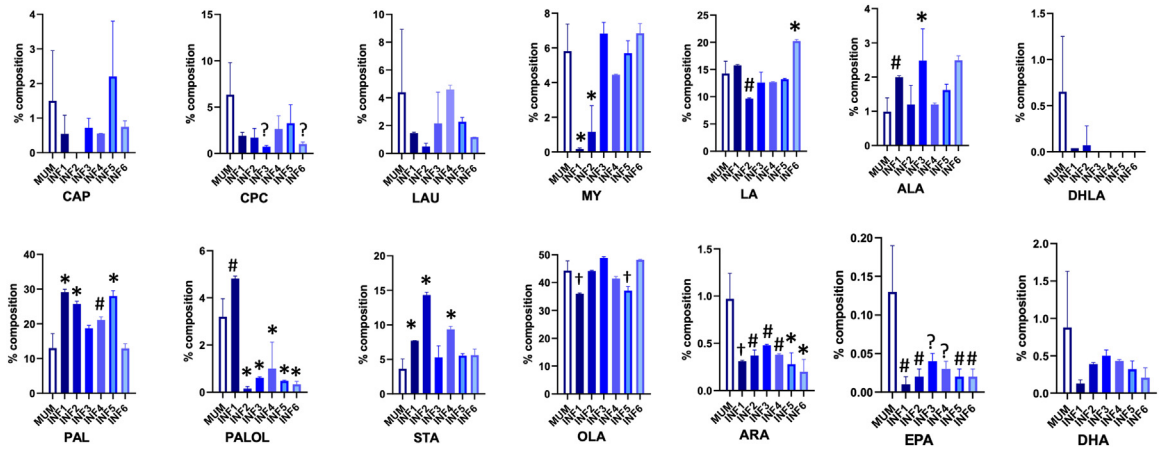

Fig. 1. Fatty acid composition of human milk (HM) and infant formulas (IF1-6) expressed as g/100 g of fatty acids. Panel A: caprylic acid (CAP, C8:0), caproid acid (CPC, C10:0), lauric acid (LAU, C12:0), myristic acid (MY, C14:0), palmitic acid (PAL, C16:0); Panel B: palmitoleic acid (PALOL, C16:1), stearic acid (STA, C18:0), oleic acid (OLA, C18:1), linoleic acid (LA, C18:2, ω-6), alpha-linolenic acid (ALA, C18:3, ω-3), di-homo-gamma-linolenic acid (DHLA, C20:3, ω-6), arachidonic acid (ARA, C20:4, ω-6), timnodonic acid (eicosapentaenoic acid, EPA, C20:5, ω-3), cervonic acid (Docosahexaenoic acid, DHA, C22:6, ω-3). One factor ANOVA p values are reported in Table 1S (supplementary), * $p<.0001$, † $p<.0005$, # $p<.001$, ? $p<.2$.

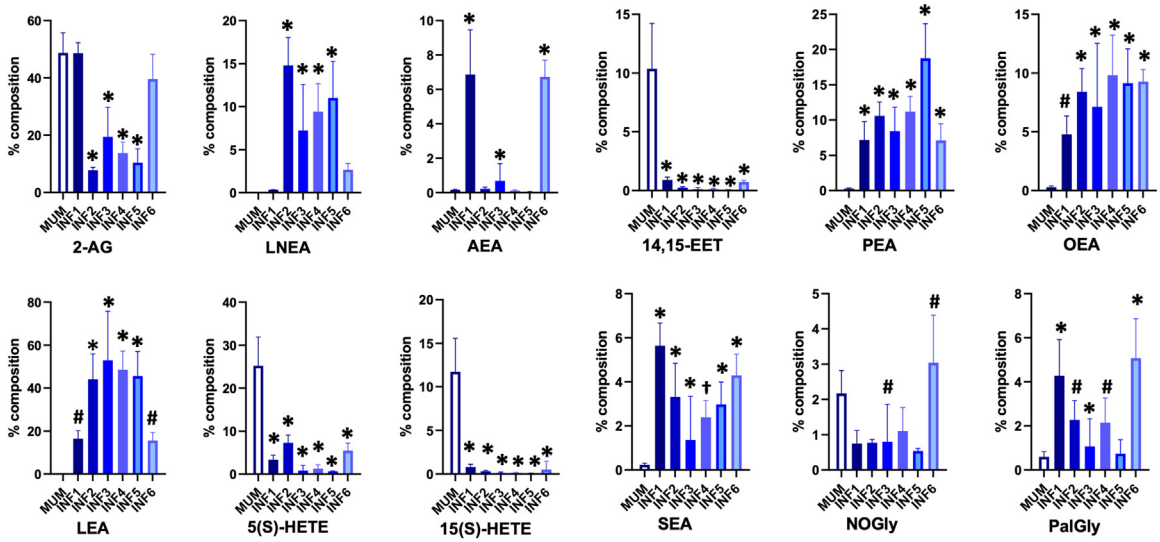

Fig. 2. Endocannabinoids and eicosanoids composition of human milk (HM) and infant formulas (IF1-6), quantification analysed as mg/100 g of milk and expressed as percentage of the total amount of this two lipid families. Panel **A:** 2-arachidonoyglycerol (2AG), *N*-linolenoylethanolamide (LNEA), *N*-arachidonoylethanolamide (AEA), *N*-linoleoylethanolamide (LEA), 5-hydroxyeicosatetraenoic acid (5(S)-HETE), 15-hydroxyeicosatetraenoic acid (15(S)-HETE); Panel B: (±)14(15)-epoxyeicosatrienoic acid (14,15-EET), *N*-palmitoylethanolammide (PEA), *N*-oleoylethanolamide (OEA), *N*-stearoylethanolamide (SEA), *N*-oleoylglycine (OGly), *N*-palmitoylglicine (PalGly). One factor ANOVA *p* value was <.0001 for all molecules, * $p<.0001$, † $p<.0005$, # $p<.001$.

Despite the differences showed by the six IFs, the comparison of fatty acids percentage compositions between the means of IFs values and HM shows slight differences, up to 10%. Main differences regard higher content of short chain fatty acids in HM and of long chain fatty acids, as palmitic and stearic acids, in IFs (Fig. 3, panel A). Analysing the ECs and eicosanoids composition percentage, shown in Fig. 3 panel B, 2-AG (30%) and the three eicosanoids 5(S)-HETE, 15(S)-HETE and 14,15-EET are from 10% up to 25% higher in HM than in IFs. On the contrary the ECs LEA is much higher in IFs (48%). There are various factors that can affect the levels of eicosanoids and ECs of HM and IFs. In HM, these compounds are naturally produced. However, in IFs, they can come from different materials used in their preparation, such as oils, milk, and soy lecithin, (which is often used as an emulsifier). Since eicosanoids and ECs are amphipathic molecules, they can be lost during the preparation of oils, because they are removed along with phospholipids. Nevertheless, TAGs of vegetable are an important source of fatty acids in IFs. Accordingly, no correlations were observed between precursor fatty acid concentrations and the IFs composition in terms of eicosanoids and ECs. While the role of 2-AG in infant suckling initiation [11], feeding stimulation [12,13] and gut microbiota homeostasis and composition [38] has been recently studied, the role of the eicosanoids HETEs and EETs in infants is quite unexplored. EETs are produced from ARA by the cytochrome P450 and its involvement in vaso-protective and anti-inflammatory mechanisms as endogenous anti-pressor agent has been investigate in pregnancy and fetoplacental circulation [67]. HETEs, instead, are produced from ARA by the actions of lipoxygenases and are a family of inflammation and immune system mediators [68], in particular 15-HETE has been suggested to play a protective or anti-inflammatory role in asthma and higher levels of this eicosanoid have been found in very young wheezing chil-

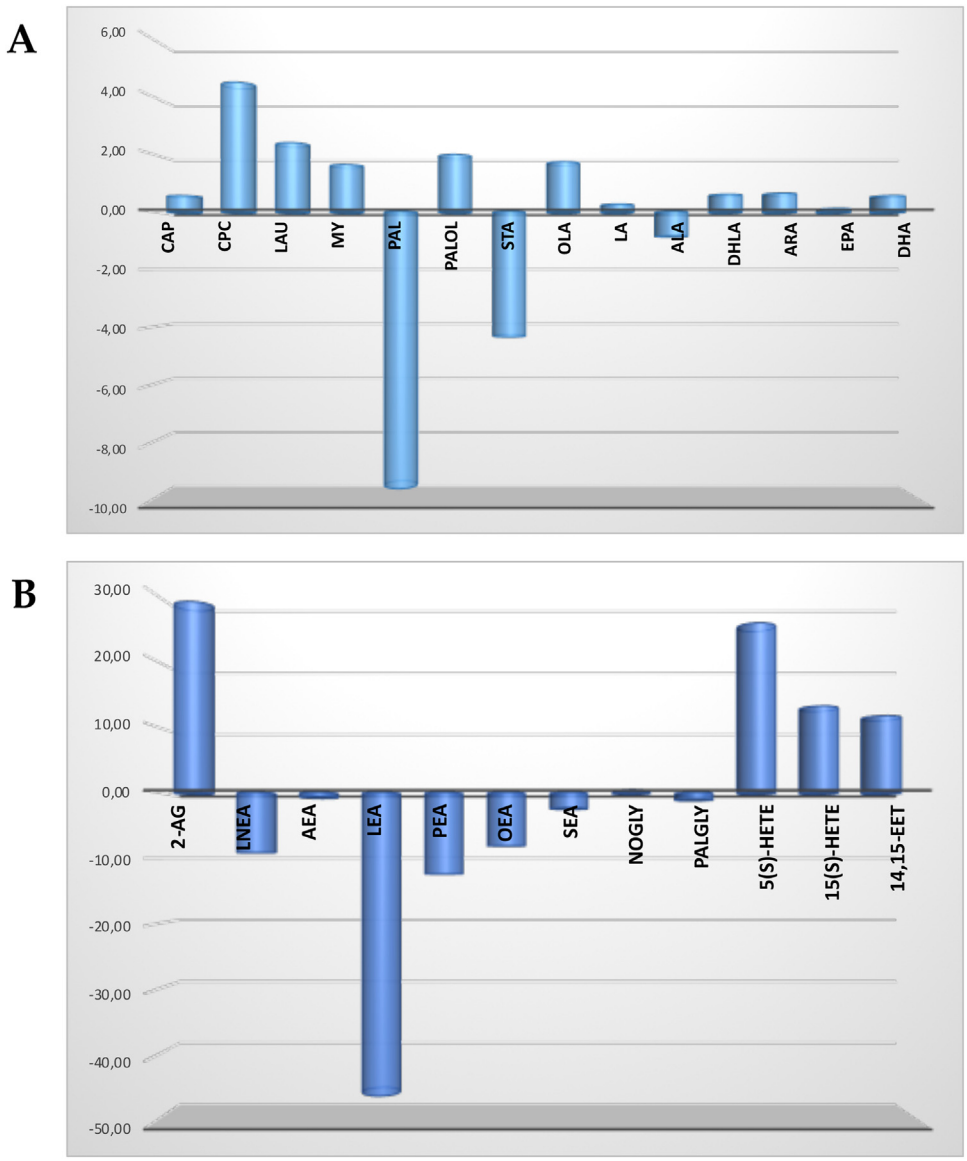

Fig. 3. Differences of percentage content of free fatty acids (panel A) or functional lipids (ECs ad eicosanoids panel B) obtained subtracting of IFs to HM. Panel A: caprylic acid (CAP, C8:0), caproid acid (CPC, C10:0), lauric acid (LAU, C12:0), myristic acid (MY, C14:0), palmitic acid (PAL, C16:0), palmitoleic acid (PALOL, C16:1), stearic acid (STA, C18:0), oleic acid (OLA, C18:1), linoleic acid (LA, C18:2, N-6), alpha-linolenic acid (ALA, C18:3, N-3), dihomo-gamma-linolenic acid (DHLA, C20:3, N-6), arachidonic acid (ARA, C20:4, N-6), timnodonic acid (eicosapentaenoic acid, EPA, C20:5, N-3), cervonic acid (Docosahexaenoic acid, DHA, C22:6, N-3). Panel B: 2-arachidonoyglycerol (2AG), *N*-linolenoylethanolamide (LNEA), *N*-arachidonoylethanolamide (AEA), *N*-linoleoylethanolamide (LEA), 5-hydroxyeicosatetraenoic acid (5(S)-HETE), 15-hydroxyeicosatetraenoic acid (15(S)-HETE), (±)14(15)-epoxyeicosatrienoic acid (14,15-EET), *N*-palmitoylethanolammide (PEA), *N*-oleoylethanolamide (OEA), *N*-stearoylethanolamide (SEA), *N*-oleoylglycine (OGly), *N*-palmitoylglicine (PalGly).

dren in respect to normal ones [69]. Comparing the compositions of eicosanoids and EC with those of fatty acids, the ARA derivatives 2-AG, 5(S)-HETE, 15(S)-HETE and 14,15-EET, higher in HM, do not reflects the ARA content which is only slightly higher in HM than in IF. Furthermore, considering also LA and DHLA, the main fatty acid precursors of ARA, they are respectively slightly higher in IFs and higher in HMs. From these considerations, we can suppose that the gap in ARA derivatives content in HM could not be filled by the infant biosynthesis starting from the IF content of ARA and their main biochemical precursors. Considering the potential role of these eicosanoids and lipid signaling molecules in general in inflammation, human immune response and other physiological processes, it could be important to address future researches to their deeper characterization in human milk ant their biological role in infants.

Fig. 4 reports HM and IFs lipid composition differences in term of saturated (SUFAs), monounsaturated (MUFAS) and polyunsaturated (PUFAs) fatty acids (panel A) or functional fatty acid derivatives (ECs and eicosanoids, panel B). The proportions of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) were significantly different (Fig. 4). Even in this case major differences are displayed by functional lipids. For fatty acids SUFAs content is major in IFs while MUFAs increase in HM. Otherwise, in functional lipids PUFAs content is higher, up to 25%, in HM than in IFs with a consequent 10% minor content in MUFA and 15% in SUFAs.

Fatty acids N-3 and N-6 percentage and ratio was also considered, even though the N-3 PUFA content of the IFs was slightly different from that of the HMs, the ratios between N-6 and N-3 PUFAs were similar in the two milk types (Supplementary,

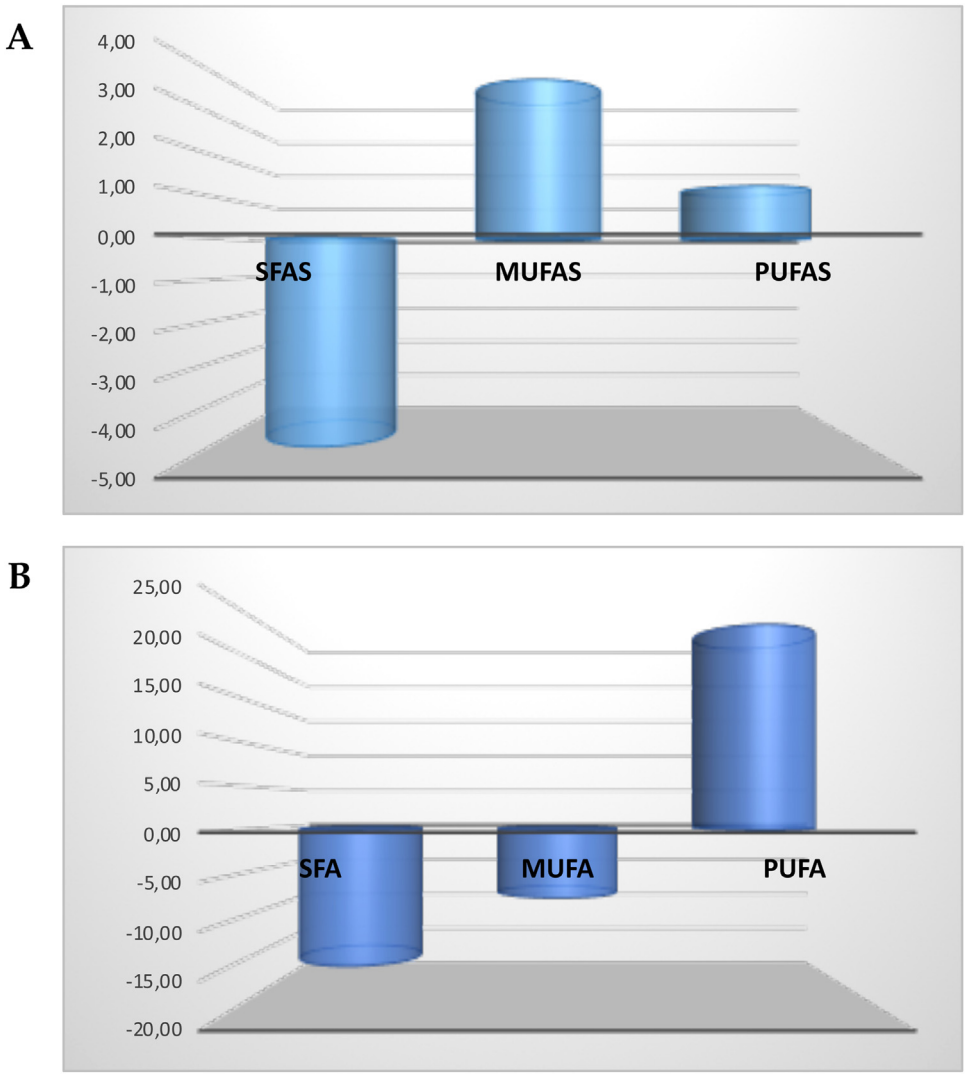

Fig. 4. Differences of percentage content of fatty acids (panel A) or functional lipids (ECs ad eicosanoids panel B), obtained subtracting of IFs to HM, grouped as saturated (SUFAs), monounsaturated (MUFAS) and polyunsaturated (PUFAs).

Table 1S). As LNEA was the only N-3 fatty acid derivative quantifiable in all samples, data calculation on N-3 and N-6 percentage and ratio were not studied.

4. Conclusions

Breastfeeding remain the gold reference standard for infant nutrition and infant formula composition optimization. Lipids are the most variable constituents of the human milk as they are not only rich in energy, but also contain essential fatty acids, fat-soluble vitamins, hormones and other bioactive components that are necessary for infants' growth. While fatty acids and vitamins human milk content has been extensively studied and, when needed those have been added to infant formulas, less is known about polyunsaturated fatty acids functional derivatives and other bioactive components. Here we describe the lipid composition of breast milk from 22 healthy volunteer Caucasian mothers and the 6 most common infant formula devoting, for the first time, particular attention to 29 signaling lipids belonging to endocannabinoids and eicosanoids. The main differences between breast milk and formulas lie in a variety of saturated and unsaturated fatty acids and in the total amount (45–95% less in infant formula) and a variety of ECs and eicosanoids. ECs and eicosanoids main differences regard the arachidonic acid derivative 2-AG and its metabolites produced by lipoxygenases HETEs and EET. Considering the contribution of 2-AG in suckling initiation and feeding stimulation and the potential role of these eicosanoids and lipid signaling molecules in general in inflammation, human immune response and other physiological processes, it could be important to address future researches to their deeper characterization in human milk ant their biological role in infants. Since it is well documented that breast milk is a dynamic bioactive fluid, its composition indeed varies from colostrum to late lactation, diurnally, between mothers and based on the mother's diet, this preliminary study can pave the way for more structured future studies aimed to deeply investigate signaling lipids in this biomatrices, fundamental for infant nutrition.

CRediT authorship contribution statement

Roberta Ottria: Conceptualization, Formal analysis, Data curation, Funding acquisition, Investigation, Project administration, Writing – original draft, Writing – review & editing. **Matteo Della Porta:** Formal analysis. **Ornella Xynomilakis:** Data curation. **Sara Casati:** Formal analysis. **Roberta Cazzola:** Data curation, Funding

acquisition, Investigation, Writing – original draft, Writing – review & editing. **Pierangela Ciuffreda:** Conceptualization, Funding acquisition, Investigation, Project administration, Writing – original draft, Writing – review & editing.

Acknowledgments

The authors are particularly grateful to Valeria Vimercati (Università degli Studi di Milano) for fatty acid analysis.

Declaration of competing interest

The Authors declare no conflict of interest.

Funding

This research was funded by Università degli Studi di Milano, Finanziamento di Ateneo - Linea 2 - funding to PC and RO.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2024.109580.

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