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Original article

NMR-based metabolomics analysis of organic and conventionally produced formula milk: preliminary results

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Proceedings

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Abstract

Nutrition in early life has important biological effects on immediate and lifetime health. In the light of these considerations, products such as specialized and standard infant formulas substitute for human milk have the potential to influence health outcomes differently depending on their composition. The recent knowledge of the long-term health benefits of breast-feeding has addressed research toward the creation of formulas ever closer to the needs of the infant both in term of nutritional and functional compounds. In this regard, metabolomics has proved to be a promising tool to investigate the metabolic composition of breast milk and the differences compared with formula milk. To the best of our knowledge, no metabolomics studies on the compositional differences between organic and conventionally produced infant milk have been performed so far. To fill this gap, the aim of the present work was to use the ^{1}H NMR-based metabolomics approach to compare the metabolome of organic and conventionally produced formula milk designed for fulfill infants' nutritional needs from birth to 12 months of age. Methionine content was found to be significantly ($p = 0.001$) higher in organic milk than in conventional formulas. For the sake of comparison, the metabolome of human milk samples was also analyzed. Although the study presents several limitations, our preliminary results further support the utility of metabolomics in research for infant nutrition.

Keywords

Metabolomics, NMR, human milk, infant formulas, organic.

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Introduction

Short-term benefits of breast-feeding on health are widely recognized so that human milk (HM) is considered the gold standard for infants' nutrition in the early stages of life [1]. Besides its content of macro- and micro-nutrients, such as carbohydrates, fats, proteins, minerals and vitamins, HM contains also various functional and biologically-active constituents, including antimicrobial substances, oligosaccharides, growth factors, immunoglobulins and specific immune cells [2]. The more recent awareness of the long-term health benefits of breast-feeding [3-4] has aimed research on formula composition to make these products ever closer to the needs of the infant both in term of nutritional and functional compounds [5]. Nowadays, several varieties of infant formulas are available world-wide and their composition vary greatly depending on the nutritional needs of the targeted infant population and on the presence of supplemental ingredients added to attain health benefits. Minimum standard global recommendations for the composition of infant formulas has been published previously and revised over the years [6], and furthermore the introduction of new functional ingredients is always under examination [7-9].

Metabolomics is a discipline finalized to the qualitative and quantitative analysis of the metabolome, i.e. the set of low molecular weight metabolites (< 1.5 kDa) of a biological system (plant, animal, human) that derives from the biochemical transformation of the substrates in the different metabolic processes and thus represents the final result of the cellular function. It is the "omics" science that provides the closest information on phenotype, considered to be the pivotal point of the etiopathogenesis of the multifactorial diseases. For this reason, the applications of metabolomics are very appropriate in the medical field.

In food science, metabolomics has been used for assessing safety and quality of food [10], monitoring compositional changes during processing and storage, selecting markers of authenticity, differentiating between organic and conventional food [11]. At the moment, there are still few metabolomics applications for infant nutrition. Most of these studies were aimed at characterizing the metabolome of HM [12-26], while, to a lesser extent, some were carried out on formula milk (FM) [12, 18, 21, 25, 26]. To the best of our knowledge, there are no metabolomics investigations performed on organic infant formula.

The aim of the present work was to use a ^{1}H NMR-based metabolomics approach to compare the metabolome of organic and conventionally produced FM designed for fulfill infants' nutritional needs from birth to 12 months of age. For the sake of comparison, HM samples collected at 7 ± 3 days post-partum were also analyzed.

Material and methods

Subject and sample collection

Overall, 15 FM samples, among which 6 organic and 9 conventionally produced, were purchased from a local supermarket. HM samples were obtained from 15 healthy mothers of term infants with a mean gestational age of 39 ± 3 weeks at 7 ± 3 days post-partum. Written informed consent was obtained by each mother before participation in the study. Samples were collected (2-3 ml) by manual expression in a sterile bottle and stored at -80°C before the analysis.

Sample preparation

Before ^{1}H NMR analysis, thawed milk samples were centrifuged at 10,000 g for 30 min at 4°C using Amicon Ultra 0.5 mL 10 kDa spin filters (Millipore, Billerica, MA, USA) in order to remove residual lipids and proteins. Each filtered sample (350 µL) was mixed with 350 µL of 0.1 M phosphate buffer solution (pH 7.4) containing sodium 3-trimethylsilyl-(2,2,3,3-2H$_4$)-1-propionate (TSP) (final concentration 2 mM) and then transferred into a 5 mm wide NMR tube.

NMR spectroscopy

^{1}H NMR experiments were performed at 300 K on a Varian UNITY INOVA 500 spectrometer

(Agilent Technologies, Inc., Santa Clara, CA), operating at a frequency of 499.83 MHz. One-dimensional (1D) ^{1}H NMR spectra were obtained using a standard pulse sequence (1D NOESY) with presaturation during relaxation and mixing time for water suppression. Milk spectra were acquired with 256 transients over a spectral width of 6,000 Hz with a total acquisition time of 1.5 s and a mixing time of 0.1 s. After Fourier transformation with 0.3 Hz line broadening, spectra were phased and baseline corrected, and the chemical shift scale was set by assigning a value of $\delta = 0.00$ ppm to the signal for the internal standard TSP.

Spectral processing and statistical analysis

Before statistical analysis, the NMR spectra were processed using MestReNova, version 12.0.1 (Mestrelab Research SL, Santiago de Compostela, Spain) and corrected for misalignments in chemical shift primarily due to pH-dependent signals. Each spectrum was integrated (binned) using 0.001 ppm integral regions between 9.5 and 0.5 ppm, excluding the portions with the residual water (δ 4.6-5.2), TSP resonances and all lactose signals except those at δ 3.30. Bins were normalized to the sum of the total spectral area to compensate for the overall concentration differences. The two final data set were automatically reduced to ASCII files and converted into an Excel® file. The NMR data sets were imported into SIMCA 14.1 (Umetrics, Umea, Sweden), Pareto-scaled, and analyzed by Principal Component Analysis (PCA). Metabolites were identified based on literature data and using the Human Metabolome Database (http://www.hmdb.ca/).

PCA is a multivariate data analysis method to summarize massive numbers of variables in a dataset into a few correlated variables. Graphically, the results of a PCA consist of score plots, giving an indication of any grouping in the data set, and loading plots, indicating which variables are important in reference to the score patterns. Separation of samples in clusters signifies differences between groups as represented by the loadings for those PCs. Generally, the relevant information of PCA analysis are retained by the first two or three principal components (PC1, PC2, and PC3).

Results

In this study, 15 commercial FM and 15 HM samples were analyzed by ^{1}H NMR spectroscopy. Commercial milk samples were intended for different targeted infant population: nine first infant FM (indicated from birth), one FM for infants 0-12 months and five follow on FM (from 6 to 12 months). Six of these formulas were produced using organic ingredients. Nutritional information of FM as labelled at milk package are reported in **Tab. 1**. HM samples were collected at 7 ± 3 days post-partum from mothers who gave birth at term.

To investigate the main variance amongst FM samples, an unsupervised approach using PCA was applied to the ^{1}H NMR data set containing only commercial products. The first two PCs of the resultant scores plot jointly explained almost 65% of the total variance (**Fig. 1**). Two main clusters of samples are observable along the PC1: one located on the right side of the plot, including five first FM, and the other comprising all the remaining FM located on the left side of the plot. The examination of PC1 loadings (**Fig. 1B**) suggested that the differences between the two groups mainly involved maltodextrins, lactose, citrate, choline and phenylalanine. In good agreement with the values reported in the nutritional facts panel, maltodextrins were more abundant in milk clustered on the left side of the plot, while lactose levels were higher on samples located on the right side. Phenylalanine was present only in the first infant milk coded F-org, F, D-org, D-org*, as also mentioned in their ingredients list. The analysis of the PC2 loadings plot (**Fig. 1C**) evidenced others differences among samples involving metabolites such as carnitine, creatinine, methionine, *N*-acetylcarbohydrates, lactate, pantothenic acid, glucose, galacto- and fructo-oligosaccharides (GOS, FOS). In particular, GOS and FOS (negative loadings) were found only in first formulas coded A, H and D-org*, and in the follow on formulas coded A, E and H (**Fig. 1A**). On the other hand, the upper side of the graph included samples with higher levels of lactate, pantothenic acid, *N*-acetylcarbohydrates, creatinine, carnitine, choline and methionine. Except for methionine, these differences might be reasonably ascribed to the manufacturing composition of FM, since these metabolites are mentioned either in the nutritional fact panel or in the ingredient list of the products. Interestingly, methionine content was found to be significantly ($p = 0.001$) higher in organic milk than in conventional formulas (**Fig. 2**).

A further exploratory analysis was performed for the comparison between the FM and HM

Table 1. Information about the formula milk composition as labelled at milk package (continues on the next page).

Milk code	Age infant population target (month)	Organic	Ingredients [a]
A	0-6	No	Demineralized water, skimmed milk, milk lactose, vegetable oils, milk GOS, whey protein concentrate, emulsifiers: mono- and diglycerides of fatty acids, FOS, citric acid, fish oil, tricalcium phosphate, potassium chloride, calcium hydroxide, potassium hydrogen carbonate, sodium L-ascorbate, tripotassium citrate, potassium dihydrogen phosphate, choline chloride, sodium chloride, ascorbic acid, potassium hydroxide, Taurine, emulsifier: soy lecithin, magnesium oxide, inositol, ferrous lactate, DL-α-tocopheryl acetate, zinc sulfate, uridine 5'-monophosphate sodium sale, cytidine 5'-monophosphate, L-carnitine, retinyl acetate, adenosine 5'-monophosphate, inosine 5'-monophosphate selling sodium, ascorbyl palmitate, cholecalciferol, nicotinamide, calcium D-pantothenate, guanosine 5'-monophosphate sodium, copper gluconate, sodium selenite, potassium iodide, folic acid, D-biotin, riboflavin, cyanocobalamin, phytomenadione, thiamine hydrochloride, pyridoxine hydrochloride, manganese sulfate.
A	6-12	No	Demineralized water, skimmed milk, lactose, palm oil, low erucic rapeseed oil, coconut oil, sunflower oil, Mortiriella Alpina oil, maltodextrin, milk GOS, concentrated milk whey protein, emulsifiers: mono- and diglycerides of fatty acids, FOS, citric acid, tricalcium phosphate, fish oil, potassium chloride, calcium hydroxide, calcium citrate, sodium hydrogen carbonate, sodium L-ascorbate, choline chloride, potassium hydroxide, taurine, ferrous lactate, emulsifier: soy lecithin, inositol, magnesium oxide, DL-α-tocopheryl acetate, trisodium citrate, zinc sulfate, retinyl acetate, uridine 5′-monophosphate sodium salt, cytidine 5′-monophosphate, adenosine 5′-monophosphate, inosine 5′-monophosphate sodium salt, ascorbyl palmitate, colecalciferol, nicotinamide, L-carnitine, calcium D-pantothenate, guanosine 5′-monophosphate sodium salt, copper gluconate, sodium selenite, L-tryptophan, potassium iodide, folic acid, D-biotin, riboflavin, phytomenadione, thiamine hydrochloride, cyanocobalamin, pyridoxine hydrochloride, manganese sulfate.
B-org	0-12	Yes	Water, whole milk[b], demineralized whey powder[b], vegetable oils[b] (canola[b], coconut[b], sunflower[b]), maltodextrin[b], lactose[b], mineral salts (hydrogen calcium phosphate, calcium carbonate, potassium chloride, trisodium citrate, sodium chloride, magnesium carbonate, ferrous lactate, potassium iodide, zinc sulfate, sodium selenite, copper sulfate, manganese sulfate), emulsifier: soy lecithin, choline bitartrate, vitamins (C, E, A, pantothenic acid, niacin, D, biotin, folic acid, K, B12, thiamine, riboflavin, B6, inositol).
C-org	0-6	Yes	Skim milk[c] (Germany), whey powder[b] partially demineralised, vegetable oil[b] (palm oil[b], canola oil[b], sunflower oil[b]), maltodextrin[b], skimmed milk powder[c], calcium carbonate, potassium chloride, sodium chloride, vitamin C, vitamin E, iron lactate, copper sulfate, vitamin A, vitamin B1, vitamin B6, manganese sulfate, potassium iodate, acid foliate, vitamin K, sodium selenate, vitamin D.
D-org	0-6	Yes	Skimmed milk[b], whey powder[b], vegetable oils[b] (palm oil[b], canola oil[b], sunflower oil[b]), lactose[b], long chain polyunsaturated fatty acids oil mixture (fish oil, vegetable oil from M. alpina), calcium carbonate, potassium chloride, L-tyrosine, vitamin C, L-phenylalanine, zinc sulfate, L-tryptophan, iron sulfate, stabilizer: lactic acid, inositol, niacin, pantothenic acid, copper-lysine complex, vitamin E, thiamine, vitamin A, vitamin K, vitamin B6, potassium iodate, folic acid, manganese sulfate, riboflavin, sodium selenate, vitamin D, biotin, vitamin B12.
D-org*	0-6	Yes	Skimmed milk[b], partially demineralized whey powder[b], vegetable oils[b] (organic palm oil, canola oil, sunflower seed oil) lactose[b], GOS from lactose, whey protein, potassium citrate, calcium chloride, long chain polyunsaturated fatty acids oil mixture (fish oil, vegetable oil from M. Alpina), l-phenylalanine, sodium citrate, calcium carbonate, magnesium sulfate, calcium orthophosphate, vitamin C, l-triptophane, ferrous sulfate, lactic ferments (*Lactobacillus fermentum hereditum*), zinc sulfate, copper sulfate, vitamin B1, vitamin B6, potassium iodate, vitamin E, magnese, sulfate, folic acid, vitamin K, sodium selenate, vitamin D, vitamin B2, vitamin B12.
E	6-12	No	Demineralized water, skimmed milk, demineralized whey powder, vegetable oils (palm, canola, sunflower), maltodextrin, GOS syrup (from milk), lactose, glucose syrup, emulsifier: mono- and diglycerides of fatty acids, calcium citrate, calcium salts of orthophosphoric acid, calcium carbonate, potassium citrate, vitamin C, potassium chloride, magnesium chloride, L-tyrosine, sodium chloride, potassium carbonate, sodium carbonate, ferric diphosphate, taurine, L-tryptophan, zinc sulfate, niacin, pantothenic acid, vitamin E, copper sulfate, vitamin A, vitamin B6, thiamine, riboflavin, potassium iodate, manganese sulfate, folic acid, vitamin K1, sodium selenate, biotin, vitamin D3, vitamin B12.

Table 1. Information about the formula milk composition as labelled at milk package (continues from the previous page).

Milk code	Age infant population target (month)	Organic	Ingredients [a]
F-org	0-6	Yes	Milk skim[b], lactose[b], vegetable oils[b] (sunflower oil[b], rapeseed oil[b]), milk protein[b] powder, maltodextrin[b], minerals (calcium, potassium, iron, zinc, copper, sodium, magnesium, manganese, iodine, selenium), emulsifier lecithin (soy), fish oil, L-phenylalanine, oil extracted from Mortierella alpina, vitamins (C, E, pantothenic acid, niacin, thiamine, A, B6, folic acid, K, biotin, D, B12), inositol, *Lactobacillus reuteri*, choline, L-histidine.
F	0-6	No	Water, skimmed milk, lactose, vegetable oil, partially demineralized and fractionated whey powder, minerals (calcium, potassium, sodium, magnesium, iron, zinc, copper, manganese, iodine, selenium), emulsifier lecithin (soy), fish oil, L-phenylalanine, vitamins (C, E, PP, pantothenic acid, A, B1, B6, B2, folic acid, K, biotin, D), taurine, inositol, L-histidine, nucleotides, L-carnitine.
F	6-12	No	Water, skimmed milk, maltodextrin, vegetable oils (palm olein, canola oil, coconut oil, sunflower oil), lactose, partially demineralized and fractionated milk serum powder, minerals (calcium, potassium, sodium, magnesium, iron, zinc, copper, iodine, manganese, selenium), emulsifier lecithin (soy), vitamins (C, niacin, E, pantothenic acid, A, thiamine, B6, folic acid, K, biotin, D).
G	0-6	No	Water, whole milk, demineralized whey powder, vegetable oils (low erucic rapeseed, coconut oil, sunflower oil), maltodextrin, milk lactose, mineral salts (calcium phosphate, calcium carbonate, potassium chloride, trisodium citrate, chloride sodium, magnesium carbonate, iron lactate, potassium iodide, zinc sulfate, sodium selenite, copper sulfate, manganese sulfate), soy lecithin emulsifier, choline bitartrate, vitamins (C, E, pantothenic acid, niacin, thiamine, riboflavin, A, B6, folic acid, K, biotin, D, B12), inositol, taurine.
G	6-12	No	Water, semi-skimmed milk, milk lactose, vegetable oils (low erucic rapeseed, coconut oil, sunflower oil), maltodextrin, demineralized whey powder, mineral salts (potassium chloride, magnesium carbonate, phosphate calcium, sodium citrate, calcium carbonate, iron lactate, sodium selenite, copper sulfate, manganese sulfate), soy lecithin emulsifier, vitamins (C, E, pantothenic acid, PP, K, folic acid, biotin, D, B12), inositol.
H	0-6	No	Water, whole milk (21%), demineralized whey powder, vegetable oils (rapeseed low in erucic acid, coconut, sunflower), maltodextrin, milk lactose, milk GOS, skimmed milk powder fermented with *Lactobacillus paracasei CBA L74* (0.3%), whey protein rich in alpha-lactalbumin, mineral salts, emulsifier: soy lecithin, vitamins, inositol, L-cysteine, choline bitartrate, taurine, nucleotides (cytidine-5'-monophosphate, adenosine-5'-monophosphate, uridine-5'-monophosphate, inosine-5'-monophosphate, guanosine-5'-monophosphate).
H	6-12	No	Water, whole milk (17%), demineralized whey powder, maltodextrin, vegetable oils (rapeseed low in erucic acid, coconut, sunflower), dairy lactose, milk GOS, skimmed milk powdered with *Lactobacillus paracasei CBA L74* (0.7%), mineral salts, whey protein rich in alpha-lactalbumin, emulsifier: soy lecithin, vitamins, inositol, nucleotides (cytidine 5'-monophosphate, adenosine 5'-monophosphate, uridine 5'-monophosphate, inosine 5'-monophosphate, guanosine 5'-monophosphate).
I-org	0-6	Yes	Skimmed milk[b], partially demineralized whey powder[b], vegetable oils[b] (palm oil[b], rapeseed oil[b], sunflower oil[b]), maltodextrin[a], skimmed milk powder[a], calcium carbonate, potassium chloride, Vitamin C, sodium chloride, Vitamin E, *Bifidobacteria* (*B. breve*, *B. bifidum*, *B. infantis*, *B. longum*), iron lactate, zinc sulphate, niacin, calcium pantothenate, copper sulphate, vitamin A, vitamin B1, vitamin B6, manganese sulfate, potassium iodate, folic acid, vitamin K1, sodium selenate, vitamin D.

[a] As stated on the label by the manufacturing companies; [b] ingredients derived from organic farming; [c] ingredients derived from biodynamic farming.
GOS: galacto-oligosaccharides; FOS: fructo-oligosaccharides.

metabolome. The PCA scores and loadings plots built with the first three principal components are depicted in **Fig. 3**. As it can be noted, a clear separation of HM (left) from commercial milk (right) samples is visible along the PC1 axis. The loadings analysis for PC1 (**Fig. 3B**) showed that the metabolites mainly responsible for this distribution were human milk oligosaccharides (HMOs), glycerophosphocholine (GPC), glutamate, glutamine and alanine more abundant in

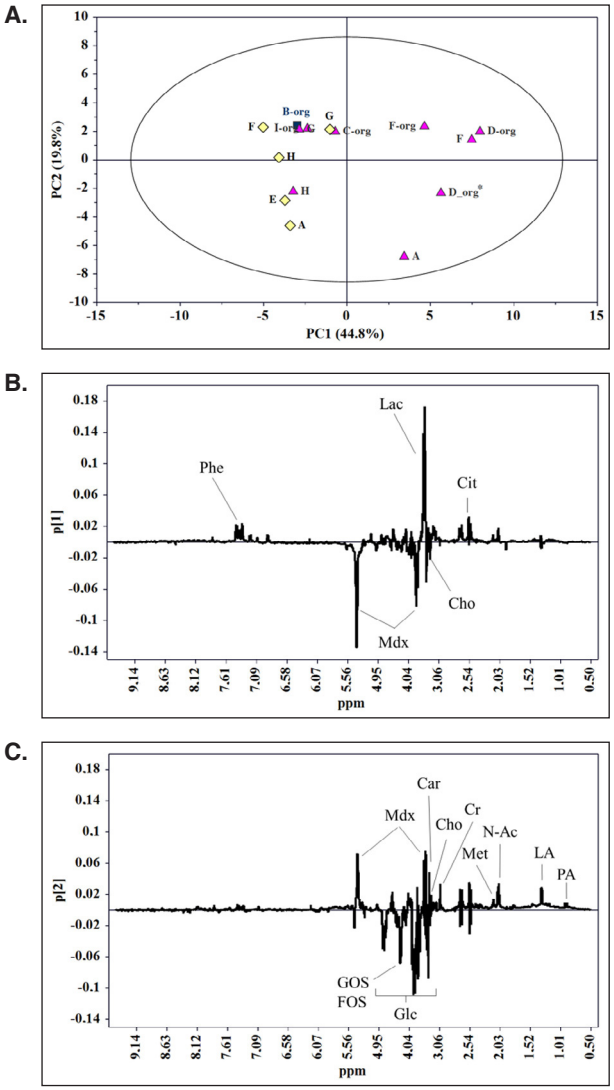

Figure 1. PCA of 15 formula milk samples: scores plot (**A**); PC1 (**B**) and PC2 (**C**) loadings plots.
■ Formula milk 0-12 months; ▲ First infant milk; ◆ Follow on milk. The suffix "org" denotes organic formula.
Metabolite abbreviations: Car: carnitine, Cho: choline, Cit: citrate, Cr: creatinine, FOS: fructo-oligosaccharides, GOS: galacto-oligosaccharides, Glc: glucose, LA: lactic acid, Lac: lactose, Mdx: maltodextrins, Met: methionine, N-Ac: *N*-acetylcarbohydrates, PA: pantothenic acid, Phe: phenylalanine.

HM, and maltodextrins, carnitine, choline, creatinine, citrate and lactate present at higher levels in FM. Moreover, the loadings analysis for PC2 (**Fig. 3C**) highlighted some minor inter- and intra-group variations. In particular, differences in the HMOs composition among HM samples drove the scores distribution along PC2, reflecting the secretor (Se^+) and non-secretor (Se^-) nature of mother milk phenotypes. Within the FM group, phenylalanine, citrate and *N*-acetylcarbohydrates were more abundant in samples located along the positive PC2 axis, while maltodextrins and lactate were higher in samples placed on the negative

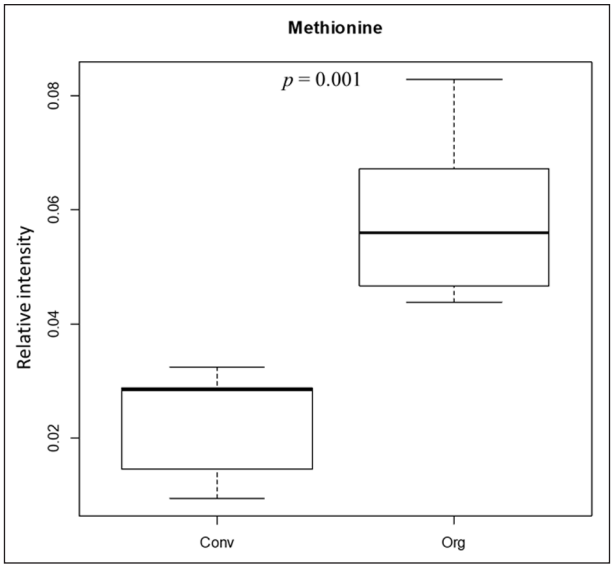

Figure 2. Box plot showing the differences in methionine relative intensity between organic (Org) and conventionally produced (Conv) formula milk.

side. Finally, a high level of lactose characterized HM and FM samples located on the upper side of the scores plot.

Discussion and conclusions

HM is considered the most suitable food in the early stages of life because it guarantees the infant not only the achievement of his nutritional needs but also the contribution of bioactive functional substances, such as the oligosaccharides that positively influence development and long-term health. In cases where breastfeeding is not possible, cow milk-based FM represents a good substitute although its composition does not completely mimic those of HM.

In the present study, a ^{1}H NMR-based metabolomics approach was used to compare the metabolome of 15 commercial FM of which 6 prepared with organic ingredients. In addition, the metabolome of these samples was compared with that of HM collected at 7 ± 3 days post-partum from mothers who gave birth at term. Lactose, the main energy supply in milk, is the primary carbohydrate in HM and FM for infants. It was the major component in the metabolome of all analyzed samples. No significant differences in lactose content were observed between FM and HM. Differently, intra-sample lactose variations were observed within the FM group, the levels being more abundant, in particular, in 5 first infant formula (coded A, F and D). Other important

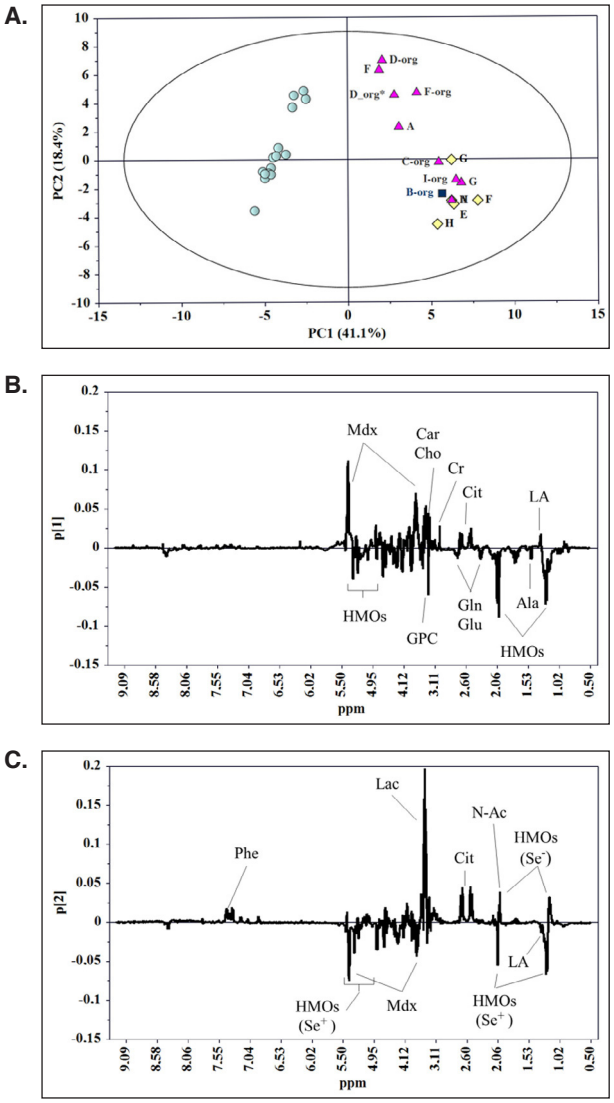

Figure 3. PCA of 15 formula milk and 15 human milk samples: scores plot (**A**); PC1 (**B**) and PC2 (**C**) loadings plots.
● Human milk; ■ Formula milk 0-12 months; ▲ First infant milk; ◆ Follow on milk.
The suffix "org" denotes organic formula.
Metabolite abbreviations: Ala: alanine, Car: carnitine, Cho: choline, Cit: citrate, Cr: creatinine, Gln: glutamine, Glu: glutamate, GPC: glycerophosphocholine, LA: lactic acid, Lac: lactose, Mdx: maltodextrins, N-Ac: *N*-Acetylcarbohydrates, HMOs: human milk oligosaccharides, Se^+: secretors phenotypes; Se^-: non-secretors phenotypes

differences among FM samples were observed for oligosaccharides and maltodextrins. The presence and the level of both types of metabolites in FM is linked to their preparation. In particular, the former are added in the prebiotic concept, while the supplementation with the latter is done as a source of digestible carbohydrates. In agreement with the nutritional information as labelled at milk package, only the NMR spectra of first formulas coded A, H and D-org*, and follow on formulas coded A, E and H exhibited signals ascribed to GOS and FOS, all these formulations being enriched with these compounds. Concerning maltodextrins, their levels were fairly variable among FM and, not surprising, discriminating against HM since many baby food companies incorporate maltodextrins in the ingredients of infant formula products in order to enhance the nutritive value.

HMOs represent a peculiar molecular component of breast milk, the third most abundant after lactose and lipids, playing an important role in the protective health effects of breast milk [27]. The secretion of HMOs in mother's milk is a complex, variable and dynamic process. Their composition varies among women and the quantity decreases during the lactation from colostrum to mature milk. As highlighted by the multivariate statistical analysis of the present study, the presence of these molecules in the HM samples under investigation provided not only the main contribution to the clustering of breast milk, but diversified also the same HM specimens based on the secretory phenotype.

It is worth noting the different form of choline present in FM and HM groups: GPC was more abundant in breast milk, while free choline in the FM. GPC (a lipid containing a molecule of glycerol bound to a phosphocholine) represents the major source of choline since, being one of the few phospholipids present in the aqueous phase, it can circulate in the blood, reaching easily all the tissues including the central nervous system and can be used for the synthesis of new phosphatidylcholine. GPC is also able to improve the performance of the cellular membranes, favoring the binding of the membrane phospholipids with the DHA and giving them that fluidity that makes the membranes more metabolically active. Briefly, even if it is not an antioxidant molecule, it protects all cells in an incomparable way [28]. Choline is the precursor of the acetylcholine as well and it is part of the membrane phospholipids. It is instable in the aqueous phase, thus its quantity is low in plasma and two of its derivatives, phosphocholine and GPC represent its reservoir to be used when necessary [28]. In breastmilk of mothers with preeclampsia there is a scarcity of phosphocholine and GPC. They originate from phosphatidylcholine by the action of phospholipase A2 (PLA2), an enzyme that has an important role in the phlogistic processes and in the oxidative stress. While in the controls the concentration of phosphocholine and GPC increases from day 3 to 6 months, only the GPC increases in preeclampsia, that however

it was lower compared to controls. It can be hypothesized that in the breast of mothers with preeclampsia there is probably a lack of PLA2 enzyme, maybe due to its massive use in the inflammatory processes and in the oxidative stress correlated to the preeclampsia [29-30].

Additional differences were observed between FM and HM: (i) higher levels of some amino acids such as alanine, glutamate and glutamine in HM than FM, in good agreement with the literature [31]; (ii) the presence of phenylalanine in FM coded F-org, F, D-org, D-org*, as also confirmed by nutritional information as labelled at milk package; (iii) high level of lactate in the follow on milk coded H, reasonably ascribable to its fermentation with *Lactobacillus paracasei* (0.7%).

As for the comparison between organic and commercially produced milks, the only metabolite significantly differing in level was methionine, more abundant in organic milk, whose presence was not directly related to the ingredients used for the preparation of formula.

The study presents several limitations: the samples pool is very small and heterogeneous, and the lack of mature breast milk samples does not allow further comparison with FM samples. Thus, the meaning of these findings needs to be further investigated. Nevertheless, we think that these preliminary data can contribute to constitute a starting point potentially useful for the optimization of infant products.

Declaration of interest

The Authors declare that there is no conflict of interest.

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