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Metabolite profiles of formula milk compared to breast milk

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ABSTRACT

Breast milk (BM) feeding is the gold standard in neonate nutrition. When BM is not available it can be substituted or integrated with commercial formula milk (FM) usually sold under different brands and formulations. In this work, the low-molecular-weight hydrophilic compounds in milk were studied by gas chromatography electronic impact mass spectrometry (GC–MS), comparing eight different FM brands with BM samples. With the aid of multivariate statistical data analysis, a marked variability among FM brands, especially driven by the presence of prebiotics in their formulation, was highlighted. Quali-quantitative differences were found between FM and BM. Orotic acid and isomaltulose were found exclusively in FM, while phenylalanine and tyrosine levels were high in two FM brands. Moreover, higher levels of malic acid, sugars (glucose, fructose and galactose), and mannitol were detected in FM. On the other hand, BM showed a higher amino acid content. In conclusion, GC–MS proved to be a very sensitive analytical technique for the study of FM, highlighting metabolite differences among FM brands, and between FM and BM, that may have a possible strong impact on neonatal nutrition.

1. Introduction

Human milk offers the best physiological nourishment to the neonate, with its composition used to estimate the nutritional requirements of infants and to guide the composition of infant formulae. The latter are specialized products designed for dietary management of infants in the first 12 months of life (Guo, 2014). Infant formulae are available on the market as powder, liquid concentrate, and ready to feed formulations. Powders and liquid concentrate formulations should be mixed with water before feeding. Moreover, FM are prepared on a cow milk base with added vegetable oils, vitamins, minerals, and iron. Other infant formulae are soy-based or specialized formulae. Generally, FM are prepared using a combination of proteins, lipids, carbohydrates, minerals, and vitamin components including novel ingredients offering nutritional benefits. Other components, such as probiotics, prebiotics, sugars and non-fermentable sugars, inositol, carnitine, taurine, nucleotides, and amino acids, can be added to mimic human milk composition and/or for functional and technological properties. Since the latter are not necessarily the same as health benefits (Hernell, 2011), new compounds have to be rigorously evaluated and their suitability proven for infant feeding.

In the United States, formula milk is the most highly regulated commercially available food controlled by the Code of Federal Regulations (Code of Federal Regulations, 2000a, 2000b). In Europe, infant formulae and follow-on formulae are specifically covered by Commission Directive (Commission of the European Communities, 2006). This Directive lays down the requirements for the composition and labeling of these products, and the annexes of the Directive give criteria for the composition. The WHO International Code of Marketing Breast Milk Substitutes was designed to control advertising on breast milk substitutes (Brady, 2012).

As many outcomes in formula-fed infants do not match those in breast-fed populations, opportunities for innovations continue to exist and further researches on breast-milk and infant formulations are needed. In our previous work, analyzing the urine metabolite profiles of neonates, we found that BM and FM nutrition regimens differently affected the neonatal metabolism during the first week of life (Dessì et al., 2016).

Many researches have studied the most abundant components, while little is known about the metabolite content of FM (Marincola et al., 2012; Longini et al., 2014). Sophisticated analytical procedures are required to investigate different milk metabolites. Among analytical hyphenated platforms, GC–MS followed by multivariate statistical data analysis (MVA) has shown a good capability for the identification and estimation of milk polar and hydrophilic low-molecular-weight metabolites (Marincola, Dessì, Corbu, Reali, & Fanos, 2015; Scano, Murgia, Pirisi, & Caboni, 2014; Klein et al., 2010; Klein et al., 2013). The aim of

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this work was to apply this combined approach to the study of the metabolite content of FM samples from different brands and to compare it with that of BM.

2. Materials and methods

2.1. Samples

Thirty-one FM samples, from 8 different brands commonly sold in Italy, were acquired from local markets. Twenty-five were ready-to-feed UHT liquid infant formulae, sold in 500 mL sterile cartons, and 6 samples were sold as powder. Seventeen were first infant formula (designed for neonates 0–6 months of age) and 14 of follow-on formula (age > 6 months). All FM samples were based on skimmed cow's milk with added vegetable oils (Table 1). Thirty-one samples of BM were donated by healthy Italian lactating women (age 30–42 years) who had delivered full-term healthy neonates. Samples were collected between the 3rd to the 26th week after parturition. From each participant informed written consent was obtained, and the protocol was approved by the local ethical committee (CA-206-18/03/2013). Ten mL of BM were stored into Falcon tubes at −80 °C until analysis.

2.2. Chemicals and reagents

Methanol, chloroform, hexane, pyridine, methoxamine hydrochloride, potassium chloride, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), and all analytical standards with purity >95% (lactic acid, succinic acid, glyceric acid, itaconic acid, malic acid, 3-phosphoglyceric acid, citric acid, alanine, valine, isoleucine, glycine, serine, threonine, aspartic acid, pyroglutamic acid, glutamic acid, phenylalanine, tyrosine, ribose, arabitinol, fucose, ribonic acid, altrose, fructose, galactose, glucose, talose, isomaltulose, mannitol, sorbitol, *scyllo*-inositol, *myo*-inositol, palmitic acid, oleic acid, stearic acid, urea, phosphate, uracil, and creatinine) were purchased from Sigma Aldrich (Milano, Italy). Bidistilled water was obtained from a MilliQ purification system (Millipore, Milano, Italy).

2.3. Extraction and derivatization

Powdered formula samples were diluted according to manufacturer's specifications. Milk samples (10 mL) were then sonicated for 15 min in a Falcon tube, with 1 mL of the suspension then transferred into a Falcon tube and 2.5 mL of methanol and 1.2 mL of chloroform added. After 1 h, 3.8 mL of chloroform and 0.9 mL of aqueous potassium chloride (0.2 M) were added. One mL of the suspension was then centrifuged at 12,000 rpm for 10 min in an Eppendorf tube. The aqueous layer (0.5 mL) was then transferred into a glass vial and dried under a gentle nitrogen stream and derivatizated with 50 μL of pyridine containing methoxamine hydrochloride at 10 mg/mL. After 17 h, 0.1 mL of MSTFA was added at room temperature and after 1 h samples were re-suspended with 0.8 mL of hexane containing 2,2,3,3-d4-succinic acid as internal standard (2 mg/L).

2.4. GC–MS analysis

Derivatized samples (1 μL) were injected splitless into a 6850 gas chromatograph coupled with a 5973 Network mass spectrometer (Agilent Technologies, Santa Clara, CA). The injector and ion source were maintained at 250 °C and 230 °C, respectively. Helium flow rate through the column was 1 mL/min. The fused silica capillary column was a 0.25 μm DB5-MS, 30 m × 0.25 mm ID (J&W scientific, Folsom, CA). The initial temperature program was as follows: 10 min of isothermal heating at 50 °C then increased to 300 °C at 10 °C/min and held at 300 °C for 4 min. Ions were generated at 70 eV with electron ionization and were recorded at 1.6 scan/s over the mass range *m/z* 50–550. Identification of metabolites was performed by co-chromatography with analytical standards and by comparison of their mass spectra with the NIST08 library of the National Institute of Standards and Technology (Gaithersburg, MD), and a library developed at the Max Planck Institute of Golm (http://gmd.mpimp-golm.mpg.de/). GC–MS spectra deconvolution was performed by the AMDIS tool in the NIST08 library. Retention indices were calculated according to Kovats, for the series of C9-C24 alkanes.

For each metabolite, GC–MS peak areas relative to the most abundant mass fragment were calculated using the MSD Chemstation (Agilent Technologies, Santa Clara, CA) and the resulting data were submitted to MVA. Absolute quantification of 11 compounds was performed with the external method using pure standards (Nollet & Toldrá, 2009). Calibration graphs were calculated by plotting peak area versus concentration (μg/mL). For all calibration curves, a good linearity was achieved between the linear range 0.1 and 100 μg/mL with correlation coefficient >0.9990, CV < 6.7.

Table 1
Table of macronutrients and some micronutrients[a], as reported in the ingredients list, for FM brands.

Brand (n of samples[b])	Protein source	Fat source	Carbohydrate source	Prebiotics[c]	Free aminoacids[d]	Inositol[e]
First infant formula						
A (n = 2r)	Skimmed milk, whey protein	Vegetable oil	Lactose	GOS, FOS		y
B (n = 2r)	Skimmed milk, whey protein	Vegetable oil	Lactose	GOS	Phe, His, Trp	y
C (n = 1r + 1p)	Skimmed milk, whey protein	Vegetable oil, fish oil	Lactose	GOS, FOS		y
D (n = 2r + 1p)	Skimmed milk, milk whey	Vegetable oil, fish oil	Lactose		Phe, His	y
E (n = 2r)	Skimmed milk, milk whey	Vegetable oil	Lactose, maltodextrin			y
F (n = 2r)	Skimmed milk, whey protein	Vegetable oil	Lactose, maltodextrin	GOS		y
G (n = 2p)	Skimmed milk, milk whey	Vegetable oil	Maltodextrin			
H (n = 2p)	Skimmed milk, milk whey	Vegetable oil	Maltodextrin			
Follow-on						
A (n = 2r)	Skimmed milk, whey protein	Vegetable oil, fish oil	Lactose, maltodextrin	GOS, FOS	Trp	
B (n = 3r)	Skimmed milk, whey protein	Vegetable oil	Lactose, glucose syrup, maltodextrin	GOS	Tyr, Trp	y
C (n = 3r)	Skimmed milk	Vegetable oil	Lactose	GOS, FOS	Trp	y
D (n = 2r)	Skimmed milk, milk whey	Vegetable oil	Lactose, maltodextrin			
E (n = 2r)	Skimmed milk, milk whey	Vegetable oil	Lactose, maltodextrin			y
F (n = 2r)	Skimmed milk	Vegetable oil	Lactose, maltodextrin	GOS		y

[a] Other compounds, such as nucleotides, vitamins, minerals etc., were reported in the ingredients list; brand H reported *Bifidobacteria*.
[b] r = ready-to-feed; p = powder.
[c] Galactooligosaccharides (GOS) and fructooligosaccharides (FOS).
[d] Standard nomenclature IUPAC-IUB (1984).
[e] y = inositol was in the ingredients list.