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Characterization of donkey milk and metabolite profile comparison with human milk and formula milk

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ABSTRACT

Donkey milk is considered a potential substitute to human milk for infants affected by cows' milk protein allergy. With the aim to widen our knowledge on this valuable food, we explored the compositional characteristics of Sardinian donkey milk. Donkey milk showed a low lipid content and high lysozyme levels compared to human milk, and a bacterial count below the recommended threshold. Hydrophilic compounds such as amino acids, organic acids and mono and disaccharides, were analyzed by GC-MS for donkey milk, formula milk and human milk. Results of the multivariate statistical analysis indicated that the metabolite profile of donkey milk is more similar to human milk than cow milk based formulae, the latter being richer in sugars and lower in amino acids. Moreover, modifications of human milk and donkey milk metabolite profiles during lactation time were studied. An increase of protein levels was observed in donkey milk, while in human milk pyroglutamic acid and *myo*-inositol levels increased and decreased, respectively.

1. Introduction

Human milk (HM) is recognized as the gold standard for infant nutrition conferring unique nutritional and non-nutritional benefits to the infant and the mother and, in turn, optimizing infant, child, and adult health as well as child growth and development (Eidelman et al., 2012; Oftedal, 2012; World Health Organization, 2009). HM was found to contribute to the development, and functions of the early immune system through a variety of mechanisms, including direct or indirect antimicrobial activity, modulating immune function, anti-inflammatory effects, and enhancing growth and development of the infant's tissues (Castellote et al., 2011). When breastfeeding is not possible, nutritionists and dietitians need to find alternatives. Commercial infant formula milk (FM), based on cows' milk, is the accepted replacement milk, designed to closely resemble HM to meet the nutritional needs of infants and young children (World Health Organization, 2009). However, IgE and non-IgE mediated cows' milk protein allergy (CMPA) and allergy to soy and enzymatically hydrolysated formulas, make the infant nutrition challenge more difficult. Potential alternatives to HM are milks from other animal species such as cows, goats, horses and donkeys. Horses' and donkey milk (DM) showed chemical composition and organoleptic characteristic similar to human milk (Guo et al., 2007; Salimei Fantuz, 2012, 2004; Vincenzetti et al., 2008). These species have a monogastric digestive system and their milk contains a lower bacterial load than cows' milk (Salimei & Fantuz, 2012). The compositional characteristics and the levels of bioactive and functional compounds in DM have been reviewed by Salimei et al. (2012). From an immunological point of view, DM was found to induce the release of inflammatory and anti-inflammatory cytokines from normal human peripheral blood lymphomononuclear cells maintaining a condition of immune homeostasis (Jirillo & Magrone, 2014). DM was also found to be able to normalize the human intestinal microbiota with a cascade of protective effects at intestinal mucosa sites triggering T regulatory cells (Jirillo & Magrone, 2014). Monti et al. 2007 studied the tolerance of DM in children with CMPA indicating DM as a valid

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alternative to cows' milk derivatives. Moreover, being rich of lactose and low in lipid content, DM was found to help the intestinal absorption of calcium (Polidori & Vincenzetti, 2013).

Foodomics approaches, i.e. the application of multivariate statistical analysis (MVA) to food analytical data, can been used to explore relationships between samples and their metabolite profiles, and to identify biomarkers for food quality amelioration (Marincola et al., 2012; Pisano, Scano, Murgia, Cosentino, & Caboni, 2016; Scano, Murgia, Pirisi, & Caboni, 2014). Among various analytical techniques, such as NMR and LC-MS, gas chromatography coupled to mass spectrometry (GC-MS) has been successfully used in the identification and quantitation of milk polar and hydrophilic metabolites (Scano et al., 2014, 2016).

Aims of this work were to study the compositional characteristics and the polar metabolite profile of DM through an untargeted metabolomics approach and to highlight metabolite differences between DM, HM and FM.

2. Materials and methods

2.1. Chemicals and reagents

Methanol, chloroform, hexane, pyridine, methoxamine hydrochloride, potassium chloride, N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), and all analytical standards were purchased from Sigma Aldrich (Milan, Italy). Bidistilled water was obtained from a MilliQ purification system (Millipore, Milan, Italy).

2.2. Samples

Forty-four samples of raw Sardinian Donkey milk were kindly provided by six different farmers located in Sardinia (Italy). Samples were collected on sterile tubes through manual milking, between the 23rd and the 34th week of lactation. Thirty-three samples of HM were obtained from healthy volunteers (age 30–42 years), between the 3rd and the 24th week of lactation. The morning milk samples were obtained by completely emptying one breast. Ten mL of milk were stored into Falcon tubes at −20 °C and analyzed within two days. Informed written consent was obtained from each participant, and the local ethical committee approved the protocol. Twenty-five ready-made UHT liquid infant formula milk samples, from different brands, were acquired from commercial sources: 11 samples of first infant formula (0–6 months) and 14 of follow-on formula (>6 months).

2.3. Extraction and GC-MS analysis

To obtain rupture of matrix micelles, 10 mL of milk were placed into a Falcon tube and sonicated for 15 min. One mL of milk was transferred in a Falcon tube adding 2.5 mL of methanol and 1.2 mL of chloroform. After 1 h, 3.8 mL of chloroform and 0.9 mL of aqueous potassium chloride 0.2 mol/L were added. Thermolabile hydrophilic compounds, such as mono and disaccharides, due to their limited volatility, were detected after methoximation of aldehyde or keto groups and silylation of polar hydroxy, thiol, carboxy, amide and amino groups. One mL of the suspension was centrifuged at 26 242 × g for 10 min in an Eppendorf tube. Exactly 0.5 mL of the aqueous layer were then transferred into a glass vial and dried using 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 were added and after 1 h the samples were resuspended with 0.8 mL of hexane.

One μL of derivatized sample was injected splitless into a 6850 gas chromatograph coupled with a 5973 Network mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The injector temperature was set at 200 °C and the gas 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, USA). 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/sec over the mass range *m/z* 50–550. GC-MS data analysis was conducted by integrating each resolved chromatogram peak. Identification of metabolites was performed using the standard NIST08 mass spectra library, a library developed at the Max Planck Institute of Golm, and by comparison with authentic standards when available. Quantitative analysis of metabolites was carried out with the external standard method using authentic standards. Calibration graphs were calculated plotting peak area versus concentration (μg/mL). A good linearity was achieved between the linear range 0.1 and 100 μg/mL with correlation coefficient >0.9990.

2.4. Statistical data analysis

For quantitative analysis, ANOVA and Tukey *post-hoc* mean comparison test were performed with OriginLab software.

For MVA, for each sample, the GC-MS peak intensities of metabolites (variables) were normalized to a total sum of 100. Each variable was mean centered and unit variance scaled over all samples. MVA refers to all statistical techniques that simultaneously analyze multiple measurements on samples under investigation. In this work, different tools were used:

a) the unsupervised Principal Component Analysis (PCA) for data set overview, where results are showed as score and loading scatter plots in two dimensions;
b) the unsupervised Hierarchical Cluster Analysis (HCA), to group samples on the basis of metabolite profiles similarities. It was performed using the Ward method on the standardized original variables and the results plotted as tree plot, where the vertical axis indicates the distance level, in arbitrary units (a.u.);
c) the supervised Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) to find discriminant metabolites. Results are shown as scatter score and column loading plots. The results of two OPLS-DA models were also compared and reported as SUS-plot (Shared and Unique Structures–plot): a 2D scatter plot of the loading correlation vectors of the predictive components of two separate models (Wiklund et al., 2008);
d) the supervised OPLS regression to study linear relationship between time and metabolite profiles, results are shown as observed *vs.* predicted score scatter plot and column loading plot.

The quality of the models and the optimum number of principal components (PC) were evaluated on the basis of the cumulative parameters R^2X and R^2Y and their analogues in cross validation Q^2X and Q^2Y (Eriksson et al., 2013). All MVA were performed with SIMCA-P+ program (Version 14.1, Umetrics, Sweden).

2.5. Composition and lysozyme activity assay

For DM, fat, protein and lactose content, expressed as g/100 g, were obtained with Milkoscan FT6000 (Foss, Denmark) following the procedures ISO 9622 IDF 141:2013 Guidance on the application of mid-infrared spectrometry. Somatic cells were counted using the procedure ISO 13366-2 IDF 148-2 2006 - Enumeration of somatic Cells, Part II, Guidance on the operation of fluoro-opto-electronic