A MALDI-TOF-MS Approach for Mammalian, Human, and Formula Milk Profiling*

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5.1 INTRODUCTION

Breast milk is the primary food source for newborn mammals, and the World Health Organization recommends that infants should be exclusively breastfed for the first six months of life [1,2]. Breast milk synthesis is subtly regulated at the local level [3], and its composition is influenced by several factors such as animal species and genetics, environmental conditions, and animal nutritional status [4]. Human milk (HM) composition varies with gestational age, lactation stage (transition from colostrum to late lactation),

* Originally published in *Nutrients* 2018, 10, 1238.

within feeds, diurnally, and among mothers [5,6]. HM provides proteins, tricalcium phosphate, lipids, vitamins, salt, and lactose; it also contains many hundreds to thousands of distinct bioactive molecules which protect against infection and inflammation and contribute to immune maturation, organ development, and healthy microbial colonization [7].

Despite many campaigns promoting breastfeeding, only 38% of infants around the world are exclusively breastfed [8,9]. When HM becomes unsuitable or inadequate, its ideal substitutions are infant formulae, defined as “a breast milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding” [10]. Unlike the dynamic composition of HM, infant formulae are standard products with a composition that is highly regulated by the authorities. To date, the most commonly recommended infant formulae are based on cow’s milk [11]. The Food and Drugs Administration (FDA) in the US and the European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) in Europe recommend that the formulae should be enriched in whey protein fractions and lowered in caseins [9,12]. The worldwide recommendations are primarily based on the chemical analysis of human milk, and manufacturers are continually modifying their products to make them more similar to human breast milk, the gold standard to estimate the needs of an infant [13], and to increase their health benefits, including iron, nucleotides, prebiotics, and compositions of fat blends [14]. In this context, the number of studies on HM and its protein composition has dramatically increased during the last half-century [15–19]. For children with a cow’s milk allergy (CMA) whose mother cannot breastfeed, milk from different mammals has been evaluated, but no milk formulae from animals other than cows have been formulated, and cross-reactivity is possible between the proteins of cows and other mammalian milk [20]. Thus, other animal milk-based formulae are currently not recommended [21].

The recommendations on substitute formulae in cases of lactation failure are based on many factors, but nutritional considerations are one of the most prominent factors. Among the nutritional factors, proteins are the most important. This study aims to investigate the potentiality of linear matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF-MS) as a tool to assess the diversity and oddness of different artificial and mammalian kinds of milk compared to the reference human milk. MALDI-TOF-MS is a platform adopted by many healthcare clinical laboratories worldwide owing to its simplicity of use, high reproducibility of the mass spectra, and low cost of analysis [22]. Recently, this technique has been proposed as a powerful tool to obtain informative fingerprints of milk proteins [23]. The identification of differences and similarities among several types of artificial and animal milk compared to the reference human milk at different stages of lactation could assist the design of infant formulae. Furthermore, a MALDI-TOF-MS-based approach, coupled with a multivariate statistical assessment of MS data, could represent a versatile workflow to evaluate the quality and safety of sample milk in blind for nonspecialized, mass-spectrometric laboratories.

5.2 MATERIALS AND METHODS

5.2.1 Milk Sampling and Pre-Treatment

Human milk samples were collected from twenty healthy breastfeeding mothers at the Department of Obstetrics and Gynecology, San Camillo-Forlanini Hospital of Rome,

Italy, at four different periods of lactation: 2 days (colostrum, HC), 30 days (HM30), 60 days (HM60), and 90 days (HM90). The study protocol was approved by the ethics committee of the San Camillo-Forlanini Hospital (protocol 460/CE; 27/03/2012) and by the institutional review board of the Bambino Gesù Children's Hospital (protocol 295 LB; 16/05/2012). Informed written consent was obtained from all mothers. Raw donkey milk (DM) from four she-donkeys belonging to the Amiata, Viterbese, and Martina Franca breeds; cow's milk (CM) from four cows belonging to the Frisona breed; buffalo's milk (BM) from seven buffalos belonging to the Mediterranean Italian breed; goat's milk (GM) from four goats belonging to the Maltese breed; and ewe's milk (EM) from five ewes belonging to the Tuscolania breed were collected from Italian farms (Lazio and Puglia). Camel's milk (CAM) from two *Camelus dromedarius* was collected from Libyan desert farms. Commercially available infant formula milk samples from four different brands were also studied: Aptamil 1 (A) (Mellin SpA, Milan, Italy), Humana 1 (H) (Humana Italia SpA), Formula 1 (F) (Dicofarm, S.A., Rome, Italy), and Nidina 1 (N) (Nestlé, S.A., Milan, Italy). For each brand, four samples were obtained from different batches that were produced over a period of two years. All the animal milk samples were mechanically milked during the middle lactation stage into sterile polystyrene containers, immediately frozen, and stored at -80°C until use to prevent undesired proteolysis. After thawing, raw milk samples were defatted by a two-step centrifugation using the Eppendorf Centrifuge 5417 R (The LabWorld, Woburn, MA). The first centrifugation was performed at $3000\times g$ for 10 min at 4°C . The skimmed milk was then centrifuged at $20,000\times g$ for 20 min at 4°C to remove bacteria and cell debris. The skimmed milk's fractions were subsequently diluted 1:100 with ultrapure water (Milli-Q Millipore) and subjected to mass spectrometry analysis [23].

5.2.2 MALDI-TOF Spectra Acquisition

An aliquot (1 μL) of each skimmed milk's fraction was directly spotted onto an MSP 96 polished steel target (Bruker Daltonics, Bremen, Germany), overlaid with 1 μL of matrix, represented by a solution of 10 mg/mL of sinapinic acid (Sigma-Aldrich, St. Louis, MO) in 50% acetonitrile (Sigma-Aldrich), containing 0.1% trifluoroacetic acid (v/v) (Sigma-Aldrich), and allowed to dry at room temperature. MALDI-TOF analysis was performed with a Microflex LT linear mass spectrometer (Bruker Daltonics) equipped with the FlexControl software package, version 3.0 (Bruker Daltonics), for spectra recording in the positive linear mode (laser frequency 20 Hz; ion source 1 voltage, 20 kV; ion source 2 voltage, 18.4 kV; lens voltage, 9.1 kV; mass range, 2000–30,000 Da). Four independent spectra (500 shots one step from different positions of the target spot, for spectrum) for each skimmed milk's fraction were manually collected, externally calibrated using Bacterial Test Standard (Bruker Daltonics), and subsequently analyzed.

5.2.3 Statistical Analysis

Before the statistical analysis was conducted, 200 mass spectra, as reported in Table 5.1, were manually acquired and visually inspected. Subsequently, each spectra was loaded into FlexAnalysis software, version 3.0 (Bruker Daltonics), to perform mass adjustment (spectra were compressed by a factor of 10 in the total mass range), smoothing (mass data were adjusted by the Savitsky–Golay algorithm with a frame size of 25 Da), baseline subtraction (was applied the minimum value for finding the baseline), normalization

TABLE 5.1 Milk Samples Analyzed and Relative Mass Spectra Acquired

Milk Source	Samples (<i>n</i>)	Spectra Replicates (<i>n</i>)	Total Spectra Analyzed (<i>n</i>)
Human milk			
Breastfeeding women at 2 days	5	4	20
Breastfeeding women at 30 days	6	4	24
Breastfeeding women at 60 days	5	4	20
Breastfeeding women at 90 days	4	4	16
Commercial brands of infant formula	4	4	16
Animal milk^a			
Cow	4	4	16
Buffalo	7	4	28
Goat	4	4	16
Ewe	5	4	20
Donkey	4	4	16
Camel	2	4	8

^a All animal milk samples were collected at the middle lactation stage.

(was applied the maximum norm to normalize the baseline subtracted data), and peak picking (was applied spectra differentiation algorithm for finding the peaks, maximum peaks 100, threshold 0.1, method Peak Fitting). The total preprocessed raw datasets of the 200 milk spectra were imported into R Bioconductor (www.bioconductor.org) [24] for Pearson’s correlation analysis and hierarchical clustering. The package pvclust was applied for bootstrapping. For each cluster generated by hierarchical computation, *p*-values (between 0 and 1) were calculated via multiscale bootstrap resampling. Two different *p*-values were provided by the package pvclust: approximately unbiased (AU) and bootstrap probability (BP). AU was computed by multiscale bootstrap resampling and represented a better approximation of an unbiased *p*-value than a BP value computed by normal bootstrap resampling. The same preprocessed raw datasets were imported into ClinProTools™ bioinformatics software, version 2.2 (Bruker Daltonics) [25], and converted into a virtual gel-like format. The mass values (*m/z*) were reported on the X axis, while the gray scale bar, reported on the Y axis, showed the relationship between the color intensity and the peak intensity. Finally, principal component analysis (PCA) was performed via ClinProTools™ software, version 2.2 (Bruker Daltonics), which employs only the statistically significant peaks after group classification for the calculation. Based on Welch’s *t*-test, a *p*-value for each peak was calculated. This value indicates the probability that the observed intensity differences among the various peaks are due to chance. These calculations have been done independently for peak heights and peak areas.

5.3 RESULTS

5.3.1 Low-Molecular-Weight Protein Profiles from Crude Milk by MALDI-TOF-MS

An analysis of the peptides and low-molecular-weight proteins (2000–30,000 Da) present in skimmed raw milk was performed using a benchtop linear MALDI-TOF mass

spectrometer. Complex mass spectra were obtained that were not affected by signal background problems. Four independent MALDI-TOF-MS protein profiles from each milk sample were recorded in order to ascertain a high level of analytical reproducibility for the analysis. Mass spectra obtained from different milk samples from the same source prepared and run on the same day were virtually indistinguishable, and the relative intensities of the protein species detected in each replicate were constant. Each spectrum was visually inspected, and the resulting flattened profiles were compared by gel-like representations with spectra from different samples. Figure 5.1 reports the MALDI-TOF-MS profile and the pseudogel view of human milk samples analyzed at 2 (colostrum, HC), 30 (HM30), 60 (HM60), and 90 (HM90) days postpartum. In all human samples, many peaks were visible in the left part of the mass spectrum. After the conversion of the obtained mass spectra to a gel-like format, it was clear that many species below a molecular weight of 5000 Da were present especially in the HC samples. In the middle of the spectra (medium-mass range), many peaks between 8500 and 16,000 Da were present in all samples, whereas no peaks were detected in the rightmost part of the spectra ($>16,000$ Da), although the sinapinic acid matrix, which is beneficial for the ionization of higher molecular weight proteins, was used during the sample deposition on the MALDI target. Only in mature milk (HM60 and HM90) was a mass value of approximately 24,000 Da detectable.

Figure 5.2 shows the MALDI-TOF-MS profile and the pseudogel view relative to four commercial infant formulae (A, H, F, and N). The distribution of molecular weights observed in the mass range between 2000 and 30,000 Da was similar in formula F, H, and N, while formula A displayed peaks only in the first part of the spectra, and no detectable signals were observed in the high-mass range, although it was not a hydrolyzate formula. A faint signal above background at approximately m/z 18,000 was detectable for both formula H and N.

FIGURE 5.1 Representative matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF-MS) profiling and pseudogel view of crude human milk at 2, 30, 60, and 90 days of lactation, indicated, respectively, as HC, HM30, HM60, and HM90. The mass-to-charge ratios (m/z) are reported on the X axis (Da), while the peak intensities are indicated as arbitrary units (a.u.) in the gray scale bar on the Y axis.

FIGURE 5.2 Representative MALDI-TOF-MS profiling and pseudogel view of commercial starting formula from four different companies (Aptamil 1, A; Formulaf 1 from Dicoform, F; Humana 1, H; Nidina 1 from Nestlé, N). The mass-to-charge ratios (m/z) are reported on the X axis (Da), while the peak intensities are indicated as arbitrary units (a.u.) in the gray scale bar on the Y axis.

Figure 5.3 reports the mass spectra and the relative pseudogel view of other mammalian milk analyzed in the present study: cow's milk, buffalo's milk, goat's milk, ewe's milk, donkey's milk, and camel's milk. All animal milk samples contained several peaks in the low-, medium-, and high-mass range.

In the region of the mass spectra $<10,000$ Da, many spectrometric signals were detectable except from EM. As in human and formula milk, a mass value of approximately 14,000 Da predominated in all animal samples. Conversely, the mass spectra of animal milk samples showed two intense peaks at approximately 18,000 (missing in human samples as well as CAM) and 24,000 Da (missing in HC, HM30 as well as in DM and CAM). Under our conditions, CAM milk showed a profile displaying a reduced number of peaks in the high-mass range compared to other milk.

5.3.2 Milk Spectra Properties and Similarities

To evaluate the similarities and differences among the peptide and protein compositions of different milk samples, we used an external statistical software (R Bioconductor) to perform a correlation analysis on spectral values (m/z and intensities) extracted after ClinProTools™ bioinformatics software preprocessing.

Figure 5.4 displays the correlation matrix obtained from all of the spectra. The figure represents three wide subgroups: the first group of animal milk (BM, CAM, CM, DM, EM, and GM), the second group of human milk (HC, HM30, HM60, and HM90), and the third group of formula milk (A, F, H, and N). From a visual analysis, animal milk does not have an appreciable correlation with human or formula milk. CAM displays a very poor correlation with all of the considered milk, suggesting that this milk might have a different protein profile compared to other animal milk.

FIGURE 5.3 Representative MALDI-TOF-MS profiling and pseudogel view of crude milk from cow's milk (CM), buffalo's milk (BM), goat's milk (GM), ewe's milk (EM), donkey's milk (DM), and camel's milk (CAM). The mass-to-charge ratios (m/z) are reported on the X axis (Da), while the peak intensities are indicated as arbitrary units (a.u.) in the gray scale bar on the Y axis.

BM has a relatively strong correlation with CM and to a lesser extent with EM and GM, which is in agreement with our previous study [26]. Human colostrum has a good correlation with mature human milk, but HM30, HM60, and HM90 are more correlated between them. Of note, although well correlated with each other, none of the milk formulae analyzed in this study displayed an appreciable correlation with animal or human milk.

These considerations were also supported by a hierarchical clustering tree (Figure 5.5) determined by the statistical software, R Bioconductor. The camel milk cluster separated from the other three clades that represent the group of formula milk, animal milk, and human milk. Formula milk from four different companies displayed a relatively homogeneous clustering, suggesting moderately common spectral characteristics. However, differences among the different brands allowed us to identify a common subcluster for each brand, even across its different batches. The group of human mature milk clustered near the colostrum clade, whereas the animal milk group was relatively well separated. The spectra were then analyzed by principal component analysis using the integrated software ClinProTools™. As shown in Figure 5.6, the milk samples of the same species clustered closely together, whereas the different milk species were well separated from each other. The 3D scatter plot image obtained from the PCA indicates that seven MALDI-TOF-MS profiles can be grouped, again corresponding to breast milk (with the two subgroups of

FIGURE 5.4 Pearson's correlation matrix of all spectral replica datasets for animal milk (BM, CAM, CM, DM, EM, and GM), human milk at 2, 30, 60, and 90 days (HC, HM30, HM60, and HM90, respectively), and infant formula (A, F, H, N). Correlation coefficients are represented with decreasing gray shades according to a scale ranging from 0 to 1, respectively.

FIGURE 5.5 Hierarchical clustering tree (bootstrap $n=1000$) of all MALDI-TOF spectral replica from animal milk (CM, BM, GM, EM, DM, and CAM), human milk at 2, 30, 60, and 90 days (HC, HM30, HM60, and HM90, respectively), and infant formula (A, F, H, N). Left values above horizontal lines are approximately unbiased (AU) p -values, right values above horizontal lines are bootstrap probability (BP) values, and values beneath are cluster labels.

FIGURE 5.6 3D scatter plot image from the principal component analysis (PCA) for human milk at 2, 30, 60, and 90 days (HC, HM30, HM60, and HM90, respectively), infant formula milk (A, F, H, and N), and animal milk (CM, BM, GM, EM, DM, and CAM). Each spot represents one milk sample.

colostrum and the other stages of breast milk), starting formulae, CM, BM, GM/EM, DM, and CAM.

5.4 DISCUSSION

In the current study, we focused our interest on protein content that serves diverse biological activities in milk, such as providing essential amino acids to growing infants, supplying newborns with enzymatic activity, and making available vitamins and hormones. Proteins are present in milk with a very large dynamic range in their concentrations [27]. After a defatting operation consisting of a two-step centrifugation, the milk fat globule membrane proteins were lost in great part together with their lipid counterpart, and the protein content of all samples resulted principally in caseins and soluble whey proteins. A qualitative MALDI-TOF-MS-based analysis of defatted milk was then conducted to evaluate the similarities and differences in the low-molecular-weight profiles of the composition of milk from a different source.

Multiple components were detected as clear signals in the mass range of 2000–30,000 Da. This region is known to represent milk proteins and peptides with pivotal roles in infants' health and development such as antimicrobial activities

(e.g., lysozyme 16 kDa and lactalbumin 14 kDa) and mineral absorption functions (e.g., caseins, between 20 and 25 kDa). Furthermore, most of the bioactive factors are peptides originally present in milk, which may exert their biological activity in the upper gastrointestinal tract regardless of digestive processes [19].

Our data indicate that the human colostrum profile (<30,000 Da) is more complex than every other kind of milk (Figures 5.1–5.3). Colostrum, secreted in the few days after birth, is reported to contain higher amount of peptides, proteins, and vitamins compared to mature milk [28]. The unique characteristics of HC, with additional nutrients and immune and growth factors, make it interesting as a therapy to promote neonatal health [28,29]. We found that HC spectra are particularly rich in low-molecular-weight proteins and are dominated by spectrometric signals with a mass <10,000 and mainly <7000 Da, a significant fraction of which may be involved in its physiological characteristics. Based on the linear MALDI-TOF-MS, our analysis does not allow us to identify any milk proteins, but it clearly indicates that the milk protein profile gradually changes over the following 30 days after birth. The amount of peptides and proteins decreases rapidly during the first month of lactation and then stabilizes in “mature” milk after 60 days (HM60 and HM90; Figure 5.1). By contrast, polypeptides of approximately 15,000 Da remain stable across colostrum and mature milk. A noteworthy finding was that the appearance of spectrometric signals with an m/z value of approximately 24,000 Da in mature milk (HM60 and HM90) was concomitant to a decrease in components with a molecular weight of <12,000 Da. In agreement with these data, proteomic studies have shed light on the dynamic composition of human milk throughout lactation stages [18,30–32]. In particular, whey proteins implicated in the modulation of the immune system and in the maturation of the gastrointestinal tract of neonates are overrepresented in human milk during the first days of lactation.

A hierarchical cluster analysis of the mass spectra was used to group milk samples according to the similarity of their spectral profiles. In this unsupervised analysis, the group assignment of the protein/peptides expression patterns was generated based on the similarities of spectral patterns in the automatically selected peaks. This analysis demonstrated that all analyzed milk samples formed four main clusters (Figure 5.5). All human samples ($n=20$) formed a cluster in which milk at 30, 60, and 90 days constituted a well-defined subcluster. These results indicate that colostrum could be clearly differentiated by the signal patterns of its MALDI-TOF mass spectra as an out-group. Even more clearly, the PCA of the qualitative characteristics generated seven principal components (PCs) (Figure 5.6). Human milk was segregated into a single PC, in which HC was identified as different from human milk samples at different stages of lactation (HM30, HM60, HM90).

The spectra relative to the commercial starting formulae reported in Figure 5.2 were from four different brands in which bovine milk was the only source of protein (e.g., casein alone and whey proteins together with caseins) while fat content was derived from a mixture of vegetable oil. Each group included four different samples of the same formula, coming from different batches. The linear MALDI-TOF-MS technique was able to identify a consistent similarity among the profiles of different milk samples. All formulae displayed a profile consistently different from cow's milk, which is their parent protein source. This is consistent with the ESPGHAN recommendations for the protein composition of infant formulae [12], which were modified; for example, the formulae studied were all enriched in whey protein fractions and lowered in caseins. However, each brand was characterized by a specific protein profile. Although all 16 samples of infant formula

formed a tight cluster (Figure 5.5), each brand could be differentiated, indicating that the proteomic asset of each formula was stable across batches and could be identified in a blinded, unsupervised analysis.

The animal milk analyzed in the present study showed similar protein peak profiles ranging from 14,000 to 18,000 Da (Figure 5.3). In particular, these spectrometric signals were conserved across CM, BM, GM, EM, and DM, but were less evident in CAM. The spectrometric signals at 18,000 and 24,000 Da correspond, respectively, to the theoretical molecular weights of lactoglobulin and casein—two components of cow's milk responsible for cow's milk allergy (CMA), the most common food allergy affecting children [33]. The absence of these spectrometric signals in the CAM sample and in all analyzed human milk is in agreement with previous studies that report the lack of these proteins in camel's milk [34,35]. For this reason, CAM, although not extensively used, was recommended by pediatricians for children with a cow's milk allergy and hence was evaluated in the present work [34,36–39].

GM and EM clearly displayed two additional peaks at 20,000 and 25,000 Da, which was less evident in CM and BM. They were absent in donkey's milk, which was characterized by a notoriously low protein content (1.3–2.8 g/100 mL) and a high whey protein/casein ratio [40]. The hierarchical cluster analysis of peak profiles indicated that 24 samples of five types of animal's milk could be subcategorized by source: a subcluster identified DM vs. Bovidae milk (buffalo, goat, ewe, cow), another included all seven BM samples, and a third was composed of the 13 samples of CM, EM, and GM. The results indicate that cow, ewe, and goat's milk have a homogeneous milk proteome, while milk proteins from donkey and cow's milk share a low-sequence similarity due to the genetic distance between the Equidae and Bovidae families. Indeed, there is evidence of cross-reactivity between cow's milk and proteins from goat, sheep, and buffalo's milk [20], while substantial differences in the immunoglobulin E (IgE)-binding epitope of cow's milk proteins and the corresponding domains of donkey's milk protein may, besides the low content in caseins, account for the demonstrated reduced allergenicity of DM [41]. Its distinctive protein composition is also evident in the results of our PCA, where we were able to differentiate between BM and CM samples and also between these and DM. The only two kinds of milk that clustered together were from ewe and goat samples, which was not a surprising finding if we consider the taxonomic proximity between goats and sheep (i.e., Caprinae subfamily) and the well-known clinical cross-reactivity among their milk [42]. Conversely, camel's milk is completely different from any other mammalian milk from a proteomic point of view. The same observation stems from the PCA reported in Figure 5.6, where CAM is segregated with a different color. These results confirm the moderately different protein composition of camel's milk compared to other animals' milk [43]. Among the mammalian species that are proposed to be suitable as a valid substitute for cow's milk-based formulae, CAM has a unique spectra profile that could have interesting properties in the nutrition of children.

These findings seem to suggest that the choice of an alternative to breast milk cannot be made exclusively on the basis of macronutrient composition, but that the proteomic profile can be a useful evaluation tool [44,45]. These results may be even more relevant if they are replicated in extensively hydrolyzed milk formulae (eHFs). These formulae are the first-choice milk substitutes in CMA, but may carry residual allergens that are able to cause reactions in sensitive infants [21]. A recent proteomic analysis revealed that the peptide profiles of commercially available eHFs also provide a descriptive and distinct signature [46].

5.5 CONCLUSIONS

MALDI-TOF-MS profiling of milk proteins in combination with statistical tools proved to be a high throughput and low-cost approach with promising applications as an analytical tool to quickly assess the similarities and differences of low-molecular-weight proteins present in milk from different sources with a high level of accuracy and sensitivity. Our data point to differences between the potential alternative sources of infant formula milk and create the basis for further proteomic investigations to achieve more conclusive results on the protein content of the milk types herein evaluated.

Moreover, a MALDI-TOF mass spectral database compilation can assist nonspecialized mass spectrometric laboratories in the rapid screening and characterization of milk samples. The screening procedures could become a powerful method for analyzing milk in a blinded way in order to evaluate animal milk adulteration, milk samples present in human donor breast milk banking, and matching between human milk formula compositions. A rapid MALDI-TOF-MS assay could also become an instrument for interpreting the individuality in the phenotypic expression of allergies to cow's milk proteins and beyond.

Author contributions: F.D.G. conceived and designed the study, acquired mass spectra, interpreted the data, and drafted the manuscript; L.D.F. and M.M. wrote the chapter with input from all authors; A.M. performed statistical analysis; G.S., F.S., G.R., E.P., M.S., B.M.G., O.M., and A.I.E. recruited samples and interpreted the data;

I.L. contributed to performing the pre-analytical procedures and to data generation; P.R., A.D., and I.L. contributed to interpreting the data and drafting the manuscript; A.F. and L.P. contributed to conceiving and designing the study and to drafting the manuscript.

Funding: This work was supported by San Camillo Hospital, Treviso, Italy Fellowship Program to Lorenza Putignani.

Conflict of interest: The authors declare no conflict of interest.

REFERENCES

1. Dewey, K. *Guiding Principles for Complementary Feeding of the Breastfed Child*. Pan American Health Organization [PAHO], Division of Health Promotion and Protection, Food and Nutrition Program: Washington, DC, 2003; p. 37.
2. Lessen, R.; Kavanagh, K. Position of the academy of nutrition and dietetics: Promoting and supporting breastfeeding. *J. Acad. Nutr. Diet.* 2015, *115*, 444–449.
3. Weaver, S.R.; Hernandez, L.L. Autocrine-paracrine regulation of the mammary gland1. *J. Dairy Sci.* 2016, *99*, 842–853.
4. Prentice, P.; Ong, K.K.; Schoemaker, M.H.; van Tol, E.A.F.; Vervoort, J.; Hughes, I.A.; Acerini, C.L.; Dunger, D.B. Breast milk nutrient content and infancy growth. *Acta Paediatr.* 2016, *105*, 641–647.
5. Ballard, O.; Morrow, A.L. Human milk composition. *Pediatr. Clin. North Am.* 2013, *60*, 49–74.
6. Roncada, P.; Stipetic, L.H.; Bonizzi, L.; Burchmore, R.J.S.; Kennedy, M.W. Proteomics as a tool to explore human milk in health and disease. *J. Proteom.* 2013, *88*, 47–57.

7. Del Chierico, F.; Vernocchi, P.; Petrucca, A.; Paci, P.; Fuentes, S.; Praticò, G.; Capuani, G.; Masotti, A.; Reddel, S.; Russo, A.; et al. Phylogenetic and metabolic tracking of gut microbiota during perinatal development. *PLoS ONE* 2015, *10*, e0137347.
8. Cai, X.; Wardlaw, T.; Brown, D.W. Global trends in exclusive breastfeeding. *Int. Breastfeed. J.* 2012, *7*, 12.
9. Office of Foods and Veterinary Medicine, Center for Food Safety and Applied Nutrition. Demonstration of the quality factor requirements under 21 CFR 106.96(i) for “eligible” infant formulas. 2014. Available online: www.fda.gov/RegulatoryInformation/Guidances/ucm400036.htm (accessed on 1 January 2020).
10. Food and Agriculture Organization of the United Nations, World Health Organization. CODEX alimentarius: Standard for infant formula and formulas for special medical purposes intended for infants. Revised standard for infant formula. 2016. Available online: www.fao.org/fao-who-codexalimentarius/sh-proxy/pt/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FMeetings%252FCX-720-38%252FReport%252FFINAL%252FREP17_NFSDUe.pdf (accessed on 20 May 2018).
11. Rossen, L.M.; Simon, A.E.; Herrick, K.A. Types of infant formulas consumed in the united states. *Clin. Pediatr. (Phila.)* 2016, *55*, 278–285.
12. Koletzko, B.; Baker, S.; Cleghorn, G.; Neto, U.F.; Gopalan, S.; Hernell, O.; Hock, Q.S.; Jirapinyo, P.; Lonnerdal, B.; Pencharz, P.; et al. Global standard for the composition of infant formula: Recommendations of an ESPGHAN coordinated international expert group. *J. Pediatr. Gastroenterol. Nutr.* 2005, *41*, 584–599.
13. Stam, J.; Sauer, P.J.; Boehm, G. Can we define an infant’s need from the composition of human milk? *Am. J. Clin. Nutr.* 2013, *98*, 521S–528S.
14. Ryan, A.S.; Hay, W.W. Challenges of infant nutrition research: A commentary. *Nutr. J.* 2015, *15*, 1–8.
15. Gura, T. Nature’s first functional food. *Science* 2014, *345*, 747–749.
16. Weber, M.; Grote, V.; Closa-Monasterolo, R.; Escribano, J.; Langhendries, J.P.; Dain, E.; Giovannini, M.; Verduci, E.; Gruszfeld, D.; Socha, P.; et al. Lower protein content in infant formula reduces BMI and obesity risk at school age: Follow-up of a randomized trial. *Am. J. Clin. Nutr.* 2014, *99*, 1041–1051.
17. Hidayat, K.; Du, H.-Z.; Yang, J.; Chen, G.C.; Zhang, Z.; Li, Z.N.; Qin, L.Q. Effects of milk proteins on blood pressure: A meta-analysis of randomized control trials. *Hypertens. Res.* 2017, *40*, 264–270.
18. Gao, X.; McMahon, R.J.; Woo, J.G.; Davidson, B.S.; Morrow, A.L.; Zhang, Q. Temporal changes in milk proteomes reveal developing milk functions. *J. Proteome Res.* 2012, *11*, 3897–3907.
19. Savino, F.; Sorrenti, M.; Benetti, S.; Lupica, M.M.; Liguori, S.A.; Oggero, R. Resistin and leptin in breast milk and infants in early life. *Early Hum. Dev.* 2012, *88*, 779–782.
20. Restani, P.; Gaiaschi, A.; Plebani, A.; Beretta, B.; Cavagni, G.; Fiocchi, A.; Poesi, C.; Velonà, T.; Ugazio, A.G.; Galli, C.L. Cross-reactivity between milk proteins from different animal species. *Clin. Exp. Allergy J. Br. Soc. Allergy Clin. Immunol.* 1999, *29*, 997–1004.
21. Fiocchi, A.; Brozek, J.; Schünemann, H.; Bahna, S.L.; von Berg, A.; Beyer, K.; Bozzola, M.; Bradsher, J.; Compalati, E.; Ebisawa, M.; et al. World Allergy Organization (WAO) Special Committee on Food Allergy. World Allergy Organization (WAO) Diagnosis and Rationale for Action against Cow’s Milk Allergy (DRACMA) Guidelines. *Pediatr. Allergy Immunol.* 2010, *21* (Suppl 21), 1–125.

22. Putignani, L.; Del Chierico, F.; Onori, M.; Mancinelli, L.; Argentieri, M.; Bernaschi, P.; Coltella, L.; Lucignano, B.; Pansani, L.; Ranno, S.; et al. MALDI-TOF mass spectrometry proteomic phenotyping of clinically relevant fungi. *Mol. BioSyst.* 2011, 7, 620–629.
23. Di Girolamo, F.; Masotti, A.; Salvatori, G.; Scapaticci, M.; Muraca, M.; Putignani, L. A sensitive and effective proteomic approach to identify she-donkey's and goat's milk adulterations by MALDI-TOF MS fingerprinting. *Int. J. Mol. Sci.* 2014, 15, 13697–13719.
24. Gentleman, R.C.; Carey, V.J.; Bates, D.M.; Bolstad, B.; Dettling, M.; Dudoit, S.; Ellis, B.; Gautier, L.; Ge, Y.; Gentry, J.; et al. Bioconductor: Open software development for computational biology and bioinformatics. *Genome Biol.* 2004, 5, R80.
25. Ketterlinus, R.; Hsieh, S.-Y.; Teng, S.-H.; Lee, H.; Pusch, W. Fishing for biomarkers: Analyzing mass spectrometry data with the new ClinProTools software. *BioTechniques* 2005, 38, 37–40.
26. Di Girolamo, F.; D'Amato, A.; Lante, I.; Signore, F.; Muraca, M.; Putignani, L. Farm animal serum proteomics and impact on human health. *Int. J. Mol. Sci.* 2014, 15, 15396–15411.
27. Bislev, S.L.; Deutsch, E.W.; Sun, Z.; Farrah, T.; Aebersold, R.; Moritz, R.L.; Bendixen, E.; Codrea, M.C. A bovine peptide atlas of milk and mammary gland proteomes. *Proteomics* 2012, 12, 2895–2899.
28. Gephardt, S.M.; Weller, M. Colostrum as oral immune therapy to promote neonatal health. *Adv. Neonatal Care* 2014, 14, 44–51.
29. Bagwe, S.; Tharappel, L.J.P.; Kaur, G.; Buttar, H.S. Bovine colostrum: An emerging nutraceutical. *J. Complement. Integr. Med.* 2015, 12, 1–11.
30. Liao, Y.; Alvarado, R.; Phinney, B.; Lönnerdal, B. Proteomic characterization of human milk whey proteins during a twelve-month lactation period. *J. Proteome Res.* 2011, 10, 1746–1754.
31. Liao, Y.; Alvarado, R.; Phinney, B.; Lönnerdal, B. Proteomic characterization of human milk fat globule membrane proteins during a 12 month lactation period. *J. Proteome Res.* 2011, 10, 3530–3541.
32. Reinhardt, T.A.; Lippolis, J.D. Developmental changes in the milk fat globule membrane proteome during the transition from colostrum to milk. *J. Dairy Sci.* 2008, 91, 2307–2318.
33. Sackesen, C.; Assa'ad, A.; Baena-Cagnani, C.; Ebisawa, M.; Fiocchi, A.; Heine, R.G.; Von Berg, A.; Kalayci, O. Cow's milk allergy as a global challenge. *Curr. Opin. Allergy Clin. Immunol.* 2011, 11, 243–248.
34. Shabo, Y.; Barzel, R.; Margoulis, M.; Yagil, R. Camel milk for food allergies in children. *Isr. Med. Assoc. J. IMAJ* 2005, 7, 796–798.
35. Merin, U.; Bernstein, S.; Bloch-Damti, A.; Yagil, R.; van Creveld, C.; Lindner, P.; Gollop, N. A comparative study of milk serum proteins in camel (*Camelus dromedarius*) and bovine colostrum. *Livest. Prod. Sci.* 2001, 67, 297–301.
36. El-Agamy, E.I.; Nawar, M.; Shamsia, S.M.; Awad, S.; Haenlein, G.F.W. Are camel milk proteins convenient to the nutrition of cow milk allergic children? *Small Rumin. Res.* 2009, 82, 1–6.
37. El-Agamy, E.I. The challenge of cow milk protein allergy. *Small Rumin. Res.* 2007, 68, 64–72.
38. Järvinen, K.M.; Chatchatee, P. Mammalian milk allergy: Clinical suspicion, cross-reactivities and diagnosis. *Curr. Opin. Allergy Clin. Immunol.* 2009, 9, 251–258.

39. Ehlayel, M.S.; Hazeima, K.A.; Al-Mesaifri, F.; Bener, A. Camel milk: An alternative for cow's milk allergy in children. *Allergy Asthma Proc.* 2011, 32, 255–258.
40. Piovesana, S.; Capriotti, A.L.; Cavaliere, C.; La Barbera, G.; Samperi, R.; Zenezini Chiozzi, R.; Laganà, A. Peptidome characterization and bioactivity analysis of donkey milk. *J. Proteom.* 2015, 119, 21–29.
41. Gallina, S.; Cunsolo, V.; Saletti, R.; Muccilli, V.; Di Francesco, A.; Foti, S.; Lorenzten, A.M.; Roepstorff, P. Sequence characterization and glycosylation sites identification of donkey milk lactoferrin by multiple enzyme digestions and mass spectrometry. *Amino Acids* 2016, 48, 1569–1580.
42. Restani, P.; Fiocchi, A.; Beretta, B.; Velonà, T.; Giovannini, M.; Galli, C.L. Meat allergy: III—Proteins involved and cross-reactivity between different animal species. *J. Am. Coll. Nutr.* 1997, 16, 383–389.
43. Sabahelkhier, M.; Faten, M.; Omer, F. Comparative determination of biochemical constituents between animals (goat, sheep, cow and camel) milk with human milk. *Res. J. Recent Sci.* 2012, 1, 69–71.
44. Roncada, P.; Piras, C.; Soggiu, A.; Turk, R.; Urbani, A.; Bonizzi, L. Farm animal milk proteomics. *J. Proteom.* 2012, 75, 4259–4274.
45. D'auria, E.; Agostoni, C.; Giovannini, M.; Riva, E.; Zetterström, R.; Fortin, R.; Greppi, G.; Bonizzi, L.; Roncada, P. Proteomic evaluation of milk from different mammalian species as a substitute for breast milk. *Acta Paediatr.* 2005, 94, 1708–1713.
46. Lambers, T.T.; Gloerich, J.; van Hoffen, E.; Alkema, W.; Hondmann, D.H.; van Tol, E.A.F. Clustering analyses in peptidomics revealed that peptide profiles of infant formulae are descriptive. *Food Sci. Nutr.* 2015, 3, 81–90.

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