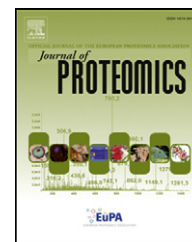


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Review

Proteomics as a tool to explore human milk in health and disease[☆]

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ABSTRACT

Proteins in milk have wide range of functions, they are carriers of minerals or chemically vulnerable and insoluble vitamins and other compounds, stabilisers of large aggregates or micelles of lipids, and components of both innate and acquired immune defence systems. Together with other components of milk, proteins may also contribute to the selection and establishment of appropriate microbiome in the gut of the infant. The proteome of mammalian milk is now known to be dynamic and changes radically with time after birth from colostrum to mature lactation. Significantly, immune and innate defence proteins appear in milk during infection of the mammary gland and possibly also during systemic infections. The understanding of the human milk proteome and how it changes with time during lactation and in disease is developing rapidly, and is to a large extent informed by proteomics of the milks of non-human mammals, domestic animals in particular. We review general methods now being applied for proteomic analysis of human milk. Moreover we place emphasis on how the milk proteome may change in different ways in response to disease, mastitis in particular, how such changes may be specific to pathogen types, and we give some insights about evolution.

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1. Introduction

The aim of this review is to discuss study design in the exploration of the milk proteome, and how currently used and developing proteomic technologies can be applied and adapted for analysis of milks. One problem with milks is that, in common with other secretions, some protein types are in such superabundance that they obscure minor components that may be equally important, albeit with different biological activities. This broad dynamic range necessitates appropriate fractionation in order to achieve satisfactory proteomic coverage. The review will follow and highlight considerations required to undertake a proteomic study, dealing with the selection of samples, study design, and the type of techniques to use. There is also some brief discussion of broader issues, particularly focussing on changes in the milk proteome during the course of lactation, comparison between different species of mammals, and the effect of maternal infection on the milk proteome and how genetic polymorphisms may have evolved in milk proteins.

1.1. Mammalian milks

The components of mammalian milks have evolved to provide an optimised source of nutrients, growth factors and immune protection to an infant, and also to establish a gut microbiome appropriate for an initial diet of milk and progression through weaning to the solid food suitable for each species [1–3]. The major constituents of milk are, broadly, lipids, for energy metabolism, membrane construction, brain development and as precursors of signalling lipids; carbohydrates for energy metabolism, protein and lipid glycosylations, and oligosaccharides as inhibitors of bacterial adhesion; and proteins.

The protein composition of milks is sensitive to the type of placenta that a species has. Humans have haemochorial placentae that engage in the translocation of, for instance, large quantities of immunoglobulins (but only IgG) during the third trimester of gestation. Species such as cows, sheep, goats and

horses have epitheliochorial placentae that do not permit this, so they acquire their immunoglobulins immediately after birth in colostrum. In the mature phase of lactation, once the colostrum phase is over, many milk proteins will have functions that are common to all mammals (e.g. the caseins), but others may have functions that are species-specific. Examples of the latter would be proteins whose operation requires the existence of specific cell surface receptors that may only operate to bind and translocate a milk protein in the same species. For instance, there are good examples of certain proteins in human milk that promote neonatal development in specific tissues, especially in brain development [4] and maturation of the gut microbiota [5,6]. It must also not be forgotten that some proteins that are abundant in the milks used as bases for artificial formulae (predominantly bovine) are absent in human milk, some of these, such as β -lactoglobulin, are also targets of allergic responses in humans [7,8].

There is an incomplete understanding of the functions of the proteins of human milk, partly due to an incomplete inventory of the human milk proteome [9]. Evidence that breastfeeding is correlated with a lower prevalence of obesity, diabetes, and cardiovascular disease in childhood and later life compared to infants fed on an artificial formulae diet [10–13], demands a fuller understanding of the proteins of human milk in addition to other essential nutrients such as lipids, carbohydrates, minerals and vitamins [14]. It is now well-known that the feeding of neonates with artificial formulae results in initial and persistent colonisation with a microbiome that is radically different from that of breast fed infants [15–17]. It is notable that breastfeeding results in enhanced colonisation by *Bifidobacterium* species whose genome sequences indicate specialisation for use of human milk oligosaccharides and similar host-derived metabolites [18,19]. In addition, the fact that bovine milk-based formula contains proteins that are inactive or not appropriately operative in humans means that refining and improving artificial formulae will ultimately be more challenging than initially anticipated. It could also be that improved understanding

of milk proteins will result in the conclusion that a true mimic of human milk is not realistically achievable, and therefore health and breastfeeding support agencies will allocate more effort towards improving breastfeeding rates and observe World Health Organisation targets more closely (<http://www.who.int/topics/breastfeeding/en/>).

One aspect of mammalian lactation that is not fully understood or appreciated is its dynamism. The proteome of human milk changes during the lactation period, presumably to fulfil the changing growth and development requirements of the infant, and also contributes to the modification of the gut microbiome as feeding patterns and diet changes [10]. A crucial aspect waiting to be fully explored is how the proteome changes with the nutritional state and health of the mother, both systemically and in localised mastitis. In mastitis, the mammary tissue has to protect and defend itself in addition to continuing the nourishment and immune protection of the infant. These two imperatives may be counteractive in that changes in milk that may be favourable for combating disease may not be conducive to optimal nutrition of the infant. The milk proteome in disease may therefore have evolved as a compromise between defence of the mammary gland and continued nutrition and immune protection of the infant. Just as at the initiation of lactation, the weaning period will involve drastic changes in the tissues of the mammary gland, involving upregulation of innate immune defensive proteins whilst maintaining, an albeit diminishing, nutritional milk supply [20].

Mastitis is a common condition usually, but not always, involving infection of the breast tissue and mammary glands. Mastitis has been shown to alter the milk proteome of ruminants, and can cause considerable economic and commercial loss [21]. It is most likely that similar changes occur in human milk during mastitis. In humans, in addition to the pain and disease involved, episodes of mastitis are known to have a negative effect on the persistence of mothers in breastfeeding [22]. So, the human milk proteome is likely to alter radically in mastitis, and possibly also in systemic diseases that do not involve the mammary gland directly. It is also conceivable that the proteome will change differently depending on the cause of the mastitis, such as infection by different pathogen types, bacteria, viruses, fungi, or due to simple blockage of milk ducts and consequent over-repletion and tissue damage.

1.2. The function of milk proteins

Milk proteins have traditionally been grouped into three crude fractions; caseins, whey proteins, and milk fat globular membrane (MFGM) proteins [11]. Knowledge of milk proteins is biased towards those that are present in high abundance, for example, β -lactoglobulin (abundant in ruminants and equids but absent in human milk), α -lactoglobulin, the caseins, and lactoferrin. A number of reviews and proteomic studies in the literature catalogue proteins identified in milk [11,14,23]. Proteomics is considerably enlarging the protein inventory of milks, which is therefore increasing our need for understanding the functions of milk proteins, especially those that may be in low abundance yet with important biological effects, as, for example, immune cytokines.

The principal function of the most abundant proteins is probably nutritional (e.g. the caseins as calcium transporters,

MFGM proteins as lipid and lipid-soluble vitamin carriers, β -lactoglobulins as fatty acid and possibly also Vitamin A/retinol carriers), and an array of functions has been attributed [11,14]. Many milk proteins are probably involved in defence and immunity to protect both the mammary tissue and the infant mammals (e.g. IgA antibody, lysozyme, lactoferrin, anti-microbial peptides, xanthine oxidase), which has led to the idea that milk first evolved in mammals to confer immune protection rather than purely to nourish [24]. Other proteins, including some of the most abundant, play a direct role in healthy growth and development of bone and other tissues, gastrointestinal tract maturation, nervous system maturation, and blood functionality. A number of proteins are signalling proteins involved in the activation of DNA repair, DNA replication and transcription pathways [14], though their function in milk, if any, remains to be elucidated. Enzymes in milk primarily play roles in protein degradation and defence. One final group of proteins acts as nutrient delivery and lipid transporters crucial for the deployment and distribution of nutrients within the neonate. Recent work by Gao et al. [25] and Liao et al. [23,26] presents interesting studies describing changes that occur in milk proteins during lactation and changes in the MFGM. They show changes in milk proteins that influence development of the immune system, central nervous system and establishment of gut microflora, during the first days of lactation, thus further extending the role for milk in host defence against pathogen challenge [9,27,28].

The degree of milk protein glycosylation and changes in this during lactation are important features; it has been reported that the different protein glycosylations can control the selection of gut commensals such that they have a pivotal role in host defence and in the stimulation of the immune system [29,30].

1.3. Conditional changes in the milk proteome

The proteins in milks will change with time for several reasons. The first is the initiation of lactation that involves both the need to produce an appropriate colostrum, but also that the mammary gland undergoes substantial modification in preparation for birth, so this and the onset of secretory processes may be reflected in the proteome. Similar considerations would apply to the weaning period when the mammary gland involutes and returns to its resting state unless a subsequent pregnancy has begun. For example, work on gene expression during weaning in mice has shown that transcription of genes encoding innate defence proteins is upregulated [20,31], presumably to compensate for an increased vulnerability of the gland to infection whilst undergoing reorganisation which might involve accumulation of apoptotic cells and the re-shaping of physical barriers to infection. The second cause of changes in the proteome will be during mastitis, during which the gland must eliminate an infection or at least limit its spread (both further within the gland and systemically), and also to increase the anti-microbial properties of the milk itself whilst still providing nutrition. So, one would expect an increase in immunoglobulins and proteins of innate defence as well as changes in the usual nutritional proteins given that some may be resources for microbial growth. Mastitis can have different aetiologies,

such as infections with pathogens that would require different immune responses (viruses, fungi, bacteria, and, within bacteria, differences between Gram positive and Gram negative types) by simple mechanical blockage and resulting tissue damage. It is entirely feasible that the local innate immune response in the mammary gland will be tuned to respond to a particular pathogen type given the different generic agonists for the different toll-like receptors (TLRs), and subsequent responses [32].

1.4. The evolution of human milk

The above reasons for investigating the proteome of human milk are mainly practical, but a final reason is speculative but of potential interest to human biology and evolution. It is already known that the oligosaccharides in human milk are not only different from those in other mammals, but also polymorphic between humans [33]. One explanation for this is that milk oligosaccharides are important in defence against colonisation of the infant gut with pathogens by, for example, acting as competitive inhibitors for attachment to the mucus and enterocytes by the adhesions of bacteria [18,33]. Balanced polymorphism in innate immune factors is a predictable consequence of the Red Queen hypothesis in the counter-evolution between pathogens and their hosts [34–36]. Thus, different populations of humans will have been exposed to different pathogens during their history, and this is likely to be reflected in the polymorphisms in anti-microbial defence factors present today. It is therefore possible that different groups of humans might also exhibit different allelomorphs of milk proteins, and even differences in the presence, absence, or abundances of different protein types. With the recent demonstration that some extant groups of humans separated from others by as many as 100,000 years [37], that interbreeding occurred between Neanderthal and ancestral Europeans [38,39], and between Denisovians with Asian and eastern groups of humans [40], it is conceivable that both selection for disease resistance and lineage of different groups of extant humans could be reflected in polymorphisms in milk proteins as is apparent with other immunity genes [36,41].

2. Sample selection for milk proteomic studies

When considering a human milk proteomic study there are, as summarised above, a number of different types and states of milk that may have altered proteomes, differences that may be advantageous or disadvantageous to the development of a neonate. And in certain circumstances, such as in mastitis, the milk proteome will presumably represent a compromise between a defence of the mammary gland and the mother's physiological integrity, and that of the infant.

During milk sample selection for proteomic analysis difficulties in sample availability can become apparent. Collection of such a material as human breast milk requires a sensitive approach and ethical considerations may have to be addressed during the process of sample selection and collection. The volume of milk sample that is required may exceed the amount that can be physically obtained, particularly in the case of colostrum which are produced in small volumes in the

immediate post-partum period where collection may be challenging. Milk from mastitic donors may also be difficult to obtain. Ethical considerations may compromise the collection of samples from untreated donors. However, the improving sensitivity of many proteomic approaches means that comprehensive analyses can be performed with relatively small sample volumes.

2.1. The milk proteome in relation to time

The milk proteome is dynamic, the most dramatic and well-known change being the transition from colostrum to mature milk (Fig. 1). There is, moreover, a developing focus on milk produced following premature births in humans in light of the extra vulnerabilities associated with prematurity. One such recent publication by De Leoz et al. explains the role of human milk oligosaccharides (HMO) in preterm infants and the crucial role of the mothers' milk in shaping the intestinal microbiota of her infant [42]. Furthermore, they report that premature delivery significantly alters the mother's lactation, and the proteome of preterm milk is also dramatically different from later milks, particularly with regard to immune defence proteins [43,44].

In addition to premature milk there is further interest in milk samples at the other end of the time line with the changes in the composition of milk during weaning [45,46]. Milk yield usually decreases as infant demands lessen, ultimately leading to cessation of lactation. Some human females continue to produce milk even after weaning, which is another phenomenon that merits attention.

There is no definitive definition of that which is meant by colostrum, or mature milk, nor in defining the time of transition between the different phases of lactation. It must also not be forgotten that timing of these phases, and the metabolome and proteome of milk, may differ between primigravida and multigravida. In humans, it is conventional to define colostrum as milk produced until about 3 days from birth, and that the transition to mature milk occurs over the subsequent 3 to 15 days [47,48]. There is, however, disagreement over these timings, and there remains insufficient information on the various components of human milk to allow a proper definition of the different phases and transition periods, and how they may vary between mothers.

2.2. Status of milk in response to disease

Mastitis is a common condition of breastfeeding mothers with 10% of mothers suffering with the condition at some point

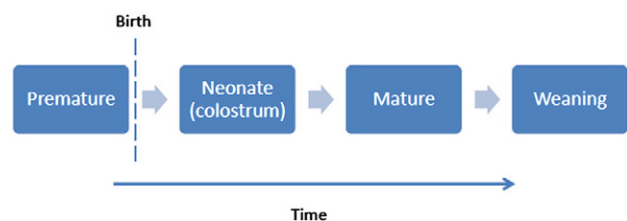


Fig. 1 – Time-line depicting the phases of lactation that will be reflected in the milk proteome.

during the period of nursing [49]. Mastitis makes breastfeeding painful and can cause concern to mothers worrying about passing infection to their child, although there is thought to be little chance of such transmission [49]. The pain and discomfort of mastitis are, however, sufficient to make the mother stop breastfeeding [22,49]. It is not known how infection changes the proteome of the milk. Previous work by Kim et al. has shown that mastitis in cows causes changes to the proteome of the milk from the infected glands [21]. The causative pathogen may contribute proteins or pathogen associated molecular patterns (PAMPs; such as bacterial lipopolysaccharide) to the milk, which may induce defensive responses in the gastro-intestinal (GI) tract of the infant [50]. Mastitis may also result in contamination of the milk with blood or pus, which may also affect the infant's gut and its microbiome. In cattle, mastitis is known to result in the deposition of innate defence proteins in milk, some of which are associated with the systemic acute phase response [51–54]. Intriguingly, some of the acute phase reactants are synthesised locally in the mammary gland, rather than in their usual site of synthesis during acute phase responses, the liver [52]. It is not known whether mastitis in humans causes similar changes, but it is an intriguing possibility that, as discussed above, different pathogen types may induce specific and detectable changes in the proteome, which can then be used to diagnose and treat appropriately. It is clearly useful, for instance, to know whether to avoid antibiotics where they would not be of benefit, because their inappropriate use could contribute to the emergence of resistance, and/or compromise the microbiome of both mother and infant.

2.3. Methods for studying the proteome of milk

Many proteomic techniques have been applied to the study of milk. An example of a typical workflow from a raw milk sample to a resolved milk proteome is depicted in Fig. 2, but a diversity of approaches has been exploited. The proteomic 'tool box' is full of ways to resolve a complex mixture of proteins but the application of proteomic technologies to milks presents particular challenges. One characteristic that

makes the milk proteome hard to resolve is the broad dynamic range (relative abundance) of proteins present. A similar problem is presented by serum, and fractionation approaches that have proven helpful in serum proteomics have potential to be applied to milk. In common with many extracellular proteomes, milk proteins carry many post-translational modifications that may serve to promote stability of secreted proteins. Other protein modifications may also serve to augment the complement of nutrients delivered by milk. For example, the high degree of phosphorylation amongst abundant milk proteins such as casein may represent an important source of phosphate for the developing infant. Other, non-protein, components such as lipids and sugars are abundant for similar reasons but can complicate sample preparation or separation. Whole milk also contains a variety of material that might be viewed as contamination, such as cell debris from the milk ducts and blood, along with other proteins often identified in milk which are not seen to be expressed by the mammary glands. These may still constitute an important component of human milk, as they have likely nutritional value at least. Leukocytes in milk have been reported possibly to play a role in providing greater pathogen defence and protection in the neonate [21]. In addition keratin, which is viewed as a troublesome contaminant in many proteomic analyses, is commonly found in milk but might derive from expression in human mammary epithelial cells [55] or from incidental contamination from skin during donation or sample handling. If keratin is not expressed in milk, it is inevitable that the baby ingests some during suckling and therefore should be considered in the composition of milk.

2.4. Fractionation techniques used to overcome protein abundance differences

Based on relative abundance, the casein isoforms (α S1, α S2, β and κ) represent 80% of the total protein content of milk and the whey proteins (β -lactoglobulin, α -lactalbumin, serum albumin, immunoglobulin's, lactoferrin and others) represent 16% [14]. It is suspected that the remaining 4% of the protein content could contain a multitude of proteins (>1000) with unique roles in

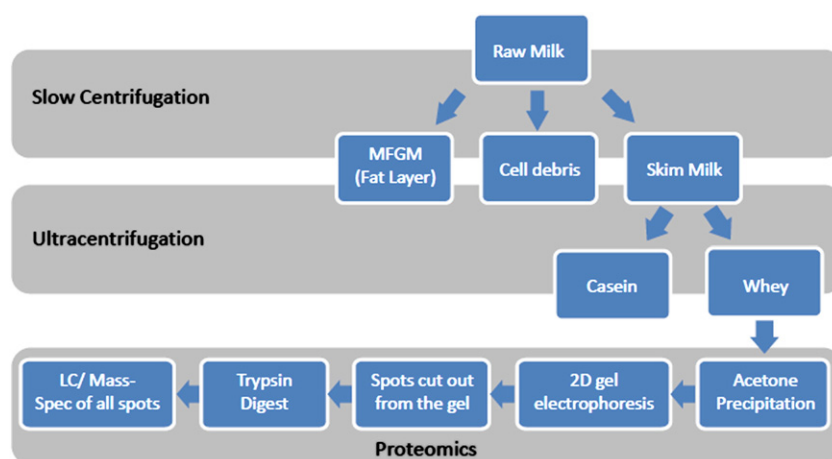


Fig. 2 – An example of a proteomic project outline to resolve the proteome of a raw milk sample. The pathway illustrates the fractionation of milk through different speed centrifugation techniques and the steps taken to resolve a proteome.

infant development [12,13,56,57]. Identification of such proteins has been a principal aim of milk proteome research [54,58].

Sample preparation has to be considered carefully to overcome problems caused by protein abundance differences. Through depleting abundant proteins in a sample of milk, other proteins that may have been masked with similar characteristics, for example molecular mass, may be revealed during analysis of the resolved proteome. Raw milk can be fractionated into fat, cell debris (from epithelial cells and leukocytes) and skimmed milk (containing whey proteins) (Fig. 2). Preliminary fractionation of milk can be achieved through slow centrifugation (<2000 g) of raw milk. A number of fractionation techniques for the depletion of super-abundant proteins have been applied to the analysis of milk, including enzymatic action, immunoprecipitation or centrifugation [12,59,60]. Ultracentrifugation ($\geq 190,000$ g), for example, can pellet much of the casein component of milk [12,59,60]. Ultracentrifugation has proven to be effective in maintaining a viable protein sample [12,59,60], but it is likely that a diverse range of less abundant proteins are precipitated, specifically or non-specifically, along with casein. Rennet coagulation is an alternative to ultracentrifugation, also acting upon casein [61], but has the additional disadvantage of potentially contributing to artefacts in addition to the presence of an added mixture of proteins.

Immunoprecipitation allows for specific proteins to be depleted, but antibodies against milk proteins can be costly when used in, for example, preparative affinity chromatography, and relies on the availability of antibodies that may only operate efficiently for milk proteins of a given species. Commercial affinity depletion systems, based on antibodies are available, mostly optimised for human serum, but might be useful if applied to specifically deplete some of the highly abundant components of human milk. There is also a concern that the removal of very abundant proteins from native milk might lead to the depletion of other, less abundant components that are complexed with the targeted protein.

The use of aptamer technology to bind protein species at equivalent stoichiometries has the potential to ameliorate the dynamic range. Such a system has been commercialised as ProteomeMiner beads (Bio-Rad Laboratories) [62,63] and has been applied to human milk as Liao et al. and Molinari et al. previously reported [23,44]. One limitation of this approach is that only a very small proportion of the starting material is eluted from the beads for subsequent analysis. Furthermore, the possibility of differential binding of post-translationally modified proteins to the aptamer population cannot be excluded.

Coupled with a fractionation procedure, further techniques are often applied to desalt the sample, break disulfide bonds and alkylate the proteins. Acetone precipitation is often used as part of such processing and has been found to cause a loss of about 22% of the total protein in a sample [64]. The overall milk protein concentration is very high so a small overall loss of protein is not a great concern although there is a risk that it will introduce a bias in the final protein profile. Previous work looking at the differences between precipitated and unprecipitated samples fractionated using two-dimensional electrophoresis (2-DE) shows better protein coverage of precipitated samples [65]. This coupled with ultracentrifugation may cause the loss of a small percentage of protein concentration, but

allows for better resolution of a complex mixture of proteins revealing a more in-depth proteome map.

2.5. Proteome analysis techniques

In order to resolve the proteome of milk, proteomic techniques usually involving one or a combination of electrophoresis, chromatography and mass-spectrometry have been applied [12,13,66]. A commonly used method to resolve a mixture of proteins is 2-DE, which employs the orthogonal characteristics of protein size and isoelectric point to achieve high resolution separation [67]. 2-DE allows for visual representation of complexity and relative abundance in the proteins that are detected. The resolution of 2-DE can be enhanced by, for example, employing discrete pH ranges for isoelectric focusing, but such strategies increase the complexity and workload of a technically challenging approach.

Once a 2-DE gel has been run it can be stained by a number of different stains, commonly Coomassie blue. This provides a visual representation of a proteome map of the resolved sample. The technique of 2-DE has been applied to the study of different fractions of human milk. Fig. 3 shows an example of a 2-DE gel of the visible proteome of a 75 day (mature) human milk sample. The figure demonstrates the clarity and resolution of spots visible on the gel allowing for accurate determination of spot location. In Table 1 are reported the identified proteins with relative functions.

Through looking at a 2-DE gel, assumptions can be made about the protein identity according to their molecular weight and isoelectric point, based on where they appear on the gel. Protein spots can be excised from the gel, fragmented into peptides and subjected to mass spectrometry analysis with the aim of identifying the proteins of interest through reference to genome sequence databases.

Chromatographic separation of peptides or combinations of electrophoresis and chromatography can also be employed to resolve complex mixtures of proteins such as milk. Chromatographic separation of tryptic peptides has advantages over gel-based separation for very large or very small proteins, or for proteins with extreme pI or hydrophobicity. Electrophoretic separation of intact proteins, in one or two dimensions, followed by chromatographic separation of peptides, generally gives the best proteomic coverage, but this can be complemented by alternative approaches such as multi-dimensional protein identification technologies [68]. However, 2-DE is particularly well suited to milk proteomic analysis because the majority of milk proteins are relatively soluble and because resolution at the intact protein level enables separation of many modified proteins. Furthermore, 2-DE is ideal for comparative analysis, often a powerful way to relate proteome to phenotype.

2.6. Comparative proteomic analysis techniques

Comparative proteomic techniques provide the tools to compare and contrast a number of different proteomes such as normal and mastitic milk. Many of these techniques involve differential labelling, either at the protein or peptide level. Difference Gel Electrophoresis (DIGE), is a 2-DE method in which up to three protein samples can be covalently labelled using different fluorophores and then resolved together in

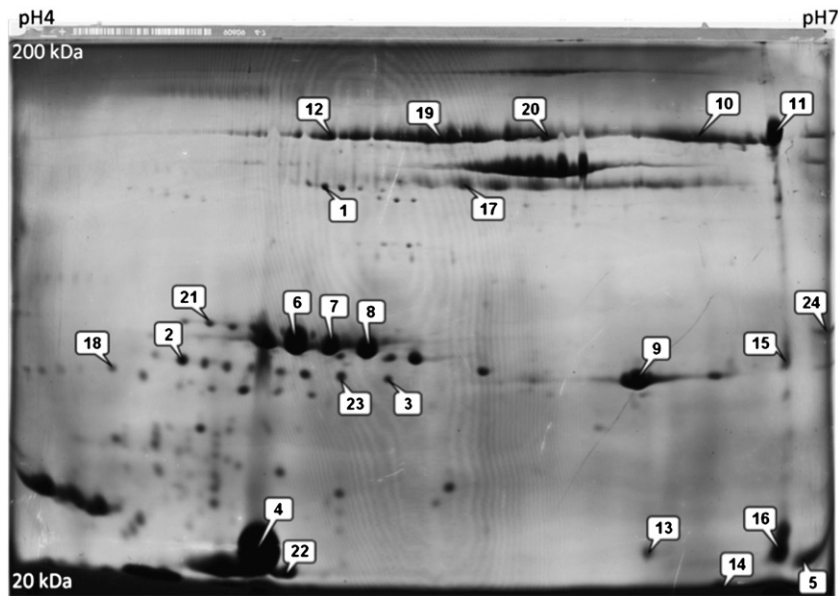


Fig. 3 – An example of two-dimensional electrophoresis (2-DE) gel of the human milk. Numbers indicate the identified protein as reported in Table 1.

one 2-DE analysis. The different tags have distinct excitation and emission spectra, such that fluorescently-labelled proteins from different sources can be discriminated. The images can then be overlaid allowing for easy and accurate identification of protein changes in the different samples [69].

Other comparative techniques are now available that could be useful. These typically involve the use of isotopic tags detectable by chromatography and mass-spectrometry applications. Such techniques that could possibly be utilised for the study of milk but would have to be adapted to fit the unique

characteristics of milk are; Isotope Coded Affinity Tagging (ICAT) [70,71], O_{18} labelling [72,73], dimethyl labelling [74,75], isotope tagging for relative absolute quantitation (iTRAQ) [76,77] and direct quantitation by comparison of ion abundance ('label-free quantitation') [78].

2.7. Post-translational modifications

As with proteins from other sources, milk proteins will be post-translationally modified in ways that are crucial to their

Table 1 – Identification of the major proteins resolved in human milk. The identity of some of the most abundant proteins resolved on the 2-DE gel is shown in Fig. 3. The proteins are classified according to their presumed biological activities, predicted from published data; these classifications are general and their functions in milks may be different.

Spot number	SwissProt accession number	Protein name	Putative roles ^a
1	P01009	Alpha-1-antitrypsin	A, C, G, I
2, 3	P47710	Alpha-S1-casein	B, I, N
4, 5	P00709	Alpha-lactalbumin	B, C, G, M, N
6, 7, 8, 9	P05814	Beta-casein	B, G, I, N
10, 11, 12	P02788	Lactotransferrin	C, I, N
13	P05413	Fatty acid-binding protein, heart	C, L, N
14	P61769	Beta-2-microglobulin	I
15	P01834	Ig kappa chain C region	I
16	P61626	Lysozyme C	I
17	P01766	Ig heavy chain V-III region BRO	I
18	P01591	Immunoglobulin J chain	I
19, 20	P01833	Polymeric immunoglobulin receptor	I, L
21	P04264	Keratin	L
22	Q13410	Butyrophilin subfamily 1 member A1	L
23	P02647	Apolipoprotein A-I	L, N
24	P02768	Serum albumin	L, N

^a Abbreviations for putative functions: A, angiogenesis and blood functionality; B, bone maturation; C, cell growth/tissue development; G, gastrointestinal tract maturation; I, defence/immunity; L, lipid transport/secretion; M, metabolism; N, nutrient delivery.

function, and these modifications may change in different stages of lactation and in different disease states. Phosphorylation, for example, can be examined by 2-DE coupled with western blotting using phosphor-specific antibodies or mass spectrometry. For example, trains of spots in a 2-D gel are readily seen in abundant milk proteins such as caseins, due to shifts (commonly by one charge unit) in isoelectric point caused by different degrees of phosphorylation [79,80]. Characterization of low abundance phosphoproteins requires selective enrichment. This can be achieved by immobilized metal or titanium dioxide affinity chromatography, applied either to intact proteins or to tryptic peptides, followed by protein identification and characterization by mass spectrometry [81].

2.8. Glycosylation

In human milk this type of modification is very important because it potentially modulates the anti-microbial functions of milk and modifies its probiotic function. A first approach for a qualitative characterization of glycosylation requires the separation of milk proteins by one or 2-DE and lectin blotting or periodic-acid Schiff staining (Pro-Q Emerald stain). Lectins are specific sugar-binding proteins useful to investigate the nature of oligosaccharide residues. Some lectins are specific for N-glycosylation (e.g. Concanavalin A and Jacalin lectin), for O-glycosylation (e.g. *Vicia villosa* lectin) and sialic acids (e.g. *Maackia amurensis* and *Sambucus nigra* lectins). So, for a global glycome analysis using lectins, it is recommended to use a mixture of these sugar-binding proteins. For a fine structural characterization of oligosaccharide structures it is necessary to implement a preliminary step of protein digestion to peptides with specific exoglycosidases for each class of glycans (PNGaseF, Endo H, O-Glycosidase). This releases glycans that can then be separated by chromatography (principally graphitized carbon or capillary electrophoresis) prior to identification by tandem mass spectrometry [82].

2.9. Bioinformatic tools to explore milk protein function

During the last few years a large number of human milk proteins from different fractions (MGFM, whey and casein) and at different time points have been identified but there is still much to learn about the biology and functions of most of these proteins in the context of milks and lactation. It is increasingly possible, however, to use recently advancing bioinformatic tools to begin to understand their roles and interactions, such as by gene ontology (GO) and network analysis.

Proteins can be classified in terms of biological processes, cellular components, and molecular functions using several online tools such as DAVID (Database for Annotation, Visualization and Integrated Discovery) [83] or PANTHER (Protein ANalysis THrough Evolutionary Relationships) [84]. Other open source software packages like Bioconductor, which utilises the R statistical programming language or BinGO as a Cytoscape plugin will be useful. Using DAVID, Gao et al. were able to infer functional roles for certain human milk proteins using well-established activities of proteins in immune protection,

carbohydrate metabolism and extracellular matrix enrichment (ECM) [25].

Switching from a single protein to functional clusters of proteins requires the investigation of protein-protein interactions, for which network analysis will be valuable. There are now many databases of known and predicted protein interactions. One such open source database is STRING 9 (Search Tool for the Retrieval of Interacting Genes/Proteins) [85]. STRING 9 includes direct (physical) and indirect (functional) associations that are derived from several sources. Analysis of protein interactions in human milk using STRING 9 has shown that several pathways were over-represented, including the ECM-receptor interaction, carbohydrate metabolism and immune activation pathways [25]. Otherwise there are also proprietary databases such as Ingenuity and MetaCore to infer interaction pathways. In 2010 D'Alessandro and colleagues analysed data sourced from the literature on human milk and found four critical overrepresented biological functions involving: immunity, cell proliferation/differentiation/movement, tissue development and lipid metabolism/nutrient transport [14].

3. Final comments

New and adapted methods for analysis of the proteome of mammalian milks are set greatly to enlarge our understanding of the functions of milk proteins, from the most abundant to those only present in trace amounts but nevertheless important to the health and development of neonates. Two major practical consequences should arise. The first is the understanding of how the proteome of milk changes in disease, and how these changes could be used to detect mastitis of different aetiologies and treat accordingly. The second is an increased perception of the changes in milk at the onset, maturation and cessation of lactation, and how the proteins and other components therein are involved in protecting a tissue, the mammary gland, that is dramatically changing its structure, metabolic activity, cellular composition, and secretions, and how the protein composition alters during the transition between the different phases of lactation. This will also have a direct bearing on how milk protects the neonate from infections in addition to a myriad of other positive influences in nutrition and developmental signals, particularly those to do with immune development of the gut. There is considerable interest now in the microbiome of infants and how it relates to present and future health. Milk will not only be a vehicle for the delivery of appropriate microbes for colonisation of the newborn's gut, but will also control and refine the nature of the microbiome. As highlighted in the [introduction](#), it is also possible that a full understanding of milk and how it changes in health and disease may either lead to improvements in the design of artificial formulae, or an increased realisation that such formulations cannot fully substitute for all of the functions of milk produced by the females of a species for its own young.

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