DOI 10.1002/mnfr.200700311 Mol. Nutr. Food Res. 2008, *52*, 1448 – 1456

Research Article

Analysis of major proteins and fat fractions associated with mare's milk fat globules

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Although several studies have aimed to identify mare's milk proteins, only the major whey proteins and some caseins have yet been characterized. Incomplete sequencing of the equine genome and the difficulty of recovering highly hydrophobic proteins mean that little is known to date about the proteins associated with milk fat globules, which have been shown to play an important role in newborns' defense mechanisms. The fat fraction, in particular the distribution of unsaturated fatty acids, has been more extensively studied, but complex lipids are only partially elucidated. This study reports a 2-DE approach combined with a powerful method for *de novo* protein sequencing, and quali-quantitative data on complex lipid composition determined by high performance TLC (HPTLC) and GC. The presence in mare's milk of long-chain highly unsaturated fatty acids, and the evidence of close similarity between equine and human milk fat globule membrane proteins, support the use of mare's milk for human nutrition.

Keywords: Fatty acid composition / Mare milk / Mass spectrometry / Milk fat globule membrane / Proteomics

Received: August 8, 2007; revised: February 8, 2008; accepted: February 11, 2008

1 Introduction

Milk is the main source of nutrition for newborn mammals during the neonatal period, and also an important means to transfer acquired immunity against pathogens from mother to newborn. The milk secreted immediately after birth (colostrum) has a unique protein composition, containing high amounts of specific Igs in addition to the major milk proteins [1]. Other constituents of milk are triglycerides, complex lipids and simple sugars, primarily lactose. The composition of mare's milk fat, in addition to the properties of its protein fraction, suggests that this product is more similar to human milk than is cow's milk [2]. For this reason, and because of the low cross-reactivity between cow's and mare's milk proteins, a clinical study has suggested that mare's milk could be used as a valid replacement for cow's milk in children with severe IgE-mediated cow's milk protein allergy [3]. Moreover, because of its high content of long-chain PUFA (LC-PUFA) [4 – 5] and vitamins, mare's milk has excellent nutritional value and good digestibility, resembling human milk in both the whey protein/casein ratio (50:50) and the low mineral salt content [2].

Harris [6] suggests dietary fat as a factor influencing human health, particularly in cardiovascular diseases. Health is improved not only by a general reduction of dietary fat intake, but by a reduction in the saturated/unsaturated fatty acids ratio. In this respect, mare's milk, which contains higher amounts of LC-PUFA than cow's milk fat [7], could be a good substitute for cow's milk. In central, Asian and eastern European countries, fresh and fermented mare's milk products are already widely consumed for their health-giving properties. Several studies have aimed to identify mare's milk proteins, although thus far only the major whey proteins and some caseins have been characterized [8 – 12]. Due to incomplete sequencing of the equine genome and the difficulty of recovering highly hydrophobic proteins, little is yet known about MFGMP (proteins associated with the milk fat globule membranes) of mare's milk. The lipid composition of fat globules of mare's milk has been studied, and changes in the fat content correlated with

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Abbreviations: GD3, disialoganglioside; **GM3,** monosialoganglioside; **HPTLC,** high performance TLC; **IPG,** immobilized pH gradient; **LC-PUFA,** long-chain PUFAs; **MFGM,** milk fat globule membranes; **MFGMP,** proteins associated with the milk fat globule membranes

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the lactation period [13, 14] and feeding regime of the lactating mares [7]. A milk fat globule normally consists of a triglyceride droplet surrounded by a protein-rich lipid bilayer, known as milk fat globule membranes (MFGM) [15]. In mare's milk, the fat is organized in globules of about 2 – 3 μm [2]. In human milk, the fat globules have an average diameter of about 4 μm and the MFGMP only accounts for 1% of the total globule mass (4% of whole milk) [15]. Study of MFGMP is of great importance because, in recent years, it has been shown that these proteins possess specific functional activities [16].

This study describes a systematic proteomic 2-DE analysis of MFGMP from mare's milk, including identification of the major proteins by MS and characterization of all classes of lipids and fatty acids in the milk fat globule.

The aim was to supply further molecular information on the fat fraction of mare's colostrum and mature milk including a qualitative evaluation of the associated proteins in comparison to human milk, for human nutritional purposes.

2 Materials and methods

2.1 Preparation of mare's milk fat globules

Mare's milk samples were collected from several mares belonging to the same farm, on day 1 and day 30 postpartum. A protease inhibitor (Complete; Roche Diagnostics, Basle, Switzerland) was added to the specimens to prevent proteolysis, and milk samples were immediately stored at – 20°C until use. Two pools, one of colostrum and the other of mature milk, were created and centrifuged at $5000 \times g$ for 30 min at 6°C to remove impurities. Whole milk was centrifuged at $189\,000 \times g$ for 70 min at 6°C: fat globules were recovered in the supernatant layer and washed three times with 0.9% NaCl [17]. The fat globule recoveries were 1% w/v and 2% of the whole milk for mature milk and colostrum, respectively.

2.2 Sample protein preparation and 2-DE

The washed globule samples were incubated at room temperature for 1 h in a buffer containing 1% w/v 3-[*N*,*N*-dimethyl(3-myristoylaminopropyl)ammonio]propanesulfonate (ASB-14) and 1% v/v Triton X-100 as detergents, 7 M urea, 2 M thiourea, 20 mM Tris and 1% w/v DTT to extract the proteins associated with the fat globule membranes. After removing the floating cream layer, the protein solution was recovered and proteins were precipitated with methanol and chloroform, as described by Wessel and Flugge [18]. Proteins were solubilized in a rehydration buffer solution (7 M urea, 2 M thiourea, 4% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 20 mM Tris, 1% DTT v/v, and 0.5% v/v immobilized pH gradient (IPG) buffer) and loaded onto 13 cm IPG Strips (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) pH linear gradient 3 – 10. The total protein amount was determined using the 2-D Quant Kit (GE Healthcare). The in-gel rehydration method at room temperature was chosen for sample application to the strips; 80 μg of total protein extract in 250 μL of rehydration solution were loaded. IEF was carried out on an IPGphor unit (GE Healthcare) at 20°C constant temperature and 8000 V for a 89 000 total V · h. After IEF, strips were incubated for 15 min at room temperature in 6 M urea, 30% v/v glycerol, 2% w/v SDS, 50 mM Tris-HCl pH 8.6 enriched with 2% w/v DTT. A second equilibration step was carried out for 15 min in the same solution, with the exception that DTT was replaced by 2.5% w/v iodoacetamide plus a trace of bromophenol blue as tracking dye.

SDS-PAGE was carried out on homogeneous running gels 11.7%T, 2.6%C (Ettan DALT II system, GE Healthcare); running conditions were 400 V, 50 mA *per* gel for about 3 h. Gels were automatically stained using the Processor Plus (GE Healthcare) with freshly prepared Blue Coomassie Colloidal stain. 2-DE gels were digitized with GS-800 Densitometer (BioRad Laboratories, Hercules, CA, USA).

Four replicates were performed for each 2-DE gel. Spot intensities were measured as normalized spot volumes. Spots intensities were statistically analyzed by means of the *t*-test: means were considered significantly different when $p < 0.05$ (PG 220 software, Nonlinear Dynamics, UK).

2.3 Protein identification

After 2-DE, the most abundant spots were excised from the gel and submitted either to direct tryptic hydrolysis and MS analysis, or to passive transfer onto a polyvinylidene fluoride membrane to determine the N-terminal amino acid sequence. For N-terminal sequencing, the spots were excised from the gel and passively eluted as described elsewhere [19]. The membrane containing the transferred protein was directly microsequenced on a Procise 492 protein sequencer (Applied Biosystem, Foster City, CA, USA). The amino acid sequences were compared with protein sequences in the UNIPROT databases, using MPsrch software (http://www.ebi.ac.uk/MPsrch/) or BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). For MS analysis, excised gel pieces were destained overnight in a solution of 50 mM ammonium bicarbonate and 40% v/v ethanol and then digested with sequencing-grade modified trypsin, as described by Speicher *et al*. [20].

The peptide mixture was desalted and concentrated using ZipTipC18 devices (Millipore, Billerica, MA, USA). After elution with 50% methanol containing 1% formic acid, 4 μL of the tryptic peptide solution was analyzed by directly applying the samples into the borosilicate capillary (New Objective, Woburn, MA) for MS/MS sequencing experiments with an IT spectrometer (LCQ, Thermo Fisher Scientific, Waltham, MA, USA) fitted with a nanoelectrospray

source operating in static mode. The spectra were automatically analyzed using SEQUEST software screening the OWL database, updated in March 2007. For the *de novo* sequencing experiments, after concentration an aliquot of the tryptic peptide mixture was derivatized by addition of acetic anhydride, as described elsewhere [21]. The MS spectra data were interpreted manually and the database search was performed by MS Homology software (http://prospector.ucsf.edu/prospector/4.0.8/html/mshomology.htm).

2.4 Extraction and analysis of fat fractions

Milk and colostrum were analyzed before lipid extraction, to quantify total sialic acid content following the colorimetric method proposed by Svennerholm and Fredman [22]. Lipid extraction was performed as described elsewhere [23]. The total organic extract was lyophilized and partitioned twice with choloform/methanol/water (3:48:47 v/v/v) to separate gangliosides from phospholipids and other nonpolar lipids. The aqueous phase containing gangliosides was lyophilized, resuspended with choloform/methanol (2:1 v/v) and fractionated by high performance TLC (HPTLC) (silica gel plates 10×10 cm^2, Merck, NJ, USA) in the following experimental conditions: solvent, choloform/methanol/water (60:40:9 v/v/v) with $CaCl_2$ 0.2% w/v; detection spray, ethanol/hydrochloric acid (4:1 v/v) containing 6 g dimethylaminobenzaldehyde (Ehrlich's spray) at 120°C for 20 min. The organic phase was purified and fractionated by silicic acid column chromatography with the following solvents: chloroform for neutral lipids, acetone/methanol, 9:1 v/v for neutral glycolipids, and methanol for phospholipids. Neutral lipids were separated and quantified by HPTLC in the following experimental conditions: solvent, hexane/diethylether/acetic acid (90:10:1 v/v/v); detection spray, 10% $CuSO_4$ in 8% phosphoric acid at 180°C for 20 min. The main classes of neutral glycolipids were separated and quantified by HPTLC in the following experimental conditions: solvent, chloroform/methanol/water (110:40:6 v/v/v); detection spray, 0.1 g diphenylamine in 9 mL acetone 1 mL sulfuric acid 0.1 mL aniline at 120°C for 20 min. The main classes of phospholipids were separated and quantified by HPTLC in the following experimental conditions: solvent, chloroform/methanol/acetic acid/water (60:45:4:2 v/v/v/v); detection spray, 0.25 mL *p*-anisaldehyde in 25 mL acetic acid and 0.5 mL phosphoric acid at 120°C for 5 min. The percentage distribution of the single molecular species was determined by densitometric scanning HPTLC using a calibration curve with pure standard (Sigma–Aldrich, St. Louis, MO, USA), spotted at three known concentrations [24]. Fatty acids of the lipid fraction were determined by GC analysis. The methylester fatty acids were obtained with sodium methoxide in methanol, 3.33% w/v, and injected into an Agilent gas-chromatograph (HP 5890, series II plus, Agilent Technologies, Palo Alto, CA, USA), equipped with a flame ionization detector HP 7673 under the following experimental conditions: capillary column AT Silar (Alltech Associates, Deerfield, IL) (5% methyl polysiloxane) length 30 m, id 0.332 mm; film thickness 0.25 μm; gas carrier: helium, flow rate 5 mL/min; temperatures: injector 250°C, detector 275°C, oven 50°C for 2 min, rate 10°C/min until 200°C for 20 min. A standard mixture (Sigma–Aldrich) containing the major fatty acids was injected to identify sample peaks [25]. The statistical analysis was performed with the ANOVA test. Five replicates were submitted to lipid fraction analysis for each sample.

3 Results and discussion

3.1 MFGM isolation

The pooled milk samples were ultracentrifuged to obtain the floating MFGM layer, the whey and the casein pellet. The casein pellet was solubilized and the proteins separated by 2-DE and analyzed by MS, to confirm the nature of the proteins in this fraction. Only caseins were detected (data not shown) demonstrating that the great majority of MFGMP remained in the supernatant layer after a single freeze/thaw cycle.

3.2 Protein extraction

Membrane protein purification is problematic due to the difficulty of extracting proteins from the bilayer, keeping them in solution after extraction, and removing other components such as lipids and carbohydrates. Intrinsic membrane proteins are frequently lost during 2-DE because of their not being in solution prior to IEF. For this reason, Reinhardt *et al*. [26] proposed a 1-D SDS gel electrophoresis combined with high performance resolution LC MS/MS to identify bovine MFGMP. In our case, this approach was not suitable, because of the lack of a complete equine genome/protein database. In order to avoid both problems, we used a 2-DE approach with an extraction step employing a buffer containing ASB-14 as detergent, combined with a powerful method for *de novo* protein sequencing [21]. After protein extraction, as suggested by Wessel and Flugge [18], a precipitation step was added to remove lipids, which are the major contaminants in MFGMP preparation and cause vertical streaks in the second dimension of 2-DE [27]. The method was found to be very effective.

3.3 Protein identification

Figures 1A and B show the spots selected for protein identification from 2-DE maps of mare's colostrum and milk, respectively. All the spots chosen for MS analysis were present in all replicate gels. Spots labeled with numbers were successfully identified while those labeled with

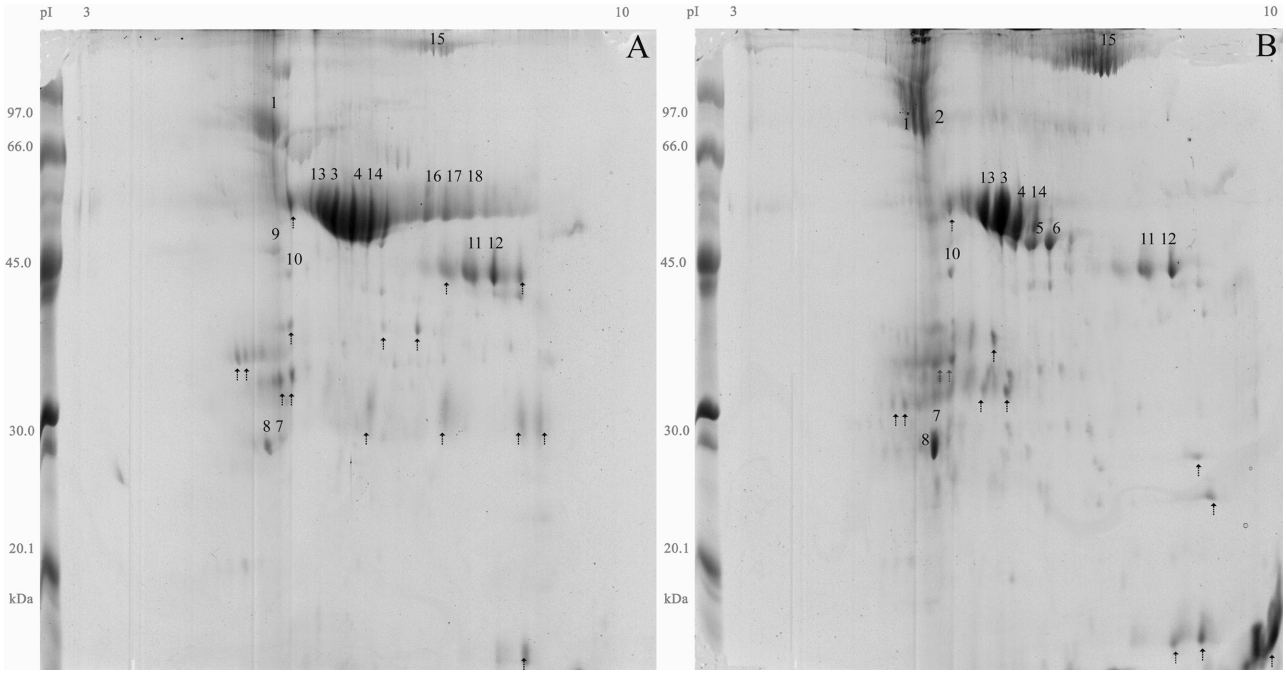

Figure 1. 2-DE maps of mare's colostrum (A) and milk (B) fat globule membrane proteins; identified proteins are marked with numbers. The same numbers are used to identify the same proteins in colostrum and milk samples.

arrows could not be identified, either because amounts were small or due to the lack of homology with known proteins. Since few equine DNA and protein sequence data are available, the PMF strategy for protein identification was not feasible; to achieve protein identification a *de novo* MS/MS sequencing approach was therefore set up. As this method requires larger amounts of protein to be effective, we first focused our attention on the larger spots expressed in the 2-DE maps; this could explain why we failed to identify some already-known MFGMP, such as CD 36 and fatty acid binding protein, which have been identified in the milk of other species [26]. Fifteen forms of eight unique proteins were identified by homology to either *Homo sapiens*, *Canis familiaris*, or *Bos taurus*, as listed in Table 1.

In Fig. 1, spots 1 and 2 correspond to butyrophilin, a type I transmembrane glycoprotein. In this case, we identified the major expressed isoform with an estimated M_r of 86 kDa, corresponding to one of the glycoforms already identified in human milk by Cavaletto *et al*. [28]. Sequence coverage of equine butyrophilin obtained by MS/MS sequencing suggests that this protein is similar to human butyrophilin, and consists of two extracellular Ig superfamily domains, a transmembrane domain and a large intracellular domain, containing a B30.2 domain that has been reported in several pathological conditions [28]. Spots 5 and 6 (Fig. 1) comprise adipophilin and spot 15 xanthine oxidoreductase. Together with butyrophilin, these two proteins are involved in the fat globule secretion mechanism. Adipophilin is a lipid droplet-associated protein thought to reside exclusively in the envelope of lipid droplets and to be involved in droplet formation [29]. The hydrophobic N-terminus of adipophilin appears to be suitable for interaction with lipid droplets [29]. Xanthine oxidoreductase, a soluble homodimeric cytoplasmic enzyme, is concentrated along the inner surface of the apical plasma membrane, where it is assumed to bind with high affinity to the cytosolic domain of plasma membrane butyrophilin [29]. Butyrophilin and xanthine oxidoreductase are understood to link milk secretory granules to the plasma membrane, for secretion by interacting with adipophilin at the milk secretory granule surface, facilitating the envelopment of the granule with plasma membrane during milk fat droplet formation [29]. Spots 3, 4, 13, and 14 (Fig. 1), with M_r ranging from 50 to 55 kDa, are probably glycosylation variants of a peripheral membrane glycoprotein called lactadherin. Lactadherin was also identified in spots 11 and 12 at lower M_r (42 kDa), probably being a truncated form of these glycoforms [30]. Lactadherin is a multidomain protein: human lactadherin has an epidermal growth factor domain in the N-terminal part of the sequence, while in other species, such as the cow, mouse and pig, there are two similar domains [16]. The C-terminal part of lactadherin from all these species contains a tandem repeated domain similar to C1, and C2 domains of blood coagulation factors V and VIII [31]. The search by homology for horse lactadherin showed approximately 74% identity with human lactadherin (while cow's/human lactadherin identity is around 65%) and excellent identity (around 97%) with an equine protein known as

Table 1. Identification of selected proteins from mare's colostrum and milk fat globule membrane 2-DE gels

Spot no.	M_r (kDa)	p*I*	MS/MS sequencing	Protein name	Organism ID Swiss-Prot or NCBI	% identity/ %homology[a)]
1 2	85.6	5.4	DVIGPP[b)]-FTVTASVVIR-FDSWPCVLGR-HYWEVEVGDR-TDWAIGVCR-TPLPLAGPPR	Butyrophilin	*H. sapiens* Q13410	97/100
3	53.4	6.3	NMFETPFLAR-VFVGNVDNSGLK-TGIVNAWTASNYDK-GDVFTQYICSCPR-IFPGNLDNNSHK-DFGHIQYVAAYK-VNMFDVPLEVQYVR-FNAWTAQSNSASEWLQVDLGSQK-VTGVVTQGASR	Lactadherin	*Equus caballus* O77718	96/97
4	51.4	6.5	NMFETPFLAR-GDVFTQYICSCPR-IFPGNLDNNSHK-DFGHIQYVAAYK-VNMFDVPLEVQYVR-FNAWTAQSNSASEWLQVDLGSQK-VAYSVDGR-DAGDSKDK-EVTGVITQGAR	Lactadherin	*E. caballus* O77718	97/99
5	49.2	6.7	NLPLVSSTYDLVSSAYLSTK-LEPQIAIANTYACK-SVVNGSINTVLGSR-QLVSSGVENALTR-KVEGFDMVQKPSYYIR-AYQQALSR-TISQLHSTVNLIEFAR-LYLSWVEWK	Adipose differentiation-related protein (ADRP) (Adipophilin)	*H. sapiens* Q99541	95/98
6	48.7	6.9	NLPLVSSTYDLVSSAYLSTK-LEPQIAIANTYACK-PILNQPNTQVVANAR-DAVTTTVTGAK-DSASTV(D/N)GVMDR-GAVTGSVEK-SVVNGSINTVLGSR-QLVSSGVENALTR-KVEGFDMVQKPSYYIR-AYQQALSR-TISQLHSTVNLIEFAR-IQDAQDK-LYLSWVEWK	ADRP (Adipophilin)	*H. sapiens* Q99541	94/98
8	25.5	5.3	QGLLPVLESLK-DLATVYVDAVK-VQPYLDDFQK-AAIDEASK-DNWDSLGTTLGK-VAQFEATGLGK	Apolipoprotein AI	Canis amiliaris P02648	88/94
7	24.6	5.4	TLTPSYYR-NDIVNMLVGNK-LAIWDTAGQER-IIQTPGLWSENQNK-LDNWLNELETYCTR-FTDDTFDPELAATIGVDFK-GAQGVILVYDVTR	Ras-related protein Rab-18	*H. sapiens* Q9NP72	94/95
9	33.5	5.4	LLLAGYDDFNCNVWDALK-VSCLGVTDDGMAVATGSWDSFLK-LIIWDSYTTNK-IYAMHWGTDSR-ADQELMTYSHDNIICGITSVSFSK-VHAIPLR-LFVSGACDASAK	Guanine nucleotide-binding protein G(I)/ G(S)/G(T) beta subunit 1	*H. sapiens* P62873	100
10	33.5	5.7	LLLAGYDDFNCNVWDALK-KACADATLSQITNNIDPVGR-VSCLGVTDDGMAVATGSWDSFLK-LLVSASQDGK-LIIWDSYTTNK-IYAMHWGTDSR-ADQELMTYSHDNIICGITSVSFSK-ACADATLSQITNNIDPVGR-ACGDSTLTQITAGLDPVGR-VHAIPLR	Guanine nucleotide-binding protein G(I)/ G(S)/G(T) beta subunit 1	*H. sapiens* P62873	100
11 12	42.3	8.1 8.4	TGIVNAWTASNYDK-VFVGNVDNSGLK-DFGHIQYVAAYK-GDVFTQYICSCPR-VNMFDVPLEVQYVR	Lactadherin	*E. caballus* O77718	97/97
13	54.6	6.0	NMFETPFLAR-VFVGNVDNSGLK-IFPGNLDNNSHK-DFGHIQYVAAYK-TGIVNAWTASNYDK-GDVFTQYICSCPR-VNMFDVPLEVQYVR-FNAWTAQSNSASEWLQVDLGSQK	Lactadherin	*E. caballus* O77718	97/98

Table 1. Continued

Spot no.	M_r (kDa)	p*I*	MS/MS sequencing	Protein name	Organism ID Swiss-Prot or NCBI	% identity/ %homology[a)]
14	54.1	6.7	VTGVVTQGASR-EVTGVITQGAR-NMFETPFLAR-VFVGNVDNSGLK-IFPGNLDNNSHK-DFGHIQYVAAYK-TGIVNAWTASNYDK-GDVFTQYICSCPR-VNMFDVPLEVQYVR	Lactadherin	*E. caballus* O77718	96/97
15	115.3	7.4	SVASVGGNIITASPISDLNPVFMASGAK-IPAFGSIPIEFR-LFQPGSMQVKELALCYGGMADR	Xanthine dehydrogenase/ oxidase (XDH)	*B. taurus* P80457	95/98
16 17 18	52.3	7.5 7.8 8.1	SQTYICNVAHPASSTK-TISKPTGQPR-FYPTDIDIEWK-YSTTPAQLDSDGSYFLYSK	Ig gamma 4 heavy chain	*E. caballus* CAC44762	100

M_r and p*I* values were deduced from 2-DE maps. Maps were calibrated using the calibration tools of PG 220 software creating a standard calibrated curve based on MW markers ranging 14–97 kDa. The XOD falls outside the calibration curve, as consequence the estimated MW in not accurate. Peptide sequences are separated by dashes.

a) Identity: % of identical amino acids found at the same position; homology: % of identical and physicochemically similar amino acids found at the same position; homology ≥ identity.

b) This peptide has been confirmed also by N-terminal sequencing.

sperm-membrane associated protein P47. This is a protein localized at the apical ridge or throughout the acrosomal region in testicular spermatozoa, suggesting a possible adhesive role in fertilization [32]. The physiological function of lactadherin is not fully known; however, in human milk a protective function against rotavirus infection at the gut level has been demonstrated [33]. The entire sequence and glycosylation sites of this protein from mare's milk are currently under investigation in our laboratory.

These identifications confirm the effectiveness of the extraction of different types of membrane protein: intrinsic (xanthine oxidase), extrisic (lactadherin), and transmembrane (butyrophilin).

3.4 Analysis of fat fractions

Table 2 shows the quantities of sialic acid and lipid in mare's milk and colostrum determined as described in Section 2. As expected, all values are higher in colostrum than in milk, reflecting the importance of these components, especially in the neonatal period. This is in line with reports by Wang *et al*. [34], who found that human milk showed a significant decline in total sialic acid concentration over time, reflecting the important role played by sialic acids in normal brain development, mainly in the first few days of life. Based on this similarity, it can be hypothesized that mare's colostrum and milk could successfully be used as substitutes for human milk. It is also well known that the diet can modify fat composition [35]; however, this factor is not relevant in our study, because all the animals providing milk or colostrum specimens ate the same diet. Sialidated oligosaccharides in human milk can act as highly specific receptors for a variety of viruses, bacteria, and parasites. Both free and bound sialidated oligosaccharides present in human milk have been shown to prevent infant diarrhea associated with rotavirus infection [36]. Table 2 highlights a difference in the concentration of membrane-associated lipids between milk and colostrum: cholesterol and phospholipid content are markedly different. Cholesterol is the major sterol, located mostly in the milk lipid globule membrane, where it influences the physical state and packing density of phospholipids. A recent study reports that fatty acids from sphingomyelin and phosphatidylcholine, by interacting with cholesterol, participate in the lipid raft formation of biological membranes [37]. The high level of cholesterol and sphingomyelin, as shown in Table 2, suggests the presence of possible lipid raft structures in MFGM of mare's milk. This hypothesis is supported by the high phosphatidylcholine content. A large number of signaling proteins are targeted to lipid rafts, where they play an important role in health/disease status [38]. Based on the fact that sphingomyelin metabolism generates anticancer molecules, such as ceramide and sphingosine, Duan [39] has suggested using MFGM or dairy products in the human diet, as therapeutics for the prevention or even for the suppression of colon tumors. Noh and Koo [40] recently showed sphingomyelin to be an effective inhibitor of intestinal absorption of cholesterol in rats, through a direct inhibiting effect of the highly saturated long chains of the acyl groups of the molecule, which influences the rate of luminal lipolysis, micellar solubilization, and transfer of micellar lipids to the enterocyte.

Table 2. Composition of mare's milk and colostrum: sialic acid and lipids

Component	Sample	
	Milk	Colostrum
Sialic acid	0.431 ± 0.042[a)*]	1.333 ± 0.254[a)*]
Triglycerides	1.033 ± 0.078[a)*]	2.045 ± 0.072[a)*]
Cholesterol	0.088 ± 0.005[a)*]	0.245 ± 0.019[a)*]
Phospholipids	47.194 ± 8.697[b)*]	92.470 ± 12.490[b)*]
Phosphatidylethanolammine	7.775 ± 0.628[c)**]	9.041 ± 1.181[c)**]
Phosphatidylinositol	2.758 ± 0.702[c)**]	1.286 ± 0.200[c)**]
Phosphatidylserine	5.533 ± 1.496[c)]	6.242 ± 2.462[c)]
Phosphatidylcholine	46.885 ± 4.456[c)]	45.654 ± 2.603[c)]
Sphingomielin	37.050 ± 2.211[c)]	37.778 ± 1.543[c)]
Clycolipids	9.446 ± 1.074[b)*]	14.207 ± 1.587[b)*]
Glucocerebroside	55.278 ± 3.928[d)]	53.562 ± 4.814[d)]
Lactocerebroside	26.105 ± 4.373[d)]	3.549 ± 1.172[d)]
Sulfatide	18.898 ± 2.490[d)*]	42.889 ± 4.064[d)*]
Gangliosides	1.647 ± 0.209[b)*]	3.223 ± 0.497[b)*]
Gm_3	35.531 ± 2.835[e)]	18.715 ± 4.111[e)*]
Gd_3	23.063 ± 4.532[e)]	53.756 ± 6.798[e)*]
Gd_{1a}	16.743 ± 4.264[e)]	5.469 ± 1.540[e)*]
Gd_{1b}	n.d.	15.193 ± 2.648[e)*]
Gt	24.663 ± 6.640[e)]	6.866 ± 2.496[e)*]

All values are expressed as means ± SD (*$p < 0.01$, **$p < 0.05$).

a) Concentration expressed in mg/mL.
b) Concentration expressed in μg/mL.
c) Concentration expressed as percentage of total phospholipids.
d) Concentration expressed as percentage of total glycolipids.

Glycolipids are also minor components of MFGM; different types of glycolipids, namely cerebrosides and sulfatide, were found in the MFGM of mare's milk, as Table 2 shows. These had different expression levels during lactation. The high proportion of sulfatide in the early stage of lactation is of great interest, as it is a major component of both the central and the peripheral nervous systems [41]. Gangliosides present in the neural tissues in relatively high concentrations are also found in mare's milk and colostrum, as Table 2 shows. They chiefly occur as GM3 and disialoganglioside (GD3), and their profiles during lactation are similar to that for human milk [42]. The concentration of GM3 increases during lactation [42], and is the major ganglioside of mare's milk, while GD3 is the major ganglioside of mare's colostrum and decreases during lactation [43] (Table 2). Milk gangliosides are considered to be bioactive components in infant nutrition, because they reduce the adhesion of pathogens to human intestinal cells, inhibiting

Table 3. Percentage distribution of phospholipid and triglyceride fatty acids in mare's milk and colostrum

Component		Sample	
		Milk%	Colostrum%
Phospholipids			
Lauric acid	12:0	1.230 ± 0.232	n.d.
Myristic acid	14:0	4.901 ± 0.265	2.107 ± 0.152*
Palmitic acid	16:0	35.003 ± 0.967	28.599 ± 0.778*
Palmitoleic acid	16:1	4.020 ± 0.424	2.457 ± 0.219*
Stearic acid	18:0	18.003 ± 3.564	19.345 ± 0.872
Oleic acid	18:1	14.227 ± 1.113	15.655 ± 0.444**
Linoleic acid	18:2	12.854 ± 1.224	21.072 ± 0.796*
Linolenic acid	18:3	5.058 ± 0.322	9.306 ± 0.530*
Triglycerides			
Caprilic acid	8:0	1.824 ± 0.013	1.163 ± 0.169*
Capric acid	10:0	4.319 ± 0.04	3.305 ± 0.468*
Lauric acid	12:0	5.671 ± 0.016	3.225 ± 0.107*
Myristic acid	14:0	7.610 ± 0.018	4.699 ± 0.029*
Myristoleic acid	14:1	0.706 ± 0.003	n.d.
Palmitic acid	16:0	25.187 ± 0.061	23.527 ± 0.164*
Palmitoleic acid	16:1	7.791 ± 0.027	5.332 ± 0.211*
Stearic acid	18:0	1.124 ± 0.068	1.974 ± 0.156**
Oleic acid	18:1	24.215 ± 0.054	20.733 ± 0.238*
Linoleic acid	18:2	6.856 ± 0.011	8.636 ± 0.080*
Linolenic acid	18:3	12.923 ± 0.033	26.775 ± 0.213*

All values are expressed as means ± SD (*$p < 0.01$, **$p < 0.05$).

the enterotoxic effects of *Escherichia coli* [44]. It has been shown that they influence the composition of the fecal flora in preterm infants and promote the growth of bifidobacteria in the infant's gastrointestinal system [45]. Table 3 shows the percentage distribution of fatty acids found in phospholipids and triglycerides of mare's milk and colostrum. Essential fatty acids, *i.e*., linoleic acid (18:2n-6) and alpha-linolenic acid (18:3n-3), are significantly higher in colostrum than in milk (Table 3). They are precursors of LC-PUFA, including eicosapentaenoic, docosahexaenoic, and arachidonic acids, essential structural components of all cell membranes, incorporated in relatively large amounts during early growth of the brain and retina. Moreover, LC-PUFA are precursors of eicosanoids, molecules with potent biological activity that modulate various cellular and tissue processes [46]. The increased interest in the potential health benefits associated with the consumption of long-chain n-3 fatty acids is testified by the number of publications on the subject, and has led to the sale of supplements and fortified foods containing these fatty acids.

4 Concluding remarks

Milk fat globules are an important part of milk as a food in all mammalian species, for both their nutritional and bioactive properties.

The presence in mare's milk of long-chain highly unsaturated fatty acids and the evidence of a close similarity between mare's and human MFGMP support the use of this product for human nutrition. Further studies will be necessary on protein characterization (*i.e*., the extent of post-translation modifications) to determine whether this milk could have the same biological activity as human milk.

This work was partially supported by the Foundation of Biotechnology of Turin, with thanks to the Business Unit Nutrition (B.U.N.) of Nestlè-Italiana, S.p.A.

The authors have declared no conflict of interest.

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