

DATASET BRIEF

Proteomics of the milk fat globule membrane from *Camelus dromedarius*

Besma Saadaoui¹, Céline Henry², Touhami Khorchani³, Mohamed Mars¹, Patrice Martin⁴ and Christelle Cebo⁴

¹ Faculté des Sciences de Gabès cité Erriadh Zrig, Tunisia

² INRA, UMR 1319 MICALIS, Plateforme PAPSSO (Plateforme d'Analyse Protéomique Paris Sud Ouest), Jouy-en-Josas, France

³ Laboratoire d'Élevage et de Faune Sauvage, Institut des Régions Arides, Medenine, Tunisia

⁴ INRA, UMR 1313 Unité Génétique Animale et Biologie Intégrative, Jouy-en-Josas, France

Camel milk has been widely characterized with regards to casein and whey proteins. However, in camelids, almost nothing is known about the milk fat globule membrane (MFGM), the membrane surrounding fat globules in milk. The purpose of this study was thus to identify MFGM proteins from *Camelus dromedarius* milk. Major MFGM proteins (namely, fatty acid synthase, xanthine oxidase, butyrophilin, lactadherin, and adipophilin) already evidenced in cow milk were identified in camel milk using MS. In addition, a 1D-LC-MS/MS approach led us to identify 322 functional groups of proteins associated with the camel MFGM. Dromedary MFGM proteins were then classified into functional categories using DAVID (the Database for Annotation, Visualization, and Integrated Discovery) bioinformatics resources. More than 50% of MFGM proteins from camel milk were found to be integral membrane proteins (mostly belonging to the plasma membrane), or proteins associated to the membrane. Enriched GO terms associated with MFGM proteins from camel milk were protein transport ($p\text{-value} = 1.73 \times 10^{-14}$), translation ($p\text{-value} = 1.08 \times 10^{-11}$), lipid biosynthetic process ($p\text{-value} = 6.72 \times 10^{-10}$), hexose metabolic process ($p\text{-value} = 1.89 \times 10^{-04}$), and actin cytoskeleton organization ($p\text{-value} = 2.72 \times 10^{-04}$). These findings will help to contribute to a better characterization of camel milk. Identified MFGM proteins from camel milk may also provide new insight into lipid droplet formation in the mammary epithelial cell.

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According to the Food and Agriculture Organization (FAO), there are about 25 million camels in the world, of which 21

million are found in Africa, mostly in the arid areas of East Africa (Somalia and Ethiopia) where camels are often the only source of livelihood. Camel milk production accounts for only 0.3% of world milk production but interest for camel milk is growing [1]. First, the ability of camels to produce milk despite the low quality forage of their hostile environment is a very attractive topic for milk producers (better efficiency in feed conversion). Camel milk could also be a way to develop the local economy. Finally, many health-promoting properties are

Correspondence: Dr. Christelle Cebo, INRA, Unité GABI, Equipe Lait, Génome et Santé (LGS), Domaine de Vilvert, Bâtiment 221, 78352, Jouy-en-Josas cedex, France
E-mail: christelle.cebo@jouy.inra.fr
Fax: +33-1-34-65-29-26

Abbreviations: DAVID, Database for Annotation, Visualization, and Integrated Discovery; ER, endoplasmic reticulum; LDH, lactadherin; MEC, mammary epithelial cell; MFGM, milk fat globule membrane

Colour Online: See the article online to view Fig. 2 in colour.

attributed to camel milk. A camel whey protein presents some similarities with the insulin family of proteins and it has been demonstrated that the consumption of camel milk in diabetic patients significantly reduces the dose required to maintain glycaemic control [2]. Due to its lower protein contents and the absence of β -lactoglobulin (a highly immunogenic lactoprotein), camel milk could be an alternative for cow milk allergy in children [3]. Also, the level of lactoferrin, an antimicrobial protein, is higher in camel milk than in cow milk [4].

Casein and whey proteins fractions of camel milk have been extensively characterized [5]. However, studies on the milk fat globule membrane (MFGM), the membrane surrounding fat globules in milk, are nearly absent in the camel species and are solely devoted to the lipid fraction of MFGM [6]. Contrariwise, large-scale studies have been published for bovine MFGM proteins [7] and more recently, for ovine [8] MFGM proteins. Thus, the aim of this study was to provide a thorough characterization of MFGM proteins from camel milk.

Individual milk samples were collected from four multiparous lactating dromedaries (*Camelus dromedarius*) at 194 ± 3 days postpartum (Experimental station of the Arid Land Institute of Medenine, Tunisia) and stored at -20°C until use. For MFGM preparation, milk samples were thawed and incubated at 37°C for 30 min. Milk samples were pooled, and a 45 mL-sample from the pool was centrifuged at $1000 \times g$ for 20 min at 20°C . Fat globules were recovered in the supernatant layer and washed twice with 40 mL of a 0.9% w/v NaCl solution to remove residual caseins and whey proteins eventually adsorbed to fat globules. MFGMs were prepared from the washed milk fat as previously described [7] with noteworthy exception of the MFGM intrinsic protein enrichment step. Protein concentration was determined using the Bio-Rad RC-DC Protein Assay (Bio-Rad, Marnes-la-Coquette, France). MFGM samples were stored at -80°C until use. Twenty-five micrograms of MFGM proteins were solubilized in the Laemmli buffer and resolved by 10% SDS-PAGE, followed by staining with Bio-Safe Coomassie (Bio-Rad).

Figure 1 shows an SDS-PAGE representative pattern of MFGM proteins from *C. dromedarius*. Caseins, which represent up to 80% of milk proteins, were efficiently removed from our MFGM preparations, although minor bands with a molecular weight between 20 and 40 kDa (casein region) were still present, being associated to fat globule membranes, as previously reported for bovine MFGM [7]. In contrast, most MFGM proteins displayed a molecular weight of 40 kDa and greater in 10% SDS-PAGE, thus demonstrating that MFGM-associated proteins were significantly enriched using our extraction protocol.

The MFGM protein profile was in good accordance with our previous studies on goat [9] or horse MFGM [10]. Fatty acid synthase (FAS), xanthine oxidase (XO), butyrophilin (BTN), and lactadherin (LDH), which are major proteins of the MFGM, were identified within MFGM proteins from camel milk by MS. Butyrophilin from camel milk was identified as a 63 kDa protein, i.e. slightly smaller in size than

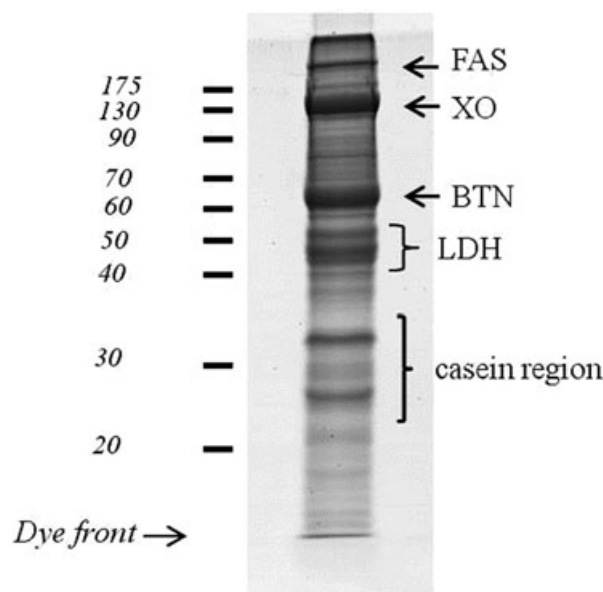


Figure 1. Representative pattern of camel milk fat globule membrane (MFGM) proteins in SDS-PAGE. FAS = fatty acid synthase; XO = xanthine oxidase; BTN = butyrophilin; LDH = lactadherin.

its bovine or caprine counterparts [11]. LDH from camel milk was characterized as two major polypeptide variants of 49 and 55 kDa whereas one, two, and up to four glycoproteins were identified for LDH from goat [9], cow [12], or horse milk [10], respectively. Interestingly, LDH from llama milk also appears as two major glycoproteins in SDS-PAGE (our unpublished data). Llamas (*Lama glama*) belong to the *Camelidae* family, along with camels. Therefore, although we have highlighted a high molecular diversity of LDH protein between species [11], it appears that LDH features are conserved between closely related species (here, llamas and camels).

Each lane of the gel was cut into 26 pieces of gel. MFGM proteins were digested by 0.1 μg of modified trypsin (Promega, sequencing grade) for 6 h at 37°C . Peptides were extracted by 5% formic acid in water/ACN v/v. Tryptic peptides were dried and resuspended in 20 μL of 0.1% v/v TFA. HPLC was performed on an Ultimate 3000 LC system (Dionex). Buffers were 0.1% formic acid, 2% ACN (A), 0.1% formic acid, and 80% ACN (B). The peptide separation was achieved with a linear gradient from 0 to 36% B for 18 min at 300 nL/min. Eluted peptides were analyzed on-line with an LTQ-Orbitrap mass spectrometer (Thermo Electron) using a nanoelectrospray interface. Ionization (1.3 kV ionization potential) was performed with liquid junction and a capillary probe (10- μm id.; New Objective). Peptide ions were analyzed using Xcalibur 2.07 with the following data-dependent acquisition steps (i): full MS scan in orbitrap (mass-to-charge ratio (m/z) 300–1600, profil mode) and (ii) MS/MS in linear trap ($q_z = 0.25$, activation time = 30 ms, and collision energy = 45%; centroid mode). Step 2 was repeated for the four major ions detected in step 1. Dynamic exclusion time was set to 90 s.

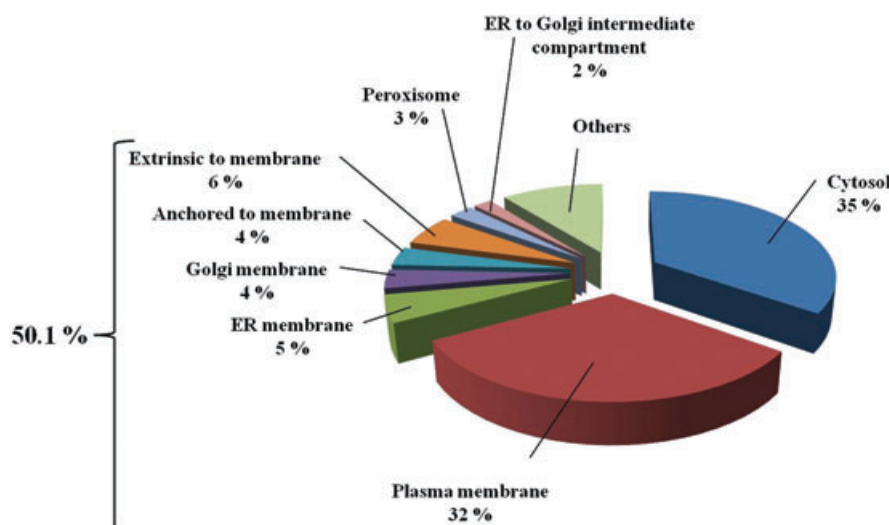


Figure 2. Cellular compartment assignment for camel MFGM proteins identified in this study.

In the absence of a fully annotated camel genome, two databases were used: the Mammalian UniprotKB database (January, 2012; 827, 782 protein entries) and the camel EST database (<http://camel.kacst.edu.sa/>) translated in six frames with ORF. These databases, in conjunction with protein to peptide reverse database (created by Database Manager v2.0) and contaminant databases were searched by algorithm: X!Tandem (version 2010.12.01.1). The precursor mass tolerance was 10 ppm and the fragment mass tolerance was 0.5 Da. One missed cleavage was allowed for trypsin. The carbamidomethylation of cysteine residues was set as a fixed modification and the oxidation of methionine residues as a variable modification. The results were filtered using in-built X!Tandem parser (X!Tandem pipeline version 3.1.5) with peptide E value of 0.05 a protein log (E value) of -4.0 and a minimum of two peptides. Experimental data have been deposited in the ProteomXchange database (a consortium of PRIDE, PeptideAtlas and Tranche databases; <http://www.proteomexchange.org/>) under the accession number # PXD000059.

Our search strategy led us to identify 322 functional groups of proteins associated with the camel milk fat globule membrane (Supporting Information Table 1). We calculated the false discovery rate performing identification with the protein to peptide reverse database and protein false discovery rate was 0.31%. Large-scale proteomic studies on cow [7, 13], sheep [8], and human [13] MFGM revealed that comparable amounts of proteins are associated with milk fat globules.

FASTA sequences (322) of LC-MS/MS identified MFGM proteins from camel milk were blasted onto a human UniprotKB/Swiss-Prot database by using the blastp suite at the NCBI site (<http://www.ncbi.nlm.nih.gov/>). Three-hundred and nineteen unique human UniprotKB entries (Supporting Information Table 1) were retrieved and used for further analysis using DAVID (the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.7). Three-hundred and sixteen DAVID identifiers (IDs) were recovered for bioinformatic analyses [14].

Proteins were classified into functional categories as follows: biological process (BP; 89.2% of DAVID IDs), cell compartment (CC; 93.4% of DAVID IDs), and molecular function (MF; 85.1% of DAVID IDs). Full annotation results are available in the Supporting Information Table 2.

Up to 50% of MFGM proteins from camel milk were found to be integral membrane proteins (mostly belonging to the plasma membrane), or proteins associated to the membrane, as for the major MFGM protein, LDH, which is an extrinsic protein (see Fig. 2 for an overview). Other membrane proteins originated from the endoplasmic reticulum (ER) or Golgi compartments, thus accounting for the dynamic activity of lipid droplets in the cell. Indeed, lipids are released from ER as protein-coated cytoplasmic lipid droplets. Cytoplasmic lipid droplets migrate at the apical pole of the MEC and are progressively wrapped up by the plasma membrane to be released as fat globules in milk. The milk fat globule membrane (MFGM), the membrane surrounding fat in milk, therefore has a complex structure, comprised of two membranes, a phospholipid monolayer derived from the ER, and an outer bilayer, originating from the plasma membrane during budding, with variable amounts of entrained cytoplasm between the layers [15]. An emerging view is that lipid droplets are not static storage deposits, but that they are dynamic organelles interacting with other organelles within the cell, including the ER, mitochondria, or peroxisomes and that information and/or material are exchanged during these heterotypic interactions [16–18]. Cytosolic proteins account for up to 35% of all identified proteins. Cytoplasmic material including proteins may be trapped between the two membranes during the final budding of lipid droplets at the apical pole of the mammary epithelial cell (MEC) [15].

Enriched GO categories associated with MFGM proteins from camel milk are fully listed in Supporting Information Table 2. As expected, enriched biological processes associated with MFGM compartment were protein transport (p -value = 1.73×10^{-14} ; 17% of DAVID IDs; Table 1), or lipid-related pathways (p -value = 6.72×10^{-08} ; 8% of DAVID IDs;

Table 1. Enriched functional annotation terms associated with MFGM proteins from camel milk. GO categories are ranked according to increasing DAVID *p*-value.

GO	UniProtKB Accession
Protein transport (<i>p</i>-value = 1.73×10^{-14})	
14-3-3 protein beta/alpha	P31946
14-3-3 protein epsilon	P62258
14-3-3 protein gamma	P61981
14-3-3 protein theta	P27348
14-3-3 protein zeta/delta	P63104
Alpha-actinin-4	O43707
AP-1 complex subunit beta-1	Q10567
AP-1 complex subunit gamma-1	O43747
AP-1 complex subunit mu-2	Q9Y6Q5
AP-2 complex subunit alpha-2	O94973
ADP-ribosylation factor 1	P84077
ADP-ribosylation factor 6	P62330
Calreticulin	P27797
Calnexin	P27824
Platelet glycoprotein 4 (CD antigen CD36)	P16671
Charged multivesicular body protein 4b	Q9H444
Clathrin heavy chain 1	Q00610
Coatamer subunit alpha	P53621
Coatamer subunit beta	P53618
Coatamer subunit delta	P48444
Coatamer subunit gamma-1	Q9Y678
Endoplasmic (GRP-94)	P14625
PDZ domain-containing protein GIPC1	O14908
Eukaryotic translation initiation factor 5A-1	P63241
Importin subunit beta-1	Q14974
Protein ERGIC-53	P49257
Tyrosine-protein kinase Lyn (EC 2.7.10.2)	P07948
Myosin-9	P35579
Unconventional myosin-Ic	O00159
Unconventional myosin-VI	Q9UM54
Programmed cell death 6-interacting protein	Q8WUM4
Protein disulfide-isomerase A3 (EC 5.3.4.1)	P30101
Ras-related protein Rab-18	Q9NP72
Ras-related protein Rab-1A	P62820
Ras-related protein Rab-2A	P61019
Ras-related protein Rab-5B	P61020
Ras-related protein Rab-7a	P51149
Ras-related protein Rab-11B	Q15907
Ribosome-binding protein 1	Q9P2E9
GTP-binding protein SAR1b	Q9Y6B6
Selenium-binding protein 1	Q13228
Vesicle-trafficking protein SEC22b	O75396
Alpha-soluble NSF attachment protein (SNAP-alpha)	P54920
Synaptosomal-associated protein 23 (SNAP-23)	O00161
Translocon-associated protein subunit alpha (TRAP-alpha)	P43307
Translocon-associated protein subunit delta (TRAP-delta)	P51571
Syntaxin-3	Q13277
Syntaxin-7	Q15400
Syntaxin-binding protein 2 (Protein unc-18 homolog 2)	Q15833
Transitional endoplasmic reticulum ATPase	P55072
Transmembrane emp24 domain-containing protein 10	P49755

Table 1. Continued

GO	UniProtKB Accession
Vacuolar protein sorting-associated protein 26A	O75436
Synaptobrevin homolog YKT6 (EC 2.3.1.-)	O15498
Lipid biosynthetic process (<i>p</i>-value = 6.72×10^{-08})	
3 beta-hydroxysteroid dehydrogenase type 7 (EC 1.1.1.-)	Q9H2F3
5'-AMP-activated protein kinase subunit gamma-1	P54619
1-Acylglycerol-3-phosphate O-acyltransferase ABHD5 (EC 2.3.1.51) (Lipid droplet-binding protein CGI-58)	Q8WTS1
Acetyl-CoA carboxylase 1 (EC 6.4.1.2)	Q13085
Acetyl-coenzyme A synthetase, cytoplasmic (EC 6.2.1.1)	Q9NR19
Long-chain-fatty-acid-CoA ligase 3 (EC 6.2.1.3)	O95573
Apolipoprotein A-I	P02647
Estradiol 17-beta-dehydrogenase 11 (EC 1.1.1.62)	Q8NBQ5
Estradiol 17-beta-dehydrogenase 12 (EC 1.1.1.62)	Q53GQ0
3-Keto-steroid reductase (EC 1.1.1.270)	P56937
7-Dehydrocholesterol reductase (7-DHC reductase) (EC 1.3.1.21)	Q9UBM7
Squalene monooxygenase (EC 1.14.13.132)	Q14534
Lanosterol synthase (EC 5.4.99.7)	P48449
Fatty acid-binding protein, heart	P05413
Fatty acyl-CoA reductase 1 (EC 1.2.1.n2)	Q8WVX9
Fatty acid synthase (EC 2.3.1.85)	P49327
Glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49)	P11413
Bifunctional UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (EC 5.1.3.14) (EC 2.7.1.60)	Q9Y223
Glycerol-3-phosphate acyltransferase 4 (EC 2.3.1.15)	Q86UL3
Lipoprotein lipase (LPL) (EC 3.1.1.34)	P06858
Lysophospholipid acyltransferase 5 (EC 2.3.1.-)	Q6P1A2
Myelin proteolipid protein (lipophilin)	P60201
NADH-cytochrome b5 reductase 3 (EC 1.6.2.2)	P00387
Sterol-4-alpha-carboxylate 3-dehydrogenase (EC 1.1.1.170)	Q15738
Sialic acid synthase (EC 2.5.1.56)	Q9NR45
Actin cytoskeleton organization (<i>p</i>-value = 2.72×10^{-04})	
Actin, cytoplasmic 2	P63261
Alpha-actinin-4	O43707
Fructose-bisphosphate aldolase A (EC 4.1.2.13)	P04075
ADP-ribosylation factor 6	P62330
Calreticulin	P27797
Adenylyl cyclase-associated protein 1	Q01518
F-actin-capping protein subunit beta	P47756
Cofilin-1	P23528
Filamin-B	Q75369
Gelsolin	P06396
Myosin-9	P35579
Plastin-2	P13796
Ras-related C3 botulinum toxin substrate 1	P63000
Ras-related protein Ral-A	P11233
Transforming protein RhoA	P61586

Table 1). The actin cytoskeleton organization GO category was also over-represented (p -value = 2.72×10^{-04} ; 5% of DAVID IDs; Table 1). Lipid droplets move along the microtubule machinery to reach the apical pole of the MEC. The presence of cytoskeleton components may thus account for the unique secretion process of lipids within the MEC. Other top-ranked biological processes were translation (p -value = 1.0×10^{-11} ; 10% of DAVID IDs) and hexose metabolic process (1.89×10^{-04} ; 4% of DAVID IDs). Since they are not directly involved in the secretion process, cytoplasmic proteins belonging to these two latter categories must probably be entrained with lipid droplets during the final budding process and the release of lipids as fat globules into milk.

In conclusion, we provide here for the first time a thorough description of proteins associated with the milk fat globule membrane (MFGM), the membrane surrounding milk fat globules in the camel (*C. dromedarius*) species. Major MFGM proteins including fatty acid synthase, xanthine oxidase, butyrophilin, and LDH were identified within MFGM proteins from camel milk. In addition, a 1D-LC-MS/MS approach led to the identification of more than 320 proteins associated with the MFGM in the dromedary. Due to the secretion process, the protein composition of MFGM reflects those of the ER and apical plasma membrane. Our MFGM proteomic dataset also contains a large number of cytoplasmic proteins as found in other studies. Thus, the MFGM can reflect dynamic changes within the MEC and may provide a “snapshot” of mammary gland biology under particular conditions [19]. We hope this camel MFGM proteome will contribute to a better description of camel milk. By the identification of genuine partners of lipid droplet secretion, we also hope to contribute to a better understanding of lipid secretion in the MEC.

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