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Comprehensive proteomic analysis of camel milk-derived extracellular vesicles

Abstract: Extracellular vesicles were recovered by optimized density gradient ultracentrifugation from milk of *Camelus (C.) dromedarius*, *C. bactrianus* and hybrids reared in Kazakhstan, visualized by transmission electron microscopy and characterized by nanoparticle tracking analysis. Purified extracellular vesicles had a heterogeneous size distribution with diameters varying between 25 and 170 nm, with average yield of 9.49 x 108 – 4.18 x 1010 particles per milliliter of milk. Using a comprehensive strategy combining classical and advanced proteomic approaches an extensive LC-MS/MS proteomic analysis was performed of EVs purified from 24 camel milks (C. bactrianus, n=8, C. dromedarius, n=10, and hybrids, n=6). A total of 1,010 unique proteins involved in different biological processes were thus identified, including most of the markers associated with small extracellular vesicles, such as CD9, CD63, CD81, HSP70, HSP90, TSG101 and ADAM10. Camel milk-derived EV proteins were classified according to biological processes, cellular components and molecular functions using gene-GO term enrichment analysis of DAVID 6.8 bioinformatics resource. Camel milk-derived EVs were mostly enriched with exosomal proteins. The most prevalent biological processes of camel milk-derived EV proteins were associated with exosome synthesis and its secretion processes (such as intracellular protein transport, translation, cell-cell adhesion and protein transport, and translational initiation) and were mostly engaged in molecular functions such as Poly(A) RNA and ATP binding, protein binding and structural constituent of ribosome. Proteomic studies of camel milk and sub-fractions thereof, such as casein, whey, or the milk fat globule membrane (MFGM) have revealed a plethora of bioactive proteins and peptides beneficial for developing immune and metabolic systems (Casado et al., 2008; Kussmann and Van Bladeren, 2011). By contrast, camel milk-derived EVs are still a largely uncharted proteomic terrain, although we know that milk-derived EVs carry cell origin-specific cargo and transport both bioactivity and information between cells (de la Torre Gomez et al., 2018).
Key words: milk, camel, exosomes, extracellular vesicles, proteome, tetraspanins.

Introduction

Milk is usually considered as a complex biological liquid in which supramolecular structures (casein micelles and milk fat globules) are found beside minerals, vitamins and soluble proteins (whey proteins) as well as cells. In addition to these components, it was recently shown that milk contains also extracellular vesicles that are released by cells as mediators of intercellular communication. Indeed, cell communicate with neighboring cells or with distant cells through the secretion of extracellular vesicles [1]. Phospholipid bilayer-enclosed extracellular vesicles (EVs) are naturally generated and released from several cell domains of life (*Bacteria, Archaea, Eukarya*) into the extracellular space under physiological and pathological conditions [2]. EVs are commonly classified according to their sub-cellular origin into three major subtypes, such as microvesicles, exosomes, and apoptotic bodies. Contents of vesicles vary a with respect to mode of biogenesis, cell type, and physiologic conditions [3]. Exosomes represent the smallest population among EVs ranging in size from 30 to 150 nm in diameter [4]. They are generated inside multivesicular bodies in the endosomal compartment during the maturation of early late endosomes and are secreted when these compartments fuse with the plasma membrane [5]. Found in all biofluids exosomes harbor different cargos as a function of cell type and physiologic state [3].

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Milk is the sole source of nutrients for the newborn and very young offspring, as well as being an important means to transfer immune components from the mother to the newborn of which the immune system is immature [6]. Milk is therefore thought to play an important role in the development of the immune system of the offspring [7]. Milk is also a source of delivers molecules, via exosomes and/or microvesicles, acting on immune modulation of neonates due to their specific proteins, mRNA, long noncoding RNA and miRNA contents. Exosomes have come in the limelight as biological entities containing unique proteins, lipids, and genetic material. It was shown that the RNA contained in these vesicles could be transferred from one cell to another, through an emerging mode of cell-to-cell communication [8]. RNAs conveyed by exosomes are translated into proteins within transformed cells (mRNA), and/or are involved in regulatory functions (miRNA). For this reason, exosomes are recognized as potent vehicles for intercellular communication, capable for transferring messages of signaling molecules, nucleic acids, and pathogenic factors [9].

Over the last decade, exosomes were widely explored as biological nanovesicles for the development of new diagnostic and therapeutic applications as a promising source for new biomarkers in various diseases [10]. For example, exosomes secreted by dendritic cells have been shown to carry MHC-peptide complexes allowing efficient activation of T lymphocytes, thus displaying immunotherapeutic potential as promoters of adaptive immune responses [11]. Recently, cell culture studies showed that bovine milk-derived exosomes act as a carrier for chemotherapeutic/chemopreventive agents against lung tumor xenografts *in vivo* [12]. Nevertheless, their physiological relevance has been difficult to evaluate because their origin, biogenesis and secretion mechanisms remained enigmatic.

Despite a significant number of publications describing the molecular characteristics and investigating the potential biological functions of milk-derived exosomes [13; 14], there are only one dealing with exosomes derived from camel milk [15]. These authors report for the first time isolation and characterization using proteomic (SDS-PAGE and western blot analysis) and transcriptomic analyses exosomes from dromedary milk at different lactation stages. However, there is no comprehensive investigation on exosomal protein variations and variability in composition between individual camels. Milk-derived EVs from Bactrian and hybrid milks have never been explored before. Therefore, to gain insight into the protein diversity of camel milk-derived EVs, we herein provide results of isolation and in-depth morphological and protein characterization of milk-derived EVs from *C. dromedarius*, *C. bactrianus* and hybrids from Kazakhstan using a comprehensive strategy combining classical (SDS-PAGE) and advanced proteomic approaches (LC-MS/MS).

Materials and methods

Milk sample collection and preparation. Raw milk samples were collected during morning milking on healthy dairy camels belonging to two species: *C. bactrianus* (n=72) and *C. dromedarius* (n=65), and their hybrids (n=42) at different lactation stages, ranging between 30 and 90 days postpartum. Camels grazed on four various natural pastures at extreme points of Kazakhstan: Almaty (AL), Shymkent (SH), Kyzylorda (KZ), and Atyrau (ZKO). Whole-milk samples were centrifuged at 3,000 *g* for 30 min at 4°C (Allegra X-15R, Beckman Coulter, France) to separating fat from skimmed milk. Samples were quickly frozen and stored at -80°C (fat) and -20°C (skimmed milk) until analysis.

Selection of milk samples for analysis. In total 24 camel milk samples (*C. bactrianus*, n=8, *C. dromedarius*, n=10, and hybrids, n=6) were selected for isolation of camel milk-derived EVs, based on lactation stages and number of parities of each camel group composed by the species and grazing regions. It should be emphasized that data available on animals: breed, age, lactation stage and calving number, were estimated by a local veterinarian, since no registration of camels in farms is maintained. Six samples of camel milk-derived EVs (*C. bactrianus*, n=2, *C. dromedarius*, n=2, and hybrids, n=2) were selected randomly for transmission electron microscopy (TEM) with negative staining (uranyl acetate). Then, 15 milk samples including the 6 examined by TEM (*C. bactrianus*, n=5, *C. dromedarius*, n=5, and hybrids, n=5) were analyzed by SDS-PAGE and LC-MS/MS analysis using a QExactive (Thermo Fischer Scientific, USA) Mass Spectrometer after a tryptic digestion of excised gel bands.

Isolation of camel milk-derived EVs. First, skimmed milk samples (40-45 mL) were incubated at 37°C for 30 min in a water bath to enhance free β-casein adsorption to casein micelles. Then, acetic acid was added to the total volume of milk, to obtain a final concentration of 10% and thus acidified milk was incubated at 37°C for 5 min for precipitation of

caseins. Finally, 1M sodium acetate was added to obtain a final concentration of 10% for salting out at RT for 5 min, followed by centrifugation at 1,500 *g* for 15 min at 20°C (Beckman Coulter, Allegra X-I5R Centrifuge, France). After being passed through sterilized vacuum-driven filtration system Millipore Steritop, 0.22 µm, the supernatant, namely the filtrated whey, was concentrated by centrifugation at 4,000 *g* and 20°C using Amicon 1,000K ultracentrifuge tube until to obtain 3 mL of concentrate remaining. The retentate thus obtained was ultra-centrifuged for pelleting the EVs at 33,000 *g* for 1h10 at 4°C (Beckman Coulter, Optima XPN-80, 50TI rotor, France). Next, the pellet was suspended in 500 µL of PBS and added to pre-prepared 11 mL of sucrose gradient 5-40% and ultra-centrifuged at 34,000 *g* for 18h at 4°C (Beckman Coulter, Optima XPN-80, SW41 rotor, France). In total, 12 fractions of 1 mL were collected. Fractions previously demonstrated to be enriched in exosomes (10, 11 and 12) were finally suspended into 50 µL of PBS and stored at -80°C, until further analyses.

Transmission electron microscopy (TEM). The EVs were analyzed as whole-mounted vesicles deposited on EM copper/carbon grids during 5 min and contrasted 10 sec in 1% uranyl acetate. Grids were examined with Hitachi HT7700 electron microscope operated at 80kV (Elexience – France), and images were acquired with a charge-coupled device camera (AMT).

Nanoparticle tracking analysis. The size distribution and concentration of EVs were measured by NanoSight (NS300) (Malvern Instruments Ltd, Malvern, Worcestershire, UK) according to manufacturer's instructions. A monochromatic laser beam at 405 nm was applied to the diluted suspension of vesicles. Sample temperature is fully programmable through the NTA software (version 3.2 Dev Build 3.2.16). A video of 30 sec was taken with a frame rate of 30 frames/s and particle movement was analyzed by NTA software.

Proteomic analysis. To estimate the concentration of total EVs, the Coomassie Blue Protein Assay was used [43]. Absorbance at 595 nm was measured using the UV-Vis spectrophotometer (UVmini-1240, Shimadzu, France). The reference standard curve was done with 1 mg/mL commercial bovine serum albumin (BSA, Thermo Fischer Scientific, USA).

In order to identify proteins, mono dimensional electrophoresis (1D SDS-PAGE) followed by trypsin digestion and LC-MS/MS analysis, was used. Ten µµg of each individual skimmed camel milk sample were loaded onto 4-15% Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad, Marne-la-Coquette, France) and subjected to electrophoresis. Samples were prepared with Laemmli Lysis-Buffer (Sigma-Aldrich, USA) with β-mercapto ethanol and denatured at 100°C for 15 min. Separations were performed in a vertical electrophoresis apparatus (Bio-Rad, Marne-la-Coquette, France). After a short migration (0.5 cm) of samples, gels were stored at -80°C until LC-MS/MS analysis.

Reduction of disulfide bridges of proteins was carried out by incubating at 37°C for one hour with dithiothreitol (DTT, 10 mM, Sigma, USA), meanwhile the alkylation of free cysteinyl residues with iodoacetamide (IAM, 50 mM, Sigma, USA) at RT for 45 min in total obscurity. After gel pieces were washed twice, first, with 100 µµL 50% ACN/50 mM NH4HCO3 and then with 50 µµL ACN, they were finally dried. The hydration was performed at 37°C overnight using digestion buffer 400 ng lys-C protease + trypsin. Hereby, peptides were extracted with 50% ACN/0.5% TFA and then with 100% ACN. Peptide solutions were dried in a concentrator and finally dissolved into 70 µL 2% ACN in 0.08% TFA.

The identification of peptides was obtained using UltiMate™ 3,000 RSLC nano System (Thermo Fisher Scientific, USA) coupled to a QExactive (Thermo Fischer Scientific, USA) mass spectrometer.

Four µL of each sample were injected at a flow rate of 20 µL/min on a precolumn cartridge (stationary phase: C18 PepMap 100, 5 µm; column: 300 µm x 5 mm) and desalted with a loading buffer 2% ACN and 0.08% TFA. After 4 min, the precolumn cartridge was connected to the separating RSLC PepMap C18 column (stationary phase: RSLC PepMap 100, 2 µm; column: 75 µm x 150 mm). Elution buffers were A: 2% ACN in 0.1% formic acid (HCOOH) and B: 80% ACN in 0.1% HCOOH. The peptide separation was achieved with a linear gradient from 0 to 35% B for 34 min at 300 nL/min. One run took 42 min, including the regeneration and the equilibration steps at 98% B.

Peptide ions were analyzed using Xcalibur 2.1 with the following machine set up in CID mode: 1) full MS scan in QExactive with a resolution of 15,000 (scan range [m/z] = 300-1,600) and 2) top 8 in MS/MS using CID (35% collision energy) in Ion Trap. Analyzed charge states were set to 2-3, the dynamic exclusion to 30 s and the intensity threshold was fixed at 5.0 x 102.

Raw data were converted to mzXML by MS convert (ProteoWizard version 3.0.4601). UniProtKB Cetartiodactyla database was used (157,113

protein entries, version 2015), in conjunction with contaminant databases were searched by algorithm X!TandemPiledriver (version 2015.04.01.1) with the software X!TandemPipeline (version 3.4) developed by the PAPPSO platform (http://pappso.inra.fr/bioinfo/). The protein identification was run with a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.5 Da. Enzymatic cleavage rules were set to trypsin digestion ("after R and K, unless P follows directly after") and no semi-enzymatic cleavage rules were allowed. The fix modification was set to cysteine carbamide methylation and methionine oxidation was considered as a potential modification, Results were filtered using inbuilt X!TandemParser with peptide E-value of 0.05, a protein E-value of -2.6 and a minimum of two peptides.

Bioinformatics and functional enrichment analysis. Functional enrichment analyses on camel milk-derived EV protein was performed using online software for gene annotation "The Database for Annotation, Visualization and Integrated Discovery (DAVID)" version 6.8 (https://david.ncifcrf.gov/home.jsp/), as described by [16].

Ethics statements. All animal studies were carried out in compliance with European Community regulations on animal experimentation (European Communities Council Directive 86/609/EEC) and with the authorization of the Kazakh Ministry of Agriculture. Milk sampling was performed in appropriate conditions supervised by a veterinary accredited by the French Ethics National Committee for Experimentation on Living Animals. No endangered or protected animal species were involved in this study. No specific permissions or approvals were required for this study except for the rules of afore-mentioned European Community regulations on animal experimentation, which were strictly followed.

Results and discussion

Isolation of camel milk-derived EVs. EVs are complex and delicate systems requiring optimized isolation and characterization adapted to each fluid type of origin [41], which may be achieved by a variety of methods, including ultracentrifugation, filtration, immunoaffinity isolation and microfluidics techniques [17]. Choice of method should be guided by the required degree of EVs purity and concentration. General protocols to isolate EVs from cell culture supernatants and body fluids involve steps of differential ultracentrifugation and further purification on a sucrose density gradient [18]. Commercially produced kits for exosome isolation are nowadays available; however, they are not adapted to milk samples. Due to highly variable composition between different body fluids and even within milks of different species, special optimization steps are required. Isolating milk-derived EVs is complicated by milk composition that differs significantly across species, lactation stage, physiological and health status of individuals. In addition, the recovery of purified exosomes from milk for subsequent analysis requires, according to research objectives, to increase sample volume that is not compatible with classical protocols.

In our study, for milk-derived EVs isolation, the "gold standard method", including differential ultracentrifugation with sucrose density gradient, was performed (Krupova et al., unpublished results). However, to achieve efficient and quantitative recovery of EVs from camel milk, commonly used protocol was modified. First, milk fat, cells and cellular debris were removed by differential ultracentrifugation. The resuspended pellet was loaded on top of a sucrose gradient and ultra-centrifuged to allow for the separation and concentration of EVs. After ultracentrifugation, individual fractions were collected, and EVs enriched fractions (10 to 12) were pooled.

Morphology of isolated camel milk-derived EVs. The method comprising differential ultracentrifugation with density gradient ultracentrifugation was described as being suitable for efficient isolation and purification of higher quality EVs with intact native morphology [19]. To visualize and characterize the morphology and size distribution of camel milk-derived EVs, TEM and NTA analyses were performed. In all 6 milk samples analyzed, a high abundance of homogenous population of EVs enriched in spherical exosomes with average yield of $9.49 \times 10^8 - 4.18 \times 10^{10}$ particles per milliliter was observed. The average sizes varied between 25 and 170 nm in diameter. A classical EV-like morphology has been noticed with no significant differences between *C. dromedarius*, *C. bactrianus* and hybrids samples (Figure 1).

Results confirmed that we have isolated both higher purity and higher quality EVs with intact morphological structures. Based on earlier observations described for dromedary milk [15] and on milk of other species, such as bovine [13], porcine [20], equine [21] and human [22], obtained characteristics for *Camelus* milk appear common to EVs across species. So, we can conclude that the method of differential ultracentrifugation with sucrose density gradient ultracentrifugation resulted in efficient and reliable isolation of camel milk-derived EVs.

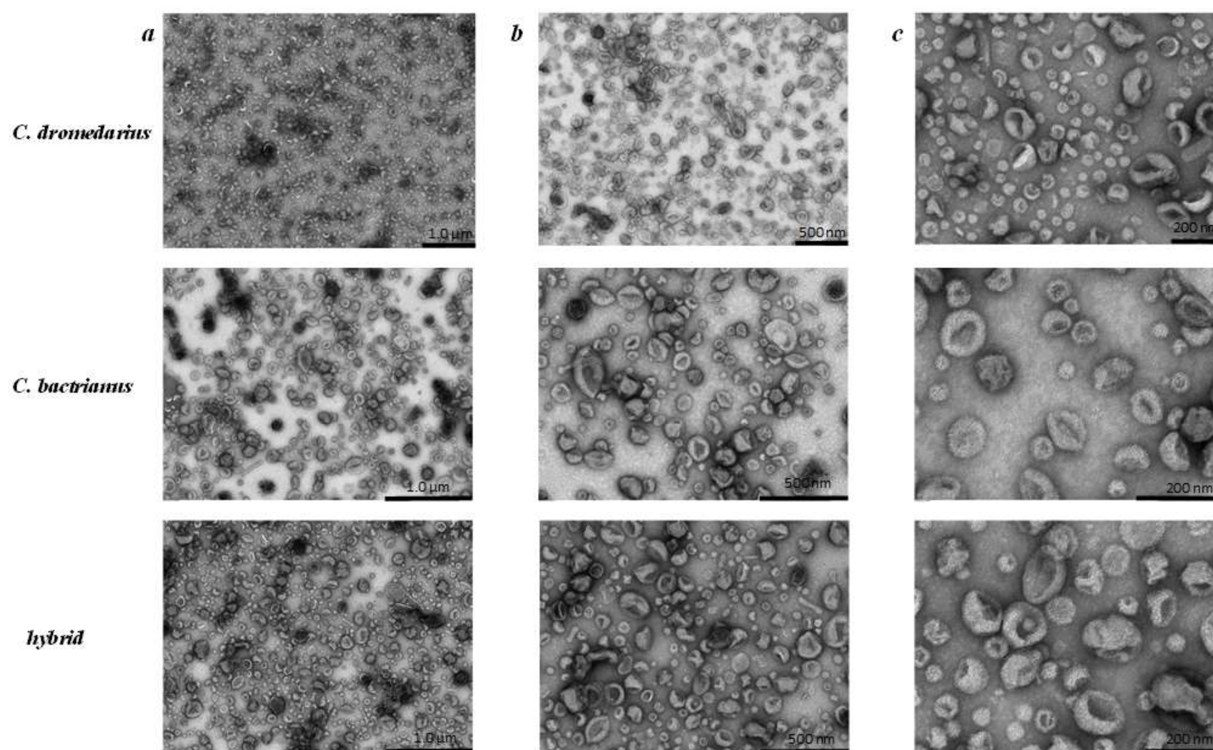

Figure 1 – Representative electron micrographs of *C. dromedarius, C. bactrianus* and hybrid camel milk-derived EVs. Scale bar represents: a) 1 µm, b) 500 nm, c) 200 nm.

In-depth proteomic analysis of camel milk-derived EVs. Apart from the morphology, specific protein composition enables to characterize EVs. To identify the proteins present in camel milk-derived EVs, extensive analyses involving trypsin digestion, LC-MS/MS (Q Exactive, Thermo Fisher Scientific, USA) and database searches were performed. Recently, using a similar approach, a total of 1,963 proteins were identified in human milk-derived EVs [14], and 2,107 unique proteins were described in bovine milk-derived EVs [23]. Here, from EV samples derived from 15 camel milks (*C. bactrianus,* n=5, *C. dromedarius,* n=5, and hybrids, n=5), a total of 1,010 functional groups of proteins (proteins belonging to a same group share common peptides) were detected (S1). About 890 proteins were common between the three camel (Figure 2a), while there are several proteins indicated as unique to *C. bactrianus* (31)*, C. dromedarius* (5)*,* and hybrids (12). Using UniprotKB taxonomy Cetartiodactyla (SwissProt + Trembl) database, proteins were identified as authentically matching with proteins in *Camelus* protein databases (*C. dromedarius, C. bactrianus,* and *C. ferus)*, and with the other mammalian species such as*, Lama glama, Lama guanicoe, Bos taurus, Bos mutus, Sus scrofa* and *Ovis aries* protein databases and others. Including the major exosomal protein markers identified, the higher number of low abundant and several differentially expressed proteins enhance the opportunity for revealing the crucial proteins, which can affect exosome synthesis and secretion pathways. By comparison, the proteome of camel milk-derived EVs identified in this study is relatively larger compared to the camel milk proteome reported in a previous study [24]. A total of 391 functional groups of proteins have been identified from 8 camel milk samples using a less sensitive LC-MS/MS (LTQ Orbitrap XL™ Discovery, Thermo Fisher Scientific), of which 235 proteins were observed as common across camel species. We cannot exclude that there may be several reasons for the significant difference in the number of proteins identified in camel milk-derived EVs, comparatively to previously published data on camel milk proteome. First and foremost the instruments (Q Exactive vs LTQ Orbitrap), since the Q Exactive analyzer was reported to provide significant improvement over the Orbitrap mass spectrometers [25] in terms of sensitivity. Comparing the proteomes between camel milk and camel milk-derived EVs, 222 proteins were identified as common (Figure 2, b), the list of which are provided as a supplementary data (S2[1]).

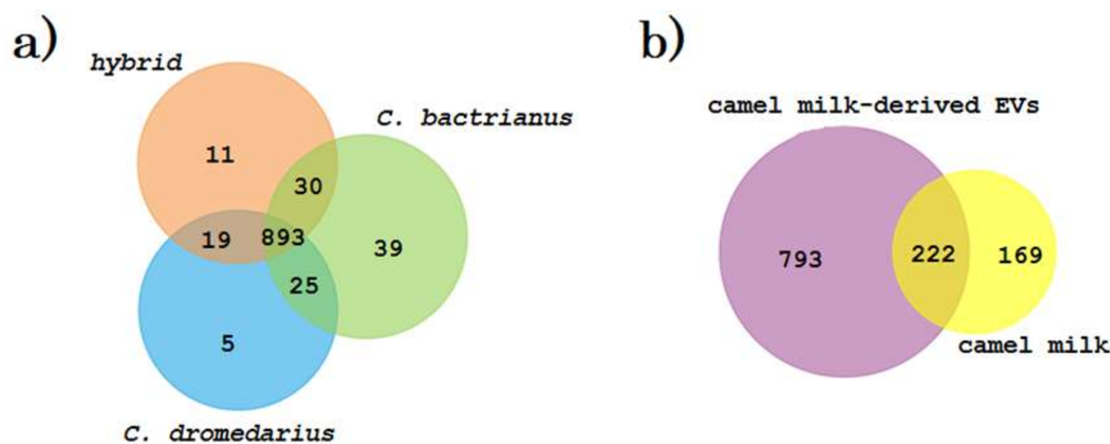

Figure 2 – Общее название. **a)** Venn diagram comparing proteins identified in *C. dromedarius, C. bactrianus* and hybrids milk-derived EVs. The diagram illustrates common and unique EV proteins between the three species **b)** Venn diagram comparing proteins identified in camel milk-derived EVs and proteins detected in camel milk reported in our previous study [24]

To get more insight into the subcellular origin of proteins identified, gene-GO term enrichment analysis was performed using DAVID bioinformatics resources 6.8. This analysis helps to understand the function of proteins and addresses them into different biological pathways [26]. In total 890 and 235 common proteins expressed in camel milk-derived EVs and camel milk, respectively, have been classified according to cellular components. However, despite the limitations of the gene annotations not all camel proteins have been annotated, therefore only 517 exosomal and 96 milk proteins could to be converted to DAVID gene IDs. Thereby, 463 exosomal and 84 milk proteins matched to GO terms under the cellular components' headings. As shown in Figure 3, both milk-derived EVs and milk samples were mostly enriched with extracellular exosomal proteins (31.09% vs 35.41%, respectively), the specific subset of cellular proteins that are targeted specifically to exosomes. These results coincide with data reported previously on human milk and milk-derived EVs, where a high percentage of proteins linked to GO terms like "exosomes" [14]. The next biggest group represented a large number of cytoplasmic proteins (19.58% EVs *vs* 14.58% milk) found in milk-derived EVs and nucleus proteins (13.24% EVs vs 15.62% milk) in camel milk. Cytoplasmic proteins might originate from "cytoplasmic crescents", which are trapped between the membrane layers of the MFGM during the budding process when the fat globule leaves the epithelial cell [27]. Thus, the MFGM can reflect dynamic changes within the MEC and may provide a "snapshot" of mammary gland biology, under specific patho-physiological conditions. About 13.24% and 12.50% were reported to be membrane proteins identified in camel milk-derived EVs and milk samples. Membrane trafficking proteins represent Rab proteins, which belong to the Ras superfamily of small GTPase. Function of these proteins is central regulation of vesicle budding, motility and fusion. They play a role in endocytosis, transcytosis and exocytosis processes [26]. In addition, some membrane proteins from intracellular organelles such as cytosol, mitochondrion and Golgi apparatus were highly expressed in camel milk-derived EVs.

Next, we classified proteins expressed in camel milk-derived EVs according to biological processes, molecular functions, and KEGG pathways. Camel milk-derived EV proteins observed were involved in twenty-six GO biological process terms (Figure 4). The most prevalent biological processes of camel milk-derived EV proteins were associated with exosome synthesis and its secretion processes, such as intracellular protein transport (5.57%), translation (3.45%), cell-cell adhesion and protein transport (3.26%), and translational initiation. Exosomes are increasingly recognized as mediators of intercellular communication due to their capacity to merge with and transfer a repertoire of bioactive molecular content (cargo) to recipient cells [11]. In addition, EV proteins were mostly engaged in cellular functions such as Poly(A) RNA and ATP (9.60%) binding, protein binding and structural component of ribosome (3.65%). About 3.84% proteins are associated with GTP binding function (Figure 4), regulating membrane-vesicle trafficking process. Proteins expressed in camel milk-derived EVs were categorized into 34 different KEGG pathways.

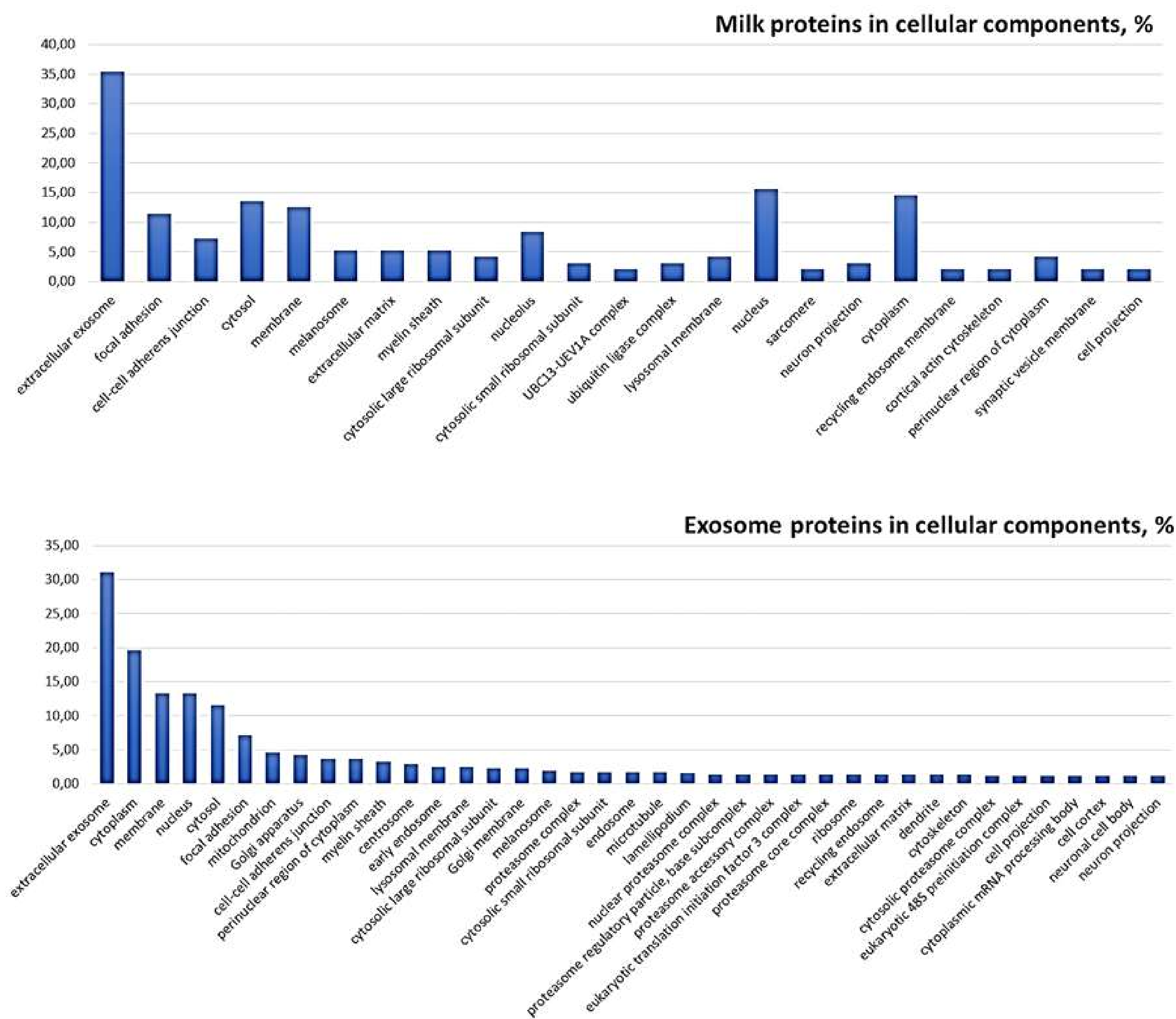

Figure 3 – Functional annotations of camel milk and milk-derived EV proteins classified into cellular components using DAVID bioinformatics resources 6.8

As shown in Table 1, camel milk-derived EV proteins were mostly associated with endocytosis (5.57%), Epstein-Barr virus infection (4.03%), ribosome (3.84%), proteasome (3.45%), RNA transport and viral carcinogenesis (2.50%) KEGG pathways. It is known that exosomes display a wide variety of immuno-modulatory properties. This is highlighted by findings showing that exosomes secreted by Epstein-Barr virus (EBV)-transformed B cells are able to stimulate CD4+ T cells in an antigenic-specific manner [11].

Exosomes are a rich source of potential milk biomarkers. Isolation of EVs from milk is complicated by the high lipid content of milk [17]. Lipids are released in milk as fat globules (MFGs) by mammary epithelial cells. These MFGs are droplets of lipids surrounded by a complex phospholipid trilayer containing proteins and glycoproteins [28], and thus are a type of EVs. MFGs are largely heterogeneous in size, and their buoyant densities are different from those of EVs. Because of their plasma membrane origin, vesicular nature, and high abundance in milk, however, MFGs may be co-isolated with other EVs populations present in milk [23]. As expected, camel milk-derived EVs analyzed were mostly enriched with MFGM-enriched proteins associated with milk, such as fatty acid synthase (FAS), MFG-E8 (also termed lactadherin), butyrophilin (BTN) and xanthine dehydrogenase. FAS, BTN and MFG-E8 are negative co-stimulatory molecules inhibiting antitumor immune responses, which have become novel target pathways for cancer- and immunotherapy development [29-31].

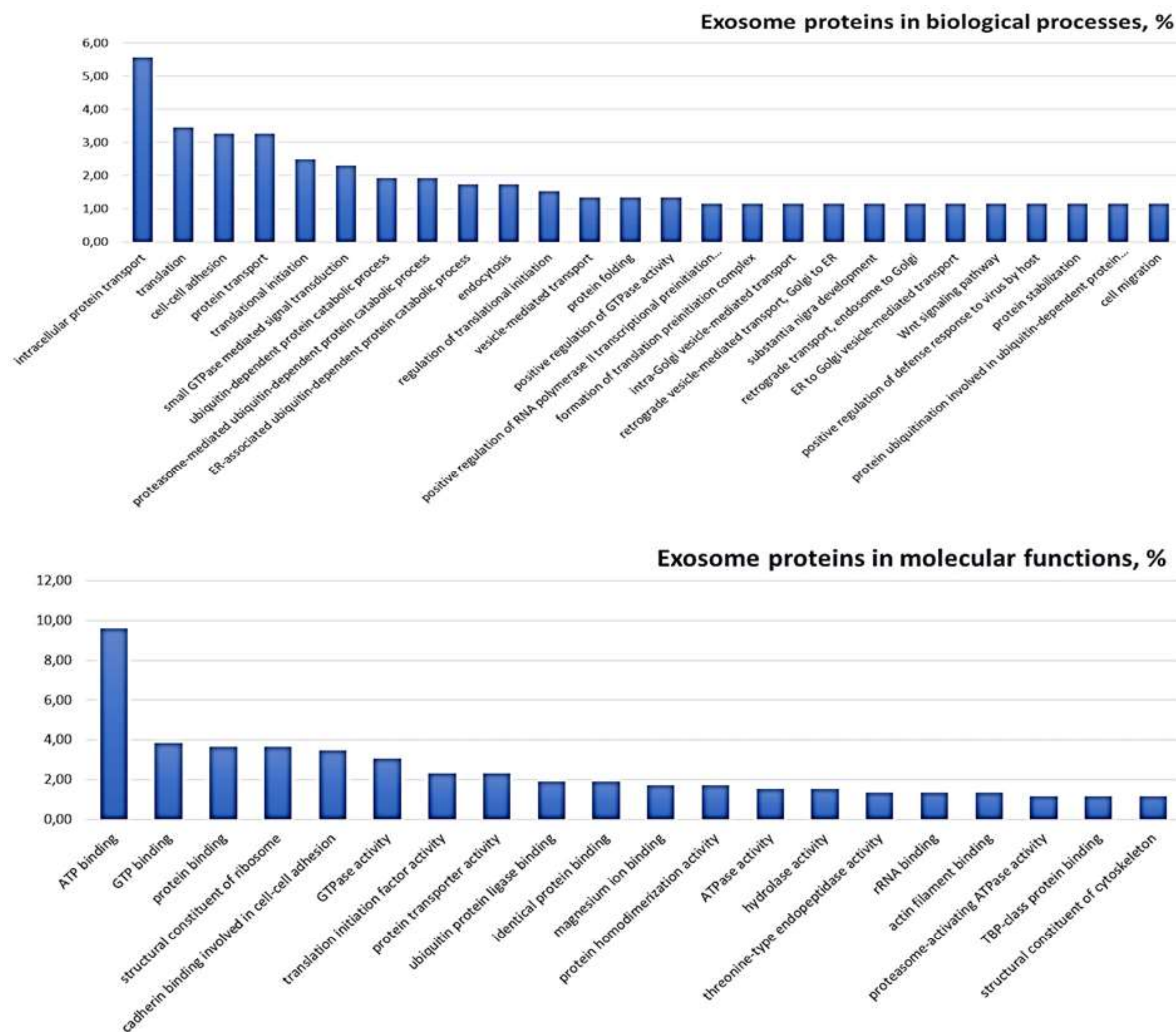

Figure 4 – GO enrichment analysis of camel milk-derived EV proteins classified into biological processes and molecular functions using DAVID bioinformatics resources 6.8

Table 1 – KEGG pathway analysis of camel milk-derived EVs

KEGG pathway term	%	PValue	Fold enrichment
Endocytosis	5.57	8.0E-11	4.32
Epstein-Barr virus infection	4.03	8.7E-8	4.23
Ribosome	3.84	1.1E-9	5.77
Proteasome	3.45	4.1E-16	15.14
RNA transport	2.69	2.2E-4	3.41
Bacterial invasion of epithelial cells	2.11	2.5E-5	5.53
Tight junction	2.11	2.7E-3	3.11
Vasopressin-regulated water reabsorption	1.92	1.9E-6	8.41
Synaptic vesicle cycle	1.92	2.9E-5	6.14

Continuation of table 1

KEGG pathway term	%	PValue	Fold enrichment
Adherens junction	1.92	5.4E-5	5.69
Salmonella infection	1.73	1.3E-3	4.19
Fc gamma R-mediated phagocytosis	1.34	2.2E-2	3.19
Legionellosis	1.15	1.5E-2	4.07
mTOR signaling pathway	1.15	1.7E-2	3.93
Biosynthesis of amino acids	1.15	4.1E-2	3.14
Endocrine and other factor-regulated calcium reabsorption	0.96	2.2E-2	4.61
Collecting duct acid secretion	0.77	3.1E-2	5.73

Camel milk-derived EVs analyzed were highly enriched with ubiquitous, cell-specific and cytosolic proteins, including proteins associated with the endosomal pathway, involved in mechanisms responsible for exosome biogenesis. All populations of EVs analyzed expressed in abundance the small Rab GTPases, such as RAB1A, RAB11B, RAB5C, RAB18, RAB2A, RAB7A and RAB21. Rab GTPases are key regulators of intracellular membrane trafficking, from the formation of transport vesicles to their fusion with membranes. Additionally, exosomes derived from all camel milk analyzed were significantly enriched with certain multifunctional proteins, such as Alix (programmed cell death 6 interacting protein PDCD6IP) and TSG101 (tumor susceptibility gene 101). These Endosomal Sorting Complexes required for Transport (ESCRT) protein components of vesicular trafficking process are believed to be a specific exosome-segregated biomarker during its biogenesis [15, 32]. Recently, it was reported that syndecan-syntenin-ALIX is an important regulator of membrane trafficking and heparan sulphate-assisted signaling, which influences pathological processes, including cancer, the propagation of prions, inflammation, amyloid deposition and neurodegenerative disease [33]. Moreover, HSP70 and HSP90 proteins implicated in innate immune responses and antigen presentation [34], involved in signal transduction protein kinases and 14-3-3 proteins, and metabolic enzymes such as peroxidases, pyruvate kinases, and α-enolase were also observed in camel milk-derived EVs. Cell membrane proteins, such as MHC I and MHC II, demonstrating vesical nature of the analyzed materials, were identified as well in all camel milk-derived EVs analyzed, as well as, cytosolic proteins such as tubulin, actin, and actin-binding proteins were highly expressed.

As a consequence of their endosomal origin, most of exosomes are composed of proteins involved in membrane transport and fusion, in multivesicular body biogenesis, in processes requiring heat shock proteins, integrins and tetraspanins [35]. While some of the proteins that are found in the proteome of many exosomal membrane preparations may merely reflect the cellular abundance of the protein, others are specifically enriched in exosomes and can therefore be defined as exosome-specific marker proteins. Apart from providing nourishment to the offspring, these proteins play a role in intercellular communication via transfer of biomolecules between cells. However, it is currently unknown whether exosomes found in milk originate from immune cells present in milk, from mammary epithelial cells, from circulating cells coming from elsewhere in the body or from bacterial species present in the mammary gland under mild permanent infection (sub-clinical mastitis).

Available proteomic studies define specific markers of the EVs (membrane and cytosolic proteins) and a specific subset of cellular proteins that are targeted specifically to exosomes, the functions of some of them still remain unknown [36]. This is particularly interesting in relation to their possible involvement in human diseases. The knowledge of exosome proteomics can help not only in understanding their biological roles but also in supplying new biomarkers [37]. Among the membrane proteins most enriched in exosomes are tetraspanins, which play a critical role in exosome formation and are involved in morphogenesis, fission and fusion processes [38]. Recently CD9, CD63, and CD81 tetraspanins have been defined as novel markers characterizing heterogeneous populations of EVs subtypes [39], the presence of which, including CD82 and TSPAN14 proteins, were confirmed in camel milk-derived EVs. However, some exosome samples analyzed were devoid of CD63. The absence of this tetraspanin in secreted exosomes by some cell types was previously re-

ported, and the necessity of analyzing instead either CD81- or CD9-bearing EVs was reported [39].

Even in the case of markers with strong evidence for EVs subtype specificity, the presence of such markers does not rule out that other types of vesicles are present in a preparation simultaneously [17]. Not only the desired populations must be confirmed as present; contaminants must be demonstrated to be absent. The purity of the exosomes isolated is highly variable due to the presence of contaminating particles, vesicles and molecules such as proteins and/or nucleic acids as well as other cellular components [40], which may co-purify in vesicle preparations and confound analysis [41, 17]. Minimizing contamination in the isolation of exosomes is vital in providing reliable information upon which to base new paradigms [40]. It was reported that exosomes isolated by differential ultracentrifugation with density gradient ultracentrifugation method can be used to examine the relationship of EV proteins to physiological or disease status of the host without any involvement or contamination of other free proteins in milk [19]. Density gradients add stringency by efficiently separating particles of different density, which allows removing contaminating non-vesicular particles. Thus, the purity of the camel milk-derived vesicles isolated from contaminations with other multivesicular bodies has been examined and confirmed by the absence of microvesicle surface markers such as p-selectin and CD40, an endoplasmic reticulum marker calnexin, mitochondrial protein mitofilin, and an ER-associated protein GP96. Even though, we have applied a filtration step of the milk supernatant prior to the EVs pelleting step, camel milk-derived EVs were contaminated with caseins, the expression of which have been also detected previously in dromedary [15], human [14] and bovine milk exosomes [23].

Conclusion

Using an optimized isolation protocol, we obtained milk-derived exosomes originating from 15 camel (*C. dromedarius, C. bactrianus* and hybrids) milk samples that satisfied the typical requirements for exosomal morphology, size and protein content. LC-MS/MS analyses allowed identifying a thousand of different proteins that represents to our knowledge, the first comprehensive proteome of camel milk-derived EVs that appears wider than the milk proteome. As mentioned previously in other species camel milk-derived EVs contain proteins also present in other milk components. This is particularly the case for lactadherin/MFG-E8, Ras-related proteins or CD9 that have been reported to occur in MFG. Our results strongly suggest that milk-derived exosomes have different cellular origin. Indeed, besides exosomes originating from mammary epithelial cells there are milk-derived exosomes from immune cells. If we consider that milk-derived exosomes also carry microRNAs, these vesicles have to be recognized as another important bioactive component of milk that might be involved in transmitting signals from the mother to the newborn but also represents a source of factors potentially responsible for the properties attributed to camelids milk and its health value.

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