NNT 2018SACLA016

# Exploring the fine composition of *Camelus* milk from Kazakhstan with emphasis on protein components

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**Thesis**

submitted in fulfilment of the requirements for the joint degree of doctor

at **AgroParisTech**

by the authority of the General Director, Prof. Dr G. Trystram

in the presence of the

Thesis Committee appointed by the Head of The Graduate School of Agriculture, Food, Biology, Environment and Health of the Agricultural, Veterinary and Forest Institute of France

to be defended in public

on Thursday 12 July 2018

at 2:30 p.m. at AgroParisTech, Centre de Paris Claude Bernard

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The research was conducted under the auspices of the Graduate School of Agriculture, Food, Biology, Environment and Health of the French Agricultural, Veterinary and Forest Institute.

# Abstract

The present study aimed to identify, in exploring the protein fraction of camelid milks from several regions of Kazakhstan, original molecules (peptide, proteins) potentially responsible for the properties attributed to camel milk. We initially analyzed globally the composition of camel milk protein fraction (proteomic approach), focusing mainly on caseins and their molecular diversity. Regarding whey proteins, we focused our efforts on the whey acidic protein (WAP) whose protease inhibitory properties are well established and which is an originality of camelids (the only large mammal with pig expressing this protein in milk). Finally, we started to isolate extracellular vesicles from milk, which are known to carry genetic information (mRNA and microRNA) and proteins involved in the communication between cells and organisms, in order to characterize their proteome.

Nearly 180 milk samples from two camel species (*Camelus bactrianus* and *C. dromedarius,* and their hybrids) we collected at different lactation stage, age and calving number, and submitted to different proven analytical techniques and proteomic approaches (SDS-PAGE, LC-MS/MS and LC-ESI-MS). A detailed characterization of 50 protein molecules, relating to genetic variants, isoforms arising from post-translational modifications and alternative splicing events, belonging to 9 protein families (κ-, $\alpha_{s1}$-, $\alpha_{s2}$-, β-; and γ-CN, WAP, α-LAC, PGRP, CSA/LPO) was achieved. We reported the occurrence of two unknown isoforms (i1 and i2) of camel $\alpha_{s2}$-CN arising from alternative splicing events. Using cDNA-sequencing, i1 was characterized as a splicing-in variant of an in-frame 27-nucleotide sequence, of which the presence at the genome level, flanked by canonic motifs defining an exon 13 encoding the nonapeptide ENSKKTVDM, was confirmed. Isoform i2, which appeared to be present at different phosphorylation levels ranging between 8P and 12P, was shown to include an additional decapeptide (VKAYQIIPNL), revealed by LC-MS/MS, encoded by a 3'-extension of exon 16. In addition, we reported, for the first time to our knowledge, the occurrence of a $\alpha_{s2}$-CN phosphorylation isoform with at least one phosphorylated S/T residue that does not match with the usual canonic sequence (S/T-X-A) recognized by the mammary kinase, suggesting thereby the existence of two kinase systems involved in the phosphorylation of caseins in the mammary gland.

This study also aimed to evaluate possible differences between species (genetic variability). We demonstrated that genetic variants, which hitherto seemed to be species-specific (*e.g*. β-CN A for Bactrian and β-CN B for dromedary), are in fact present both in *C. dromedarius* and *C. bactrianus*. Regarding camel β-CN we also determined a short isoform

(946 Da lighter than the full-length β-CN) arising very likely in both genetic variants (A and B) from proteolysis by plasmin.

As far as camel WAP is concerned, we identified in *C. bactrianus* a new genetic variant (B), originating from a transition G => A, leading to a codon change (GTG/ATG) in the nucleotide sequence of cDNA, which modifies a single amino acid residue at position 12 of the mature protein (V12M). In addition, we describe the existence of a splicing variant of camel WAP, arising from an alternative usage of the canonical splice site recognized as such in the other mammalian species expressing WAP in their milk. We also report that the WAP isoform predominantly present in camelids milk, first described by Beg et al. (1986) as displaying an additional sequence of 4 amino acid residues (56VSSP59) in the peptide segment connecting the two 4-DSC domains, results from the usage of an unlikely intron cryptic splice site, extending camel exon 3 on its 5' side by 12-nucleotides. In addition, we confirm that in the camel gene encoding WAP, intron 3 is a GC-AG intron, with a GC donor site showing a compensatory effect in terms of a dramatic increase in consensus at the acceptor exon position.

Finally, using an optimized protocol, we isolated camel milk-derived EVs satisfiying the typical requirements for exosomal morphology, size and protein content. We identified a thousand of different proteins representing the first comprehensive proteome of camel milk-derived extracellular vesicles that appears wider than camel milk proteome, including markers associated with small extracellular vesicles, such as CD63, CD81, HSP70, HSP90, TSG101 and ADAM10. We also identified proteins 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.

# Résumé

La présente étude visait à identifier, en explorant la fraction protéique des laits de camélidés provenant de plusieurs régions du Kazakhstan, des molécules originales (peptides, protéines) potentiellement responsables des propriétés attribuées au lait de chamelle. Nous avons d'abord analysé globalement la composition de la fraction protéique des laits (approche protéomique), en nous concentrant principalement sur les caséines et leur diversité moléculaire. S'agissant des protéines du lactosérum, nous avons concentré nos efforts sur la WAP dont les propriétés « inhibiteur de protéase » sont bien établies et qui est une originalité des camélidés (seul gros mammifère avec le porc exprimant cette protéine dans le lait). Enfin, nous avons commencé à isoler des vésicules extracellulaires du lait, qui sont connus pour porter des informations génétiques (ARNm et microARN) et des protéines impliquées dans la communication entre les cellules et les organismes, afin de caractériser leur protéome.

Près de 180 échantillons de lait de 2 espèces de camélidés (*Camelus bactrianus*, *C. dromedarius* et leurs hybrides) ont été collectés à différents stades de lactation, âge et nombre de vêlages, et soumis à différentes techniques analytiques et approches protéomiques (SDS-PAGE, LC-MS/MS et LC-ESI-MS). Cinquante molécules protéiques correspondant à des variants génétiques, des isoformes issues de modifications post-traductionnelles et d'épissages différentiels, appartenant à 9 familles de protéines (κ-, $\alpha_{s1}$-, $\alpha_{s2}$-, β- et γ-CN, WAP, α-LAC, PGRP, CSA / LPO) ont été caractérisées. L'existence de deux isoformes inconnues (i1 et i2) de la caséine αs2 a été observée dans les deux esèces. Ces isoformes sont des variants d'épissage consécutif pour l'un à l'intégration d'une séquence de 27 nucléotides « in frame », codant pour le nonapeptide ENSKKTVDM, dont la présence a été confirmée au niveau génomique, flanquée de motifs canoniques définissant une structure exonique. La seconde isoforme, présente à différents niveaux de phosphorylation compris entre 8P et 12P, comporte un décapeptide supplémentaire (VKAYQIIPNL), révélé par LC-MS/MS, codé par une extension 3 'de l'exon 16. En outre, nous rapportons, pour la première fois à notre connaissance, l'existence d'une isoforme de phosphorylation de la caséine αs2 présentant au moins un résidu S/T phosphorylé n'appartenant pas à la séquence canonique habituelle (S/T-X-A) reconnue par la kinase mammaire, suggérant ainsi l'existence de deux systèmes impliqués dans la phosphorylation des caséines, dans la glande mammaire.

Cette étude visait également à évaluer les différences entre espèces. Nous avons démontré que les variants génétiques, qui jusqu'ici semblaient être spécifiques d'espèce (β-CN A pour

Bactrian et β-CN B pour dromadaire), sont présents chez *C. dromedarius* et *C. bactrianus*. En ce qui concerne la caséine β, nous avons également pu identifier dans les deux variants génétiques (A et B) une isoforme courte (946 Da plus légère que la caséine β) résultant très probablement d'une protéolyse par la plasmine.

S'agissant de la WAP, nous avons identifié chez *C. bactrianus* un nouveau variant génétique (B), issue d'une transition G => A conduisant à un changement de codon (GTG/ATG) dans la séquence nucléotidique de l'ARNm, qui entraine un changement d'acide aminé en position 12 de la protéine mature (V12M). Un variant résultant de l'usage du site d'épissage canonique, reconnu comme tel chez les autres mammifères exprimant la WAP dans leur lait, a été identifié. La forme majoritaire de la WAP cameline, décrite pour la première fois par Beg et al. (1986) qui présente une insertion de 4 résidus d'acides aminés (56VSSP59) dans le segment peptidique reliant les deux domaines 4-DSC, résulte de l'utilisation d'un site d'épissage cryptique intronique improbable, prolongeant l'exon 3 du gène de 12 nucléotides sur son extrémité 5 '. De plus, nous confirmons que chez les camélidés, l'intron 3 du gène spécifiant la WAP, est un intron rare de type GC-AG, avec un site donneur faible qui s'accompagne d'un effet compensatoire au site consensus de l'exon accepteur.

Finalement, en utilisant un protocole optimisé, nous avons isolé les vésicules extracellulaires dérivés du lait de camélidés présentant les caractéristiques morphologiques, de taille et de contenu en protéines des exosomes. Nous avons identifié un millier de protéines différentes représentant le premier protéome des VE dérivés du lait de chamelle qui semble plus étendu que le protéome du lait de chamelle, incluant notamment les marqueurs associés aux VEs, tels CD63, CD81, HSP70, HSP90, TSG101 et ADAM10. Nous avons également identifié des protéines présentes dans d'autres compartiments du lait. C'est notamment le cas pour les protéines apparentées à Ras, MFG-E8, ou CD9 qui sont également présentes dans les globules gras du lait. Nos résultats suggèrent par ailleurs fortement que les VEs dérivés du lait de chamelle ont des origines cellulaires différentes.

*To my family, especially, my grandmother Zylikha, much appreciation*

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# Abbreviations

| | |
|---|---|
| α-LAC | α-lactalbumin |
| aa | Amino Acid |
| AL | Almaty region |
| B | Bactrian |
| bp | Base pair |
| BTN | Butyrophilin |
| CN | Casein |
| CSA | Camel serum albumin |
| D | Dromedary |
| Da | Dalton |
| EVs | Extracellular vesicles |
| FAS | Fatty acid synthase |
| GlyCAM1 | Glycosylation-dependent cell adhesion molecule 1 |
| H | Hybrid |
| i1, i2 and i3 | Isoforms 1, 2 and 3 |
| KZ | Kyzylorda region |
| LDH | Lactadherin |
| LC-MS/MS | Liquid chromatography coupled to tandem mass spectrometry |
| LC-ESI-MS | Liquid chromatography-electrospray ionization-tandem mass spectrometry |
| LPO | Lactoperoxidase |
| LTF | Lactoferrin |
| MEC | Mammary epithelian cells |
| MFG | Milk fat globule |
| MFGM | Milk fat globule membrane |
| P | Phosphat group |
| PCR | Polymerase chain reaction |
| PGRP | Peptido Glycan Recognition Protein |
| PP3 | Proteose peptone component 3 |
| PTM | Post-translational modifications |
| RP-HPLC | Reversed-Phase High-Performance Liquid Chromatography |
| SDS-PAGE | Sodium dodecyl sulfate–polyacrylamide gel electrophoresis |
| SH | Shymkent region |
| SIBLING | Small integrin-binding ligand N-linked glycoproteins |
| UP1 and UP2 | Unknown proteins 1 and 2 |
| WAP | Whey Acidic Protein |
| WP | Whey protein |
| XO | Xanthine oxidase |
| ZKO | Atyrau region |
| 4-DSC | Four-disulfide cores |

# Chapter 1

# General Introduction

## 1.1 Camelids: the other non-cattle dairy species of arid and semiarid rangelands

According to the most recent statistics, the world camel population is estimated to be about 29 millions (FAO, 2017). *Camelus dromedarius* is the most frequent and widespread domestic camel species, with 90% of the total camel population (Mohandesan et al., 2017). Camels have been domesticated in a number of arid regions, including Northern and Eastern Africa, the Arabian Peninsula and Central and South West Asia. *Camelus bactrianus* forms numerical inferiority, mostly inhabits in Mongolia, China, and Central Asia. Alternatively, there are also crossed camels (hybrids) which are found mainly in Russia, Iran, Turkmenistan, and in Kazakhstan. Kazakhstan is a specific region where both domesticated species (*Camelus dromedarius* and *Camelus bactrianus*) along with wild Bactrian camels (*Camelus ferus*) and their hybrids have been maintained in mixed herds (Nurseitova et al., 2014).

Dairy camel farming is a well-established part of a local economy in many arid and semiarid rangelands (Tulgat & Schaller, 1992). Camel dairy products provide not only food, but also give nomadic herders a rich source of income. The adoption of non-cattle species for milk provision was nevertheless significant and their importance can be associated with the adaptation of such species to specific geographical areas and also to local cultural beliefs and behaviors (Faye & Konuspayeva, 2012). Camel milk production accounts for only 0.34% (2.8 millions of tons) of world milk production (Faye & Konuspayeva, 2012), but interest for "white gold of the desert" is growing (Wernery, 2006). Situated in Central Asia, Kazakhstan dairy camel farming represents a vivid example of the vital adaptation of the regional economy. Camel milk is consumed as a fresh milk and as a traditional fermented drink called *shubat*, which is very popular in Central Asia countries. However, suitability for cheese production of camel milk is being low, in spite of the availability of a specific chymosin nowadays on the market and camel cheese making is in development. To our knowledge, there is no national statistical data available on camel milk industry in Kazakhstan; on average there are 35,000 of total 180,000 camel heads reared for milk production, and about 35,000 tons (1.25% of the camel milk produced in the world) of fresh and fermented milk per year are consumable (FAO, 2017). Besides its nutritional qualities, camel milk have been reported to display potential health-promoting properties (Mati et al., 2017). Fresh and fermented camel milk are widely consumed in alternative medicine for prophylactic and curative purposes of cancer (Korashy et

al., 2012), diabetes (Agrawal et al., 2013), autoimmune diseases (Al-Ayadhi et al., 2015), and hepatitis (El-Fakharany et al., 2017).

## 1.2 Camel milk as a source of health promoting compounds

Milk is a complete and complex food suited to the specific offspring requirements for growth and development. Camel milk has an overall composition very similar to that of bovine milk, especially as far as macro nutrients (protein, fat and lactose) are concerned. Camel milk is characterized by a high content of vitamin C.

There is increasing substantial evidence that milk contains many health-promoting compounds, influencing physiological functions or reducing disease risk. Bioactive components in milk might come from a variety of sources, such as lipids, carbohydrates and proteins as well as for minerals or vitamins. Some are synthesized and secreted by the mammary tissue, whereas others are drawn from maternal serum and carried across the mammary epithelium by receptor-mediated transport (Walther & Sieber, 2011). Furthermore, the secretion of the milk fat globule (MFG) into the acini lumen by the mammary epithelium carries with it a collection of membrane-bound proteins and lipids that are present into the milk (Figure 1.1).

Proteins are found mostly in the aqueous phase, either in soluble (whey proteins), or colloidal (caseins) states, but also in the lipid phase, associated with the milk fat globule membrane (MFGM). Over the last century, protein research has investigated mainly the importance of essential amino acids and their relevance for nutrition and health. Some peptides, with particular amino acid sequences encrypted in camel milk proteins, which are inactive in the intact protein, may play a beneficial role in human health once they are released from milk either in vivo during normal digestion or by proteolysis during bacterial fermentation (Walther & Sieber, 2011). However, other compounds that may play a role in the health-promoting properties of camel milk have to be found. Extracellular vesicles, which are vectors of nucleotide sequences (small and long non-coding RNA) and proteins, could be also involved in these biological properties. Hence, to go further into the evaluation of the potential suitability of non-bovine milks, including camel milk, in human/infant nutrition, a detailed characterization of their protein fraction that contributes largely to the nutritional value and technological properties of milk, as well as the successful development of camel dairy industry, is required.

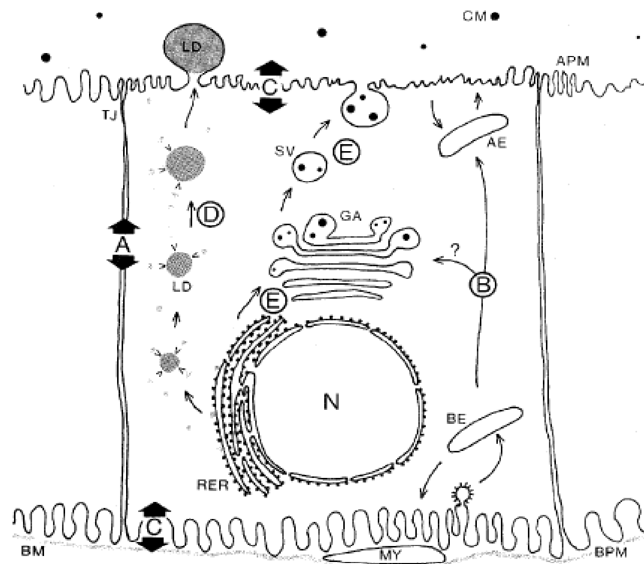


**Figure 1.1.** Major secretory pathways in mammary epithelial cells during lactation adapted from Mather & Keenan, 1998. (A) Paracellular route through leaky `tight' junctions; (B) Transcellular route through basal and apical endosomes and possibly through the secretory pathway; (C) Bidirectional transport of ions and small molecules via specific transporters in basal/lateral and apical plasma membranes; (D) Pathway for the assembly and secretion of milk-lipid droplets; and (E) Classical secretory pathway for the processing and secretion of milk proteins, lactose, water and ions. AE: apical endosome; APM: apical plasma membrane; BPM: basal plasma membrane; CM: casein micelle; GA: Golgi apparatus; LD: lipid droplet; N: nucleus; RER: rough endoplasmic reticulum; SV: secretory vesicle; BE: basal endosome; BM: basal membrane; TJ: `tight' junction.

# 1.3 The protein fractions of camel milk

Given the growing interest in camel milk, due to the health potential of its bioactive components (Al haj & Al Kanhal, 2010), the milk protein fraction of Camelids has been extensively investigated over the past 20 years and more during the last decade, with regards to casein, whey proteins and milk fat globule membrane proteins (Saadaoui et al., 2013). Whether it was on milk of one-humped Camels (*C. dromedarius)* (Alhaider et al., 2013; Elagamy et al., 1996; Ereifej et al., 2011; Erhardt et al., 2016; Felfoul et al., 2017; Hinz et al., 2012; Kappeler et al., 1999; Merin et al., 2001; Saadaoui et al., 2013; Salmen et al., 2012; Shuiep et al., 2013; Wangoh et al., 2009) or on milk of two-humped Camels (*C. bactrianus*) (Konuspayeva et al., 2007; Ochirkhuyag et al., 1997; Yang et al., 2013), all these studies from all around the world

have explored, with more or less efficient approaches, the composition of the major milk proteins.

## 1.3.1 Caseins

As in cow milk, *ca*. 80% of the total protein fraction of camel milk are represented by caseins (CN) that are synthesized under multi-hormonal control in the mammary gland. Associated with amorphous calcium phosphate nanoclusters they form large and stable colloidal aggregates, referred to as CN micelles. Casein micelles are present in the milk of all mammals. In bovine milk, still the most thoroughly studied milk to date, casein micelles are made of four distinct polypeptide chains: $\alpha_{s1}$-, β-, $\alpha_{s2}$- and κ-CN arising from the expression of four single copy autosomal genes (Figure 1.2).

Genes encoding CNs (*CSN1S1, CSN2*, *CSN1S2* and *CSN3*) are tightly linked on the same chromosome, BTA6 in cattle, CHI6 in goats (Hayes et al., 1993; Threadgill & Womack, 1990), and HSA4 in humans (Menon et al., 1992). The evolution of the CN gene cluster is postulated to have occurred by a combination of successive intra- and inter-genic exon duplications (Groenen et al., 1993; P. Martin et al., 2013; Rijnkels, 2002). In some mammals, including horse, donkey, rodents and rabbit, there are two $\alpha_{s2}$-CN encoding genes differentiating in size (*CSN1S2A* and *CSN1S2B*), which may have arisen by a relatively recent gene-duplication event in rabbit (Cosenza et al., 2010; Dawson et al., 1993). However, the existence of a second $\alpha_{s2}$-CN encoding gene in camel has not been reported so far.

CN micelles (Figure 1.3), which figure as calcium-transport vehicles, provide neonates with calcium at a very high concentration, which is achieved during their packaging in the secretion pathway (Semo et al., 2007). The CN micelle properties have a major influence on the technological properties of milk (Glantz et al., 2010). Micelles are characterized by a different size distribution in the milk from different mammals (Farrell et al., 2006). The average size of camel micelles is noticeably the largest: about 280 nm in diameter (Farah & Rüegg, 1989), 260 nm in goat, 190 nm in cow, and 180 nm in sheep milk (Park et al., 2007). The average diameter is inversely related to κ-CN and calcium phosphate concentrations; it has been established that large micelles are richer in calcium phosphate and smaller in κ-CN (Bornaz et al., 2009).

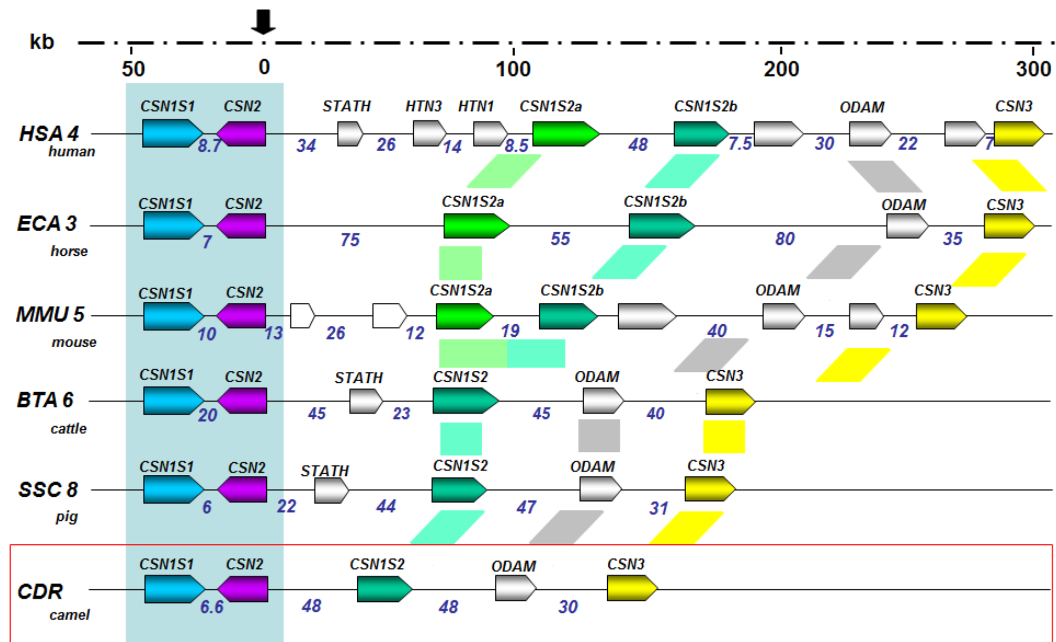


**Figure 1.2.** Evolution of the casein locus organization. Casein locus organization of human (*Homo sapiens*), horse (*Equus caballus*), mouse (*Mus musculus*), cattle (*Bos taurus*), pig (*Sus scrofa*) and camel (*Camelus dromedarius*) genomes (adapted from Martin et al., 2013 with additional genomic information from the NCBI) is compared. Genes are given by colored arrow boxes, showing the orientation of transcription. Putative genes based on similarity are indicated by empty boxes. Intergenic region sizes are given in kb.

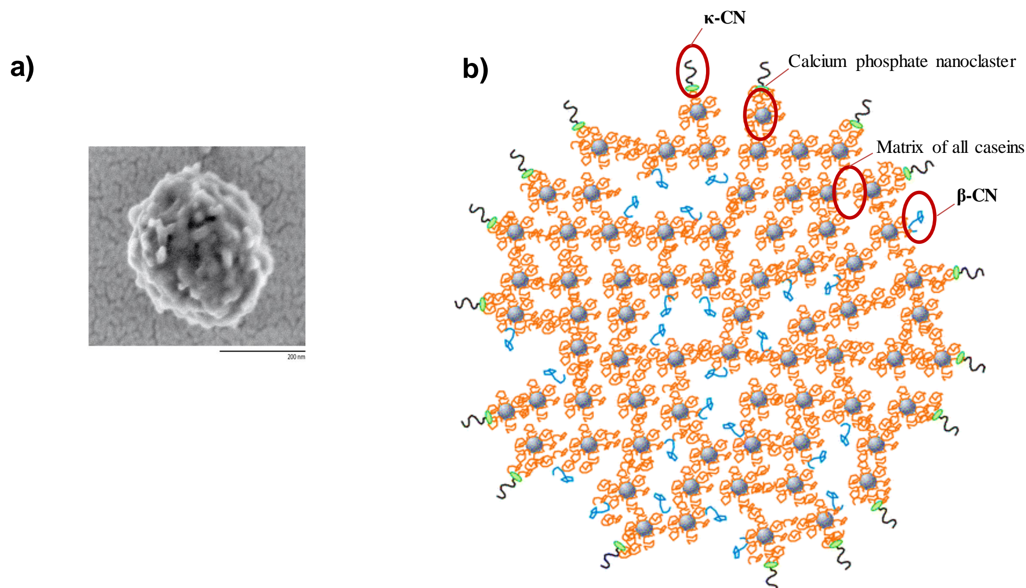


**Figure 1.3.** Field-emission scanning electron microscopy image of a casein micelle (a) and schematic representation of its structure (b) adapted from Dalgleish & Corredig, 2012. The $\alpha_s$ - and β-CNs (orange) are attached to and link the calcium phosphate nanoclusters (grey spheres). Some β-CN (blue) hydrophobically binds to other caseins and can be removed by cooling. The para κ-CN (green) and the caseinomacropeptide chains (black) are on the outermost parts of the surface.

### 1.3.2 Whey proteins

Camel whey is characterized by the presence of protective proteins, which display a wide range of bioactivities (Davoodi et al., 2016), including immuno-modulating (Legrand et al., 2004), anti-carcinogenic (Habib et al., 2013), antibacterial, and antifungal activities (Kanwar et al., 2015). The WPs of camel milk mainly consist of α-lactalbumin (α-LAC), glycosylation-dependent cell adhesion molecule 1 (GlyCAM1) or lactophorin which is closely related to the bovine proteose peptone component 3 (PP3), the innate immunity Peptido Glycan Recognition Protein (PGRP) and the Whey Acidic Protein (WAP). PGRP is an intracellular component of neutrophils, which modulates anti-inflammatory reaction of the immune response (Kappeler et al., 2004). Present at a very low level in ruminant milks (Tydell et al., 2002), PGRP has been detected in mammary secretions of porcine and camel (Kappeler et al., 2004) and was shown to participate in granule-mediated killing of gram-positive and negative bacteria (Dziarski et al., 2012). Lactoferrin (LTF) interacts with lipopolysaccharides of Gram-negative bacteria whereas lysozyme C binds and hydrolyzes peptidoglycans, preferably of Gram-positive bacteria, but with a lower affinity than PGRP (Sharma et al., 2011). PP3 plays an important immunological role in the lactating camel, to prevent the occurrence of mastitis, or for its newborn by inhibiting pathogen multiplication in the respiratory and gastrointestinal tracts of the suckling young (Girardet et al., 2000). WAP plays an important role in regulating the proliferation of mammary epithelial cells by preventing elastase-type serine proteases from carrying out extracellular matrix laminin degradation. In addition, a bacteriostatic activity of rat WAP against *Staphylococcus aureus* was reported (Iwamori et al., 2010). Whereas camel α-lactalbumin (α-LAC), a small milk calcium-binding globular protein, is known to possess noticeable anticancer activity, which is determined by the ability of this protein to form complexes with oleic acid (Uversky et al., 2017). Previously it was reported, that HAMLET (human α-LAC made lethal to tumor cells), a protein lipid complex formed by α-LAC and oleic acid, induces apoptosis-like death in tumor cells (Svanborg et al., 2003).

### 1.3.3 Milk fat globule membrane proteins

Additionally, camel milk contains proteins from milk fat globules which represent 1–4% of total protein fraction (Cavaletto et al., 2008). Due to the functional and nutritional properties, increasing attention is being paid to the components of MFGM, especially to their protein components (Yang et al. 2015). Thus, MFGM proteins are known to be involved in

many biological functions, such as inhibition of pathogen adhesion and participation in antimicrobial defense (Smolenski et al., 2007). Large-scale studies have been published for goat (Cebo et al., 2010), ovine (Pisanu et al., 2011), and bovine MFGM proteins (Reinhardt et al., 2012). More recently proteomic profiling of the MFGM from camel (*C. dromedarius*) milk has been performed by Saadaoui et al. (2013). In result, 322 proteins associated with the dromedary MFGM, such as major MFGM proteins including fatty acid synthase (FAS), xanthine oxidase (XO), butyrophilin (BTN), and lactadherin (LDH/MFG-E8), were identified. Due to the secretion process, the protein composition of MFGM reflected those of the ER and apical plasma membrane. The MFGM proteomic dataset also contained a large number of cytoplasmic proteins as found in other studies. Thus, the MFGM can reflect dynamic changes within the mammary epithelian cells (MEC) and may provide a “snapshot” of mammary gland biology under particular conditions.

## 1.4 Factors responsible for the molecular complexity of milk proteins

Milk protein polymorphism is a unique biological paradigm, which helps to understand protein transport, micelle formation and organization, biodiversity and evolution, the release of bioactive peptides with implications in human health. Therefore, there is a need to obtain insight into the primary structure and the way of modifications of proteins, characterize the polymorphism at the protein and mRNA levels for better understanding the genetic basis of milk quality in dairy animals (Claverol et al., 2003). Genetic variants and post-translational modifications (PTM) of some camel milk proteins were reported by several previous studies (Pauciullo et al., 2013; Shuiep et al., 2013).

### 1.4.1 Genetic variants

Genetic differences can be due to point mutations such as single nucleotide polymorphisms or due to DNA rearrangements such as structure variations, insertions or deletions. When changes occur in regulatory region, alteration may occur at the transcription level, and may result in different levels of expression and different amounts of proteins. When non-synonymous changes occur in coding regions, this will result in aa substitutions. One or more differences in aa sequence result in different protein variants, which may possess different physical and chemical qualities. Recently, the impact of milk protein variants on milk

composition, production and on technological properties has been reported (Lodes et al., 1996). Effects of milk proteins on human nutrition and health have further increased the interest in milk protein variants and genetic determination. In camel milk, genetic variants of the two caseins ($\alpha_{s1}$- and β-CNs) arising from single-point mutations have been detected so far. Shuiep et al. (2013) demonstrated the genetic variation of camel $\alpha_{s1}$-CN at the protein level leading (E30D) to the expression of two distinct variants called A and C. Pauciullo et al. (2014) described two genetic variants A and B of camel β-CN resulting from aa exchange in position 186 (M186I).

## 1.4.2 Alternative splicing

In mammals, alternative splicing is a major mechanism for the enhancement of transcriptome and proteome diversity that greatly expands the repertoire of protein function (Keren et al., 2010). Splicing of precursor mRNA (pre-mRNA) is a crucial regulatory stage in the pathway of gene expression: introns are removed and exons are ligated to form mRNA. The inclusion of different exons in mRNA - alternative splicing - results in the generation of different isoforms from a single gene (Keren et al., 2010). The sequences required for splicing in higher eukaryotes consist of conserved elements at the 5' and 3' splice sites and a weakly conserved element, the branch point sequence, at the site of lariat formation (Martin & Leroux, 1992). Each of these elements seems to play multiple roles in the splicing reaction, which takes place in a large ribonuclear protein complex called the spliceosome, and progress through a two-step pathway. First, cleavage occurs at the 5' splice site and the intron 5' end is joined to a 2' OH of an adenosyl phosphate residue (A*) at the branch point sequence: YTRA*Y, generating the lariat intermediate (Harris & Senapathy, 1990; Zhuang & Weiner, 1989). Second, cleavage occurs at the 3' splice junction with concomitant ligation between the two contiguous exons. In addition, exon sequences play a role in splice site selection (Reed & Maniatis, 1986). However, the weak conservation of abovementioned elements in higher eukaryotes does not usually allow the prediction of some sequences, which are recognized and used.

Such an alternative splicing event has been first demonstrated in goat $\alpha_{s1}$-CN, the alternatively processed transcript of which was lacking of exons 9, 10 and 11 together encoding 37 aa residues (Leroux et al., 1992). Multiple forms of $\alpha_{s1}$-CN have been also reported in sheep (Chianese et al., 1996; Ferranti et al., 1999), in camel (Kappeler et al., 1998) and later in lama (Pauciullo & Erhardt, 2015). In camel $\alpha_{s1}$-CN, two cDNAs (short and long) encoding two

protein isoforms of 207 and 215 aa were described (Kappeler et al., 1998). The nucleotide sequence of the most frequent variant transcript was shown to be deleted of an octapeptide (EQAYFHLE) encoded by exon 16.

### 1.4.3 Post-translational modifications - Phosphorylation

The term PTM (Post-Translational Modifications) denotes changes in the polypeptide chain due to either the addition or removal of distinct chemical moieties to amino acid residues, proteolytic processing of the protein termini, or the introduction of covalent cross-links between domains of the protein. PTMs are involved in most cellular processes including the maintenance of protein structure and integrity, regulation of metabolism and defense processes, and in cellular recognition events and morphology changes (Larsen et al., 2006). Phosphorylation of proteins is one of the most frequent PTM in eukaryotic cells. It has become a common knowledge that phosphorylation of CN occurs at S or T aa residues in tripeptide sequences S/T-X-A where X represents any aa residue and A is an acidic aa residue (Mercier, 1981). This consensus sequence is recognized by FAM20C, a Golgi CN-kinase, which phosphorylates secreted phosphoproteins, including both CN and members of the small integrin-binding ligand N-linked glycoproteins (SIBLING) protein family, which modulate biomineralization (Ishikawa et al., 2012). PTM, occurring in the endoplasmic reticulum and/or Golgi complex after synthesis of the polypeptide chain, play a critical role in micelle formation and stability (Holland, 2008).

## 1.5 Extracellular vesicles

Milk is usually consider 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. 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 (Tkach & Théry, 2016). 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 (Delcayre et al., 2005; G. Raposo, 1996). 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 with respect to mode of biogenesis, cell type, and physiologic

conditions (Abels & Breakefield, 2016). Exosomes represent the smallest population among EVs, ranging in size from 30 to 150 nm in diameter (Hromada et al., 2017). 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 (Figure 1.4) (van der Pol et al., 2012). Found in all biofluids exosomes harbor different cargos as a function of cell type and physiologic state (Abels & Breakefield, 2016).

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 (Abels & Breakefield, 2016; Hromada et al., 2017). Milk is therefore thought to play an important role in the development of the immune system of the offspring. 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 non-coding RNA and miRNA contents. Exosomes and wider EVs 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 (Colombo et al., 2014; Simons & Raposo, 2009). RNAs conveyed by EVs are translated into proteins within transformed cells (mRNA), and/or are involved in regulatory functions (miRNA). For this reason, EVs are recognized as potent vehicles for intercellular communication, capable for transferring messages of signaling molecules, nucleic acids, and pathogenic factors (Kabani & Melki, 2016).

Over the last decade, EVs 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 (Kanada et al., 2015). 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 (Keller et al., 2006). Recently, cell culture studies showed that bovine milk-derived EVs act as a carrier for chemotherapeutic/chemopreventive agents against lung tumor xenografts *in vivo* (Munagala et al., 2016). 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 (Reinhardt et al., 2012; van Herwijnen et al., 2016), there are only one dealing with exosomes derived from camel milk (Yassin et al., 2016). 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.

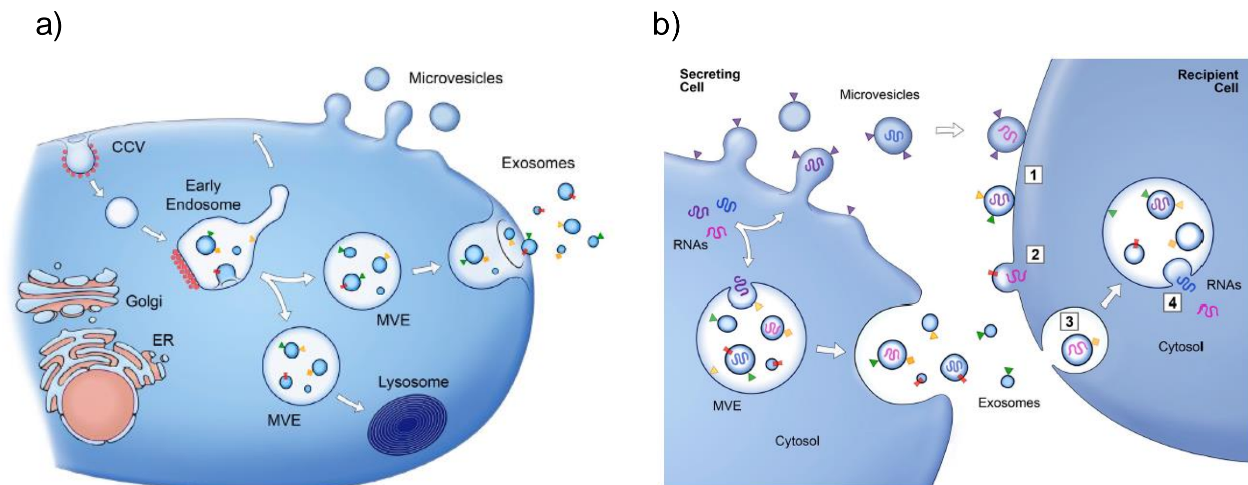


**Figure 1.4. a)** Release of microvesicles (MVs) and exosomes adapted from Raposo & Stoorvogel, 2013. MVs bud directly from the plasma membrane, whereas exosomes are represented by small vesicles of different sizes that are formed as the intraluminal vesicle by budding into early endosomes and MVEs and are released by fusion of multivesicular endosome (MVEs) with the plasma membrane. **b)** Schematic of protein and RNA transfer by EVs adapted from Graça Raposo and Stoorvogel (2013). Membrane-associated (triangles) and transmembrane proteins (rectangles) and RNAs (curved symbols) are selectively incorporated into the intraluminal vesicle of MVEs or into MVs budding from the plasma membrane. MVEs fuse with the plasma membrane to release exosomes into the extracellular milieu. MVs and exosomes may dock at the plasma membrane of a target cell (1). Bound vesicles may either fuse directly with the plasma membrane (2) or be endocytosed (3). Endocytosed vesicles may then fuse with the delimiting membrane of an endocytic compartment (4). Both pathways result in the delivery of proteins and RNA into the membrane or cytosol of the target cell.

# 1.6 Aim and outline of this study

The main objective of this thesis was to investigate the fine protein composition of *Camelus* (Bactrian, dromedary and hybrids) milks coming from different regions of Kazakhstan and of milk-derived extracellular vesicles with special emphasis on their protein contents, combining the most innovative proteomic and molecular biology approaches. We expected: i) identifying known and eventually unknown proteins from camel milk exhibiting potentially

bio-activities properties; ii) providing a solid foundation for health allegations and to get a better understanding of the mechanisms involved in the true or expected effect of camel milk on human health; iii) asserting or not the originality of camel milk amongst the other dairy species. Thus, to gain an insight into the molecular diversity of camel milk proteins, we report in **Chapter 2** a complete profiling of the milk protein fraction, including in-depth characterization of the camel caseins and whey proteins comprising variants related to genetic polymorphisms, splicing defects, phosphorylation levels. In **Chapter 3**, we report the characterization of two unknown camel $\alpha_{s2}$-CN splicing isoforms, resulting from translation of mRNAs yielded during the processing of primary transcripts encoding $\alpha_{s2}$-CN. In **Chapter 4**, we describe a new genetic variant of camel WAP (variant B) and a splicing variant, arising from the usage of an unlikely intron cryptic splice site. In addition, we report the occurrence of a GC-AG intron (intron 3) in the camel gene encoding WAP. In **Chapter 5,** we provide results on the isolation and in-depth morphological and proteome characterization of camel milk-derived extracellular vesicles. **Chapter 6** was devoted to a general discussion of the results obtained during the course of this thesis focusing onto the consequences in terms of bioactive properties of camel milks.

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# Chapter 2

# Combining different proteomic approaches to resolve complexity of the milk protein fraction of dromedary, Bactrian camels and hybrids, from different regions of Kazakhstan

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*PLOS ONE 13(5):e0197026. (2018)* https://doi.org/10.1371/journal.pone.0197026

# Abstract

Nutritional suitability of milk is not only related to gross composition, but is also strongly affected by the microheterogeniety of the protein fraction. Hence, to go further into the evaluation of the potential suitability of non-bovine milks in human/infant nutrition it is necessary to have a detailed characterization of their protein components. Combining proven proteomic approaches (SDS-PAGE, LC-MS/MS and LC-ESI-MS) and cDNA sequencing, we provide here in depth characterization of the milk protein fraction of dromedary and Bactrian camels, and their hybrids, from different regions of Kazakhstan. A total 391 functional groups of proteins were identified from 8 camel milk samples. A detailed characterization of 50 protein molecules, relating to genetic variants and isoforms arising from post-translational modifications and alternative splicing events, belonging to nine protein families (κ-, $\alpha_{s1}$-, $\alpha_{s2}$-, β-; and γ-CN, WAP, α-LAC, PGRP, CSA/LPO) was achieved by LC-ESI-MS. The presence of two unknown proteins UP1 (22,939 Da) and UP2 (23,046 Da) was also reported as well as the existence of a β-CN short isoform (946 Da lighter than the full-length β-CN), arising very likely in both genetic variants (A and B) from proteolysis by plasmin. In addition, we report, for the first time to our knowledge, the occurrence of a $\alpha_{s2}$-CN phosphorylation isoform with 12P groups within two recognition motifs, suggesting thereby the existence of two kinase systems involved in the phosphorylation of caseins in the mammary gland. Finally, we demonstrate that genetic variants, which hitherto seemed to be species- specific (*e.g.* β-CN A for Bactrian and β-CN B for dromedary), are in fact present both in *Camel dromedarius* and *C. bactrianus*.

**Key words:** *Camelus dromedarius, Camelus bactrianus,* hybrids, milk, casein, whey proteins, post-translational modifications, splicing, genetic polymorphism, phosphorylation, proteomics

## 2.1 Introduction

According to the most recent statistics, the world camel population is estimated to be about 29 million (FAO, 2017). *Camelus dromedarius* is the most frequent and widespread domestic camel species composing 90% of the total camel population (Mohandesan et al., 2017). Camels have been domesticated in a number of arid regions, including Northern and Eastern Africa, the Arabian Peninsula and Central and South West Asia. *Camelus bactrianus* forms numerical inferiority, mostly inhabits in Mongolia, China, and Central Asia. Alternatively, there are also crossed camels (hybrids) which are found mainly in Russia, Iran, Turkmenistan, and in Kazakhstan.

Kazakhstan is a specific region where both domesticated species (*C. dromedarius* and *C. bactrianus*) along with wild Bactrian camels (*Camelus ferus*) are maintained in mixed herds (Nurseitova et al., 2014). There are about 35,000 camel heads reared in this country for milk production (FAO, 2017). Camel milk is consumed as fresh milk and as a traditional fermented drink called *shubat*, which is very popular in Central Asia countries. Besides nutritional qualities, camel fresh and fermented milk have been reported to display potential health-promoting properties (Agrawal et al., 2003; Al-Ayadhi & Elamin, 2013; El-Fakharany et al., 2017; Korashy et al., 2012; Manaer et al., 2015; Sboui et al., 2010) which depend very heavily on its unique protein content.

Advanced improvement in proteomic techniques allow nowadays obtaining a precise image of the protein fraction of milk. Recently, proteomic approaches, based on mass spectrometry (Alhaider et al., 2013) and isobaric tag for relative and absolute quantification (Yang et al., 2013), have been used to analyze the proteome of dromedary camel milk and Bactrian camel milk whey, respectively. These techniques were useful to gain knowledge on the detection, quantification and characterization of camel milk proteins. These studies confirm that camel milk is a rich source of biologically active proteins and peptides (Hsieh et al., 2015; Mati et al., 2017).

Whey proteins which were reported to display a wide range of bioactivities (Davoodi et al., 2016), including immuno-modulating (Legrand et al., 2004), anti-carcinogenic (Habib et al., 2013), antibacterial, and antifungal activities (Kanwar et al., 2015), account for 20% of total camel milk proteins. Pattern-recognition proteins, such as the peptidoglycan recognition protein (PGRP), an intracellular component of neutrophils, modulate anti-inflammatory reaction of the immune response (Kappeler et al., 2004). LTF interacts with lipopolysaccharides of Gram-negative bacteria whereas lysozyme C binds and hydrolyzes peptidoglycans, preferably of

Gram-positive bacteria, but with a lower affinity than PGRP (Sharma et al., 2011). Present at a very low level in ruminant milks (Tydell et al., 2002), PGRP has been detected in mammary secretions of porcine and camel (Kappeler et al., 2004) and was shown to participate in granule-mediated killing of gram-positive and negative bacteria (Dziarski et al., 2012). Proteose peptone component 3 (PP3 or Lactophorin or GlyCAM1) plays an important immunological role in the lactating camel, to prevent the occurrence of mastitis, or for its newborn by inhibiting pathogen multiplication in the respiratory and gastrointestinal tracts of the suckling young (Girardet et al., 2000). Likewise, camel milk contains the whey acidic protein (WAP), also found in rodents and lagomorphs (Hennighausen & Sippel, 1982). The biological function of this protein is unknown. However, proteins such as elafin and antileukoproteinase 1, containing WAP domains, are known to function as protease inhibitor involved in the immune defence of multiple epithelia and has been identified as candidate molecular markers for several cancers (Bouchard et al., 2006).

As in cow milk, *ca*. 80% of the total protein fraction of camel milk are represented by caseins (CN) that are synthesized under multi-hormonal control in the mammary gland of mammals. Associated with amorphous calcium phosphate nanoclusters they form large and stable colloidal aggregates, the so-called CN micelles, which figure as calcium-transport vehicles. These CN micelles provide neonates with calcium at a very high concentration, which is achieved during their packaging in the secretion pathway (McMahon & Oommen, 2013). Recently it was reported that $\alpha_{s1}$- and $\alpha_{s2}$-CN display molecular chaperone-like activity inhibiting CN aggregation and triggering micelle structure (Sakono et al., 2011).

However, there is no comprehensive investigation on milk protein variations and variability in composition between individual camels. In addition, proteomic studies did not consider the molecular diversity of each type of protein, arising from genetic polymorphisms (mutations), defects in the processing of primary transcripts and post-translational modifications (PTM) such as phosphorylation, factors that significantly have a pronounced impact on protein structure, and finally on milk properties. Milk protein polymorphism is a unique biological paradigm that could help to understand CN intracellular transport, micelle formation and organization, biodiversity and evolution (Martin et al., 2013), the release of bioactive peptides with implications in human health (Balteanu et al., 2013).

Therefore, to gain an insight into the molecular diversity of camel milk proteins, we design a comprehensive strategy combining classical (SDS-PAGE) and advanced proteomic approaches (LC-MS/MS, LC-ESI-MS), as well as cDNA sequencing. Here we report a complete profiling of the milk protein fraction of Bactrian and dromedary camels from

Kazakhstan, including a detailed characterization of camel CN and WPs including variants related to genetic polymorphisms, splicing defects, phosphorylation levels. In addition, we introduce a reference point for further investigation in camel milk protein polymorphism.

# 2.2 Materials and Methods

## 2.2.1 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 with the exception of the rules of afore-mentioned European Community regulations on animal experimentation, which were strictly followed.

## 2.2.2 Milk samples collection and preparation

In total 181 raw milk samples (Table 2.1.) were collected during morning milking on healthy dairy camels belonging to two camel 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. Bactrian camels were originating from Kazakh type whereas dromedary camels were from Turkmen Arvana breed. Unfortunately, the information about the nature and the level of hybridization of hybrids was not available. All species are well adapted to the local environment of Kazakhstan.

Camels grazed on four various natural pastures with the distance more than 3,500 kms between the regions at extreme points of Kazakhstan: Almaty (AL) at the foot of Tien Shan Mountain, Shymkent (SH) along deserts Kyzylkum and Betpak-Dala, Kyzylorda (KZ) on the edge of the steppe, and Atyrau (ZKO) at the mouth of the Caspian Sea (Figure 2.1). Whole-milk samples were centrifuged at 2,500 *g* for 20 min at 4ºC (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.

**Table 2.1.** Camel milk samples collected (n = 181) in the 3 species of the 4 regions of Kazakhstan

| ID | Region | Coding | Bactrian (B) | Dromedary (D) | Hybrid (H) | Total number of camels for each region |
|---|---|---|---|---|---|---|
| 1 | Almaty | AL B/D/H | 13 | 20 | 1 | 34 |
| 2 | Shymkent | SH B/D/H | 20 | 21 | 20 | 61 |
| 3 | Kyzylorda | KZ B/D/H | 18 | 16 | 20 | 54 |
| 4 | Atyrau | ZKO B/D/H | 21 | 8 | 3 | 32 |

**Figure 2.1.** Geographical location of camel milk sampling

## 2.2.3 Selection of milk samples for analysis

Of the 181 milk samples collected, 63, including *C. bactrianus* (n=19), *C. dromedarius* (n=20), and hybrids (n=24) from four different regions of Kazakhstan were selected for SDS-PAGE analysis (Figure 2.2). Each Bactrian and dromedary camel group formed by 5 animals, except Bactrians of Atyrau regions (n=4). For hybrids, there were 4 groups comprising 10 animals (Kyzylorda and Shymkent regions), whereas there were only 1 and 3 animals for Almaty and Atyrau regions, respectively. This selection was based on lactation stages and number of parities (from 2 to 14) 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. Due to the lack of sufficient information, dromedary milk samples (n=5) from Almaty region were excluded from subsequent analyses. Then, 8 of the 58 remaining milk samples from three different regions (*C. bactrianus*, n=3, *C. dromedarius*, n=3, and hybrids, n=2) exhibiting the most representative SDS-PAGE patterns were analyzed by LC-MS/MS after a tryptic digestion of excised gel bands. Additionally, 30 milk samples (*C. bactrianus*, n=10; *C. dromedarius*, n=10; hybrids, n=10), taken from the 63 milks analyzed by SDS-PAGE, were analyzed by LC-ESI-MS (Bruker Daltonics).

## 2.2.4 Coomassie blue (Bradford) protein assay

To estimate the concentration of total protein in a milk sample the Coomassie Blue Protein Assay was used (Bradford, 1976). Absorbance at 590 nm was measured using the UV-Vis spectrophotometer (UVmini-1240, Shimadzu). The reference standard curve was done with commercial bovine serum albumin (BSA) powder dissolved in MilliQ water and diluted to a concentration of 1 mg/mL. Series of dilutions (0.1, 0.2, 0.4, 0.6, and 0.8 μg/μL) were prepared from the stock solution, in duplicate to ensure the protein concentration is within the range of the assay.

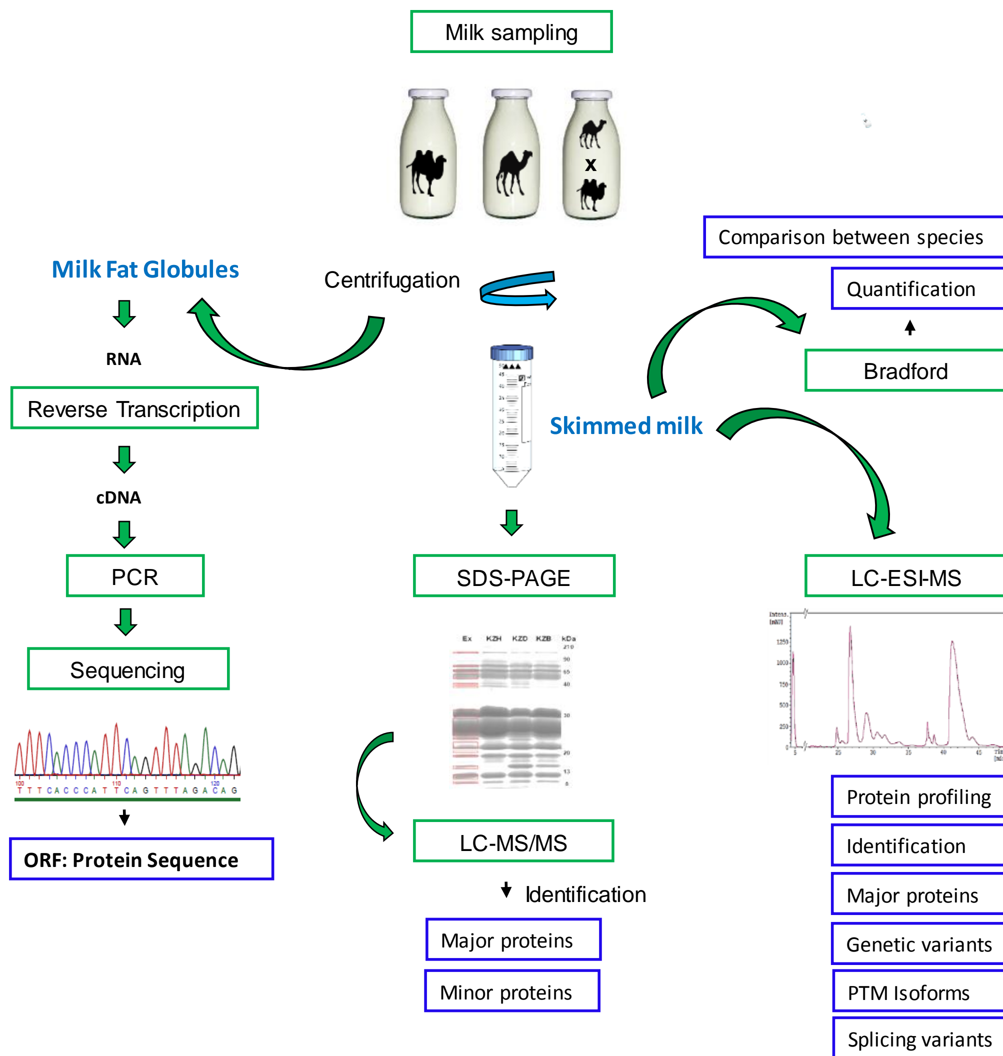


**Figure 2.2.** Diagram of the experimental scheme designed for quantification and identification of camel milk proteins

## 2.2.5 1D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Both major and low-abundant proteins resolved by SDS-PAGE were identified after excision by mass analysis of the tryptic hydrolysate. The method used in the study was based on that from Laemmli (Laemmli, 1970). Twenty-five micrograms of each individual skimmed milk sample were loaded into 12.5% acrylamide resolving gel and subjected to electrophoresis. Samples were prepared with Laemmli Lysis-Buffer (Sigma-Aldrich). Separations were performed in a vertical electrophoresis apparatus (Bio-Rad, Marnes-la-Coquette, France). After

GelCode Blue Safe Protein staining and gel scanning using Image Scanner iii (Epson Expression$^{TM}$ 10,000 XL, Sweden), resolved bands were excised from the gel and submitted to digestion by trypsin. Thereafter, tryptic peptides were analyzed by LC-MS/MS.

## 2.2.6 Identification of proteins by LC-MS/MS analysis

In order to identify the main protein contained in each electrophoretic band, mono dimensional electrophoresis (1D SDS-PAGE) followed by trypsin digestion and by LC-MS/MS analysis, was used essentially as described (Saadaoui et al., 2014). Briefly, after a 10 cm migration of samples in such an 1D SDS-PAGE, the 16 main electrophoretic bands (1.5 $mm^3$) were cut on each gel lane, transferred into 96-well microtiter plates (FrameStar, 4titude, 0750/Las). Reduction of disulfide bridges of proteins was carried out by incubating at 37ºC for one hour with dithiothreitol (DTT, 10 mM, Sigma), meanwhile the alkylation of free cysteinyl residues with iodoacetamide (IAM, 50 mM, Sigma) at RT for 45 min in total obscurity. After gel pieces were washed twice, first, with 100 µL 50% ACN/50 mM $NH_4HCO_3$ and then with 50 µL ACN, they were finally dried. The hydration was performed at 37ºC 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™ 3000 RSLCnano System (Thermo Fisher Scientific) coupled either to LTQ Orbitrap XL™ Discovery mass spectrometer or QExactive (Thermo Fischer Scientific). Four µL of each sample was injected with flow 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 Orbitrap with a resolution of 15 000 (scan range [m/z] = 300-1600) 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 $10^2$.

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 carbamido 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.

## 2.2.7 LC-ESI-MS

Fractionation of camel milk proteins and determination of their molecular masses, performed by coupling RP-HPLC to ESI-MS (micrOTOF$^{TM}$ II focus ESI-TOF mass spectrometer; Bruker Daltonics), were essentially as described (Saadaoui et al., 2014). In total 20 µL of skimmed milk samples were first clarified by the addition of 230 µL of clarification solution 0.1 M bis-Tris buffer pH 8.0, containing 8 M urea, 1.3% trisodium citrate, and 0.3% DTT. Clarified milk samples (25 µL) were directly injected onto a Biodiscovery C5 reverse phase column (300 Å pore size, 3 µm, 150 x 2.1 mm; Supelco, France). The mobile phase of the column corresponded to a gradient mixture of Solvent A ($H_2O$/TFA 100:0.25, v/v) and Solvent B (ACN/TFA 100:0.20, v/v). Elution was achieved using a linear gradient from 5% to 27% B in 20 min, from 27% to 33% B in 0.1 min, from 33% to 34% B in 11.1 min, from 34% to 40% B in 0.1 min, from 40% to 41% B in 14.9 min, and from 41% to 90% B in 0.1 min. This gradient elution was followed by an isocratic elution at 90% B for 4.9 min, and a linear return to 5% B in 0.1 min. The temperature of the column was adjusted to 52ºC and the flow rate to 0.2 mL/min. Eluted peaks were detected by UV-absorbance at 214 nm. The liquid effluent was introduced to the mass spectrometer. Positive ion mode was used, and mass scans were acquired over a mass-to-charge ratio (m/z) ranging between 600 and 3000 Da.

The LC/MS system was controlled by the HyStar software (Bruker Daltonics). Peak profiles from UV 214 nm and Extracted Ion Chromatograms (EIC), multicharged ion spectra, deconvoluted spectra and determination of masses were obtained with DataAnalysis Version 4.0 SP1 software (Bruker Daltonics).

## 2.2.8 Milk fat globule collection and RNA extraction

Milk was centrifuged at 2,500 *g* for 20 min to pellet somatic cells (SC) and to separate the upper milk fat globule (MFG) fraction. The MFG fraction was mixed with Trizol LS and heated briefly at 30°C while shaking, to emulsify fat. Total RNA was extracted from milk fat using Trizol (Invitrogen) following the protocol from the manufacturer, as described in Brenaut *et al.* (Brenaut et al., 2012).

## 2.2.9 First-strand cDNA synthesis and PCR amplification

First-strand cDNA was synthesized from 5 to 10 ng of total RNA primed with oligo(dT)20 and random primers (3:1, vol/vol) using Superscript III reverse transcriptase (Invitrogen Life Technologies Inc., Carlsbad, CA) according to the manufacturer's instructions. One microliter of 2 U/µL RNase H (Invitrogen Life Technologies) was then added and the reaction mix was incubated for 20 min at 37°C to remove RNA from heteroduplexes. Single-strand cDNA thus obtained was stored at -20°C. cDNA samples covering the entire coding regions of caseins were amplified. PCR was performed in an automated thermocycler GeneAmp® PCR System 2,400 (Perkin-Elmer, Norwalk, USA) with GoTaq® G2 Flexi DNA Polymerase Kit (Promega Corporation, USA). Reactions were carried out with 0.2 mL thin-walled PCR tubes with flat cap strips (Thermo Scientific, UK), in 50 µL volumes containing 5X Green or Colorless GoTag® Flexi Buffer, $MgCl_2$ Solution 25 mM, PCR Nucleotide Mix 10 mM each, GoTag® G2 Flexi DNA Polymerase (5 U/µL), 10 mM each oligonucleotide primer, template DNA and nuclease-free water, up to the final volume. Primer pairs, purchased from Eurofins (Eurofins genomics, Germany), were designed using published *Camelus* nucleic acid sequence (NCBI, NM_001303566.1). Sequencing of PCR fragments was performed with primer pairs used for PCR and sequenced from both strands, according to the Sanger method by Eurofins.

# 2.3 Results

## 2.3.1 Total protein content

Using the Bradford assay for estimating the protein concentration in milk samples, we observed that the highest protein concentration occurred with Bactrian camel milk samples, but the difference was slight comparing with crossed camel species. The total protein value in raw camel milk from Shymkent region was estimated to be *ca.* 33 g/L (33.15 ± 6.64 g/L) for *C. bactrianus* (n=5), and 31 g/L (30.83 ± 5.82 g/L) for *C. dromedarius* (n=7), whereas hybrids (n=9) displayed an intermediate value 31.5 g/L (31.43 ± 4.56 g/L). On average, Bactrian milk was considered to have a higher total protein content than that of Dromedary (Zhao et al., 2015) and hybrid milks. Our results are in agreement with data reported previously by Konuspayeva et al., (2009). No significant differences were found across species from different geographical locations.

## 2.3.2 Identification of main milk proteins from 1D SDS-PAGE by LC-MS/MS

After first adjusting protein concentrations at the same value, 63 individual camel milk samples were separated onto SDS-PAGE. The comparative analysis of whole milk samples by SDS-PAGE displayed rather similar electrophoretic profiles with related migration characteristics and the same apparent molecular weights between individual milk samples of different species and regions. A typical gel pattern from which proteins were identified in individual *C. bactrianus*, *C. dromedarius* and hybrid milk samples of Kyzylorda region is shown in Figure 2.3.

Sixteen main bands relatively well-resolved were excised from the electrophoretic pattern. The most intense band observed around 26 kDa was identified as β-CN. Quantitative analyses on camel milk proteins carried out before have demonstrated significantly higher amounts of β-CN compared to the homologous bovine CN (Kappeler et al., 2003). The most representative other bands were characterized as being: WAP (12.5 kDa), α-LAC (14.3 kDa), GlyCAM 1 (15.4 kDa and 17.2 kDa), κ-CN (20.3 kDa), PGRP (21.3 kDa), $\alpha_{s2}$-CN (22.9 kDa), $\alpha_{s1}$-CN (25.7 kDa), neutrophil gelatinase (28.3 kDa), lipoprotein lipase (46.5 kDa), perilipin-2 (47.2 kDa), butyrophilin (51.0 kDa), amine oxidase (55.3 kDa), lactadherin (56.2 kDa), heat shock protein (70.0 kDa), LTF (77.1 kDa), lactoperoxidase (87.7 kDa), and xanthine oxidase

(150 kDa). Masses mentioned above correspond to theoretical masses of proteins identified on the basis of tryptic profiles after LC-MS/MS analysis. Globally, the electrophoretic patterns of Kazakh camel milk samples agree with those reported recently for Israelian and Tunisian camel milk samples (Felfoul et al., 2017; Merin et al., 2001). However, surprisingly the prominent fact was the apparent absence in Kazakh milk samples of camel serum albumin (CSA), the major WP with a molecular mass equal to 66.0 kDa in camel colostrum (Merin et al., 2001). By contrast, this protein has been successfully identified, with the best *E*-value, in Tunisian fresh milk samples (Felfoul et al., 2017).

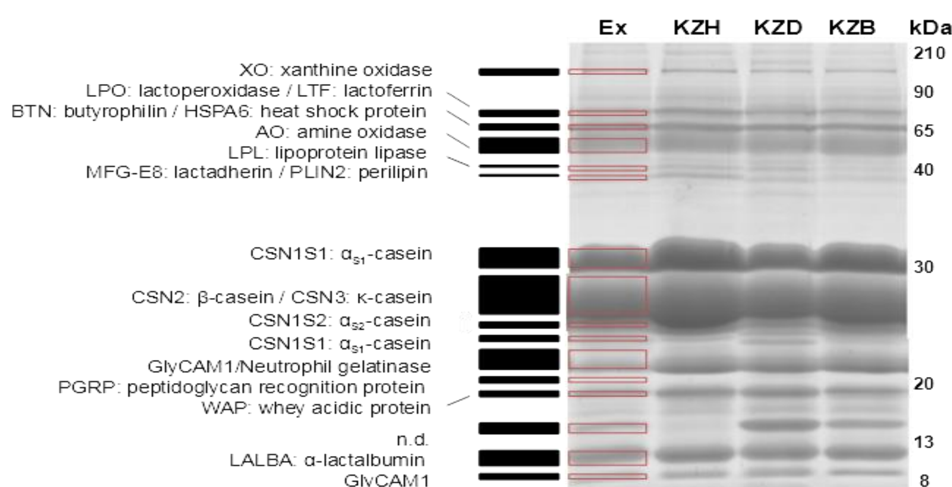


**Figure 2.3.** 1D SDS-PAGE pattern of *C. bactrianus* (KZB), *C. dromedarius* (KZD) and hybrid (KZH) skimmed milk samples of Kyzylorda (KZ) region. In the given example pattern (Ex), red frames and black boxes aligned correspond to electrophoretic bands that were excised from the gel and subsequently analyzed for protein identification, after tryptic digestion, by LC-MS/MS. Molecular weight markers from 210 to 8 kDa are indicated at the right of the gel.

## 2.3.3 Qualitative proteome of camel skimmed milk by LC-MS/MS

We took advantage of LC-MS/MS analysis to identify proteins in electrophoretic bands to go further into the description of the protein fraction of camel milk. Indeed, for each band analyzed by LC-MS/MS, between 10 and 70 different proteins were identified. In such a way, using UniprotKB taxonomy cetartiodactyla (SwissProt + Trembl) database, a total of 391 functional groups of proteins (proteins belonging to a same group share common peptides) were identified after LC-MS/MS analysis of 8 camel milk samples (S1 Table). A set of 235 proteins

was observed as common to the 8 milk samples. As example, a list of the first 70 common proteins found in milk samples of the three species from Shymkent region is given in Table 2.2.

Eight proteins were identified as authentically matching with proteins in *C. dromedarius* protein database, two with *C. bactrianus* protein database, 46 with *C. ferus* protein database, and the remaining (n=14) with the other mammalian species such as*, Lama guanicoe, Bos taurus, Sus scrofa* and *Ovis aries* protein databases. Immune-related proteins such as GlyCAM1, lactadherin (MFG-E8), and LTF, as well as milk fat globule membrane (MFGM)-enriched proteins such as xanthine oxidase (XO), butyrophilin (BTN), actin, ras-related protein Rab-18, ADP-ribosylation factor 1, tyrosine-protein kinase and GTP-binding protein SAR1b, were detected. Likewise, proteins originating from blood such as serpin A3-1, apolipoprotein A-1, α-1-antitrypsin like protein, α-1-acid glycoprotein, β-2-microglobulin, complement C3-like protein were found in all milk samples analyzed.

**Table 2.2.** Top 70 proteins identified by LC-MS/MS from individual *C. bactrianus* (B), *C. dromedarius* (D) and hybrid (H) milk samples of Shymkent region

| ID | Accession | Description | Mr | (-) log *E*-value | | | Coverage, % | | | Number of Spectra* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | kDa | B | D | H | B | D | H | B | D | H |
| 1 | O97943-2 | Short isoform of Alpha-S1-casein *(C. dromedarius)* | 25,7 | 453,63 | 535,3 | 585,79 | 88 | 92 | 92 | 574 | 679 | 842 |
| 2 | A0A077SL35 | Beta-casein *(C. bactrianus)* | 26,1 | 299,05 | 285,89 | 395,72 | 77 | 79 | 79 | 542 | 507 | 745 |
| 3 | O97944 | Alpha-S2-casein *(C. dromedarius)* | 22,9 | 285,16 | 320,65 | 276,39 | 70 | 70 | 66 | 357 | 451 | 376 |
| 4 | W6GH05 | Lactoferrin *(C. dromedarius)* | 77,1 | 723,25 | 557,5 | 1174,16 | 85 | 80 | 88 | 253 | 157 | 829 |
| 5 | P15522-2 | Isoform B of Glycosylation-dependent cell adhesion molecule 1 *(C. dromedarius)* | 15,4 | 112,39 | 189,45 | 192,31 | 64 | 70 | 69 | 220 | 359 | 358 |
| 6 | L0P304 | Kappa-casein *(C. bactrianus)* | 20,3 | 127,64 | 149,66 | 194,03 | 51 | 53 | 51 | 217 | 222 | 329 |
| 7 | S9WF76 | Lactadherin-like protein *(C. ferus)* | 45,6 | 263,03 | 350,07 | 501,73 | 51 | 53 | 57 | 162 | 236 | 436 |
| 8 | P00710 | Alpha-lactalbumin *(C. dromedarius)* | 14,3 | 288,2 | 277,9 | 329,27 | 83 | 80 | 83 | 161 | 159 | 175 |
| 9 | Q9GK12 | Peptidoglycan recognition protein 1 *(C. dromedarius)* | 21,3 | 308,94 | 359,49 | 336,8 | 73 | 73 | 79 | 112 | 156 | 140 |
| 10 | S9Z0L8 | Amine oxidase [flavin-containing] *(C. ferus)* | 55,3 | 233,97 | 231,94 | 277,32 | 74 | 70 | 75 | 110 | 138 | 190 |
| 11 | S9Y4T1 | Xanthine dehydrogenase/oxidase *(C. ferus)* | 150 | 270,07 | 435,86 | 330,26 | 40 | 45 | 41 | 79 | 138 | 121 |
| 12 | S9X3X3 | Butyrophilin subfamily 1 member A1 *(C. ferus)* | 51,09 | 95,37 | 140,19 | 162,3 | 47 | 50 | 51 | 61 | 107 | 111 |
| 13 | S9X4G0 | Neutrophil gelatinase-associated lipocalin-like protein *(C. ferus)* | 28,3 | 104,88 | 98,15 | 165,14 | 48 | 45 | 62 | 44 | 33 | 82 |
| 14 | S9X1L5 | Lipoprotein lipase isoform 3 (Fragment) *(C. ferus)* | 46,5 | 107,66 | 133,72 | 54,73 | 43 | 55 | 27 | 43 | 56 | 29 |
| 15 | S9YK74 | Perilipin *(C. ferus)* | 47,2 | 116,47 | 169,78 | 167,59 | 56 | 60 | 52 | 39 | 67 | 46 |
| 16 | P09837 | Whey acidic protein *(C. dromedarius)* | 12,5 | 42,97 | 59,92 | 82,11 | 55 | 75 | 84 | 36 | 43 | 73 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 17 | S9X4X6 | Uncharacterized protein *(C. ferus)* | 43,1 | 47,87 | 14 | 17,5 | 30 | 13 | 16 | 36 | 9 | 16 |
| 18 | S9X7Q1 | Lactoperoxidase isoform 1 preproprotein *(C. ferus)* | 87,7 | 83,1 | 62,59 | 65,16 | 26 | 22 | 20 | 25 | 19 | 17 |
| 19 | S9XDK9 | Complement C3-like protein *(C. ferus)* | 262,7 | 87,48 | 44,5 | 292,06 | 12 | 4 | 24 | 25 | 8 | 69 |
| 20 | S9XR87 | Beta-2-microglobulin *(C. ferus)* | 14,8 | 36,87 | 36,15 | 40,76 | 43 | 43 | 43 | 23 | 28 | 44 |
| 21 | O18831 | Growth/differentiation factor 8 *(S. scrofa)* | 42,7 | 44,62 | 49,29 | 67,44 | 28 | 24 | 32 | 23 | 22 | 17 |
| 22 | P68103 | Elongation factor 1-alpha 1 *(B. taurus)* | 50 | 58,23 | 42,59 | 78,3 | 31 | 27 | 31 | 20 | 12 | 24 |
| 23 | S9YCI6 | Peptidyl-prolyl cis-trans isomerase *(C. ferus)* | 23,8 | 44,54 | 43,1 | 71,24 | 54 | 54 | 57 | 20 | 21 | 22 |
| 24 | S9XP75 | Monocyte differentiation antigen CD14 *(C. ferus)* | 29,7 | 49,75 | 44,58 | 73,91 | 27 | 27 | 27 | 17 | 16 | 22 |
| 25 | S9WCV2 | Sulfhydryl oxidase *(C. ferus)* | 72,2 | 31,64 | 76,49 | 14,43 | 18 | 27 | 8 | 16 | 26 | 6 |
| 26 | S9YC53 | Alpha-1-antitrypsin-like protein *(C. ferus)* | 51,9 | 64,76 | 64,84 | 51,84 | 28 | 31 | 32 | 14 | 21 | 15 |
| 27 | S9YS49 | Putative E3 ubiquitin-protein ligase Roquin *(C. ferus)* | 158,5 | 56,65 | 90,28 | 8,84 | 10 | 14 | 2 | 14 | 29 | 3 |
| 28 | T0NN97 | Uncharacterized protein *(C. ferus)* | 151,4 | 38,86 | 53,95 | 36,72 | 12 | 13 | 11 | 13 | 16 | 15 |
| 29 | A0A0F6YEF6 | Anti-HCV NS3/4A serine protease immoglobulin heavy chain (Fragment) *(C. dromedarius)* | 13,4 | 38,24 | 35 | 52,95 | 24 | 24 | 24 | 12 | 10 | 26 |
| 30 | S9X9X0 | Vitelline membrane outer layer protein 1-like protein *(C. ferus)* | 24,2 | 44,94 | 52,11 | 75,95 | 51 | 50 | 53 | 12 | 14 | 31 |
| 31 | S9X358 | Tissue alpha-L-fucosidase *(C. ferus)* | 42,7 | 25,83 | 23,27 | 24,46 | 23 | 18 | 18 | 12 | 12 | 7 |
| 32 | S9WS72 | Sodium-dependent phosphate transport protein 2B-like protein *(C. ferus)* | 75,4 | 24,99 | 15,41 | 24,7 | 18 | 7 | 9 | 11 | 8 | 8 |
| 33 | S9X2V0 | UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase 1, membrane-bound form-like protein *(C. ferus)* | 37,3 | 30,18 | 13,09 | 38,99 | 23 | 11 | 33 | 11 | 7 | 15 |
| 34 | S9XSQ6 | Vitamin D-binding protein-like protein *(C. ferus)* | 49,3 | 41,66 | 109,25 | 18,01 | 24 | 45 | 8 | 10 | 30 | 3 |
| 35 | S9WGZ9 | Rab GDP dissociation inhibitor beta isoform 1 *(C. ferus)* | 35,7 | 27,75 | 22,32 | 55,78 | 32 | 25 | 43 | 10 | 8 | 15 |
| 36 | P19120 | Heat shock cognate 71 kDa protein *(B. taurus)* | 71,1 | 37,14 | 60,92 | 111,21 | 17 | 24 | 35 | 9 | 16 | 30 |
| 37 | S9XA25 | Ezrin isoform 5-like protein (Fragment) *(C. ferus)* | 71 | 24,32 | 28,23 | 47,46 | 13 | 10 | 20 | 9 | 7 | 13 |
| 38 | S9XI30 | Uncharacterized protein *(C. ferus)* | 22 | 25,16 | 66,23 | 20,66 | 38 | 54 | 39 | 9 | 31 | 8 |
| 39 | S9Y2X0 | Platelet glycoprotein 4 *(C. ferus)* | 38,3 | 28,14 | 40,27 | 55,15 | 15 | 17 | 29 | 9 | 8 | 17 |
| 40 | G9F6X8 | Protein disulfide-isomerase *(S. scrofa)* | 56,3 | 20,09 | 20,62 | 21,76 | 17 | 15 | 9 | 9 | 10 | 3 |
| 41 | S9WZP7 | Serpin A3-8 *(C. ferus)* | 74,9 | 15,26 | 28,06 | 72,32 | 11 | 12 | 20 | 8 | 12 | 28 |
| 42 | A7MBJ4 | Receptor-type tyrosine-protein phosphatase F *(B. taurus)* | 211,1 | 23,69 | 21,19 | 20,54 | 6 | 6 | 4 | 8 | 8 | 8 |
| 43 | S9YFG2 | Complement factor D *(C. ferus)* | 38,1 | 37,99 | 33,7 | 44,44 | 14 | 14 | 14 | 8 | 9 | 13 |
| 44 | S9WPL9 | Uncharacterized protein *(C. ferus)* | 84,6 | 31,09 | 39,23 | 57,64 | 12 | 10 | 17 | 7 | 6 | 18 |
| 45 | S9XE02 | Beta-2-glycoprotein 1-like protein *(C. ferus)* | 30,9 | 46,22 | 64,63 | 48,09 | 42 | 43 | 27 | 7 | 12 | 5 |
| 46 | B8XH67 | Na(+)/H(+) exchange regulatory cofactor NHE-RF *(S. scrofa)* | 39,2 | 20,94 | 37,33 | 32,94 | 22 | 22 | 30 | 7 | 9 | 11 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 47 | Q28452 | Quinone oxidoreductase *(L. guanicoe)* | 35,1 | 23,01 | 24,97 | 51,04 | 28 | 25 | 43 | 7 | 5 | 11 |
| 48 | S9YU13 | Glutathione S-transferase-like protein *(C. ferus)* | 27,7 | 34,68 | 8,59 | 22 | 21 | 15 | 19 | 7 | 3 | 4 |
| 49 | P00727-2 | Isoform 2 of Cytosol aminopeptidase *(B. taurus)* | 53,9 | 14,58 | 13,07 | 35,48 | 15 | 9 | 18 | 6 | 4 | 7 |
| 50 | S9WUC8 | Ig kappa chain V-II region RPMI 6410-like protein *(C. ferus)* | 26,7 | 22,72 | 12,39 | 32,25 | 19 | 10 | 27 | 6 | 4 | 15 |
| 51 | S9WRY0 | L-lactate dehydrogenase B chain isoform 1-like protein *(C. ferus)* | 30,2 | 25,51 | 28,95 | 37,66 | 22 | 22 | 22 | 6 | 7 | 6 |
| 52 | S9X5V9 | Fc of IgG binding protein (Fragment) *(C. ferus)* | 254,9 | 20,45 | 65,48 | 10,4 | 3 | 9 | 2 | 6 | 16 | 4 |
| 53 | B5B0D4 | Major allergen beta-lactoglobulin *(B. taurus)* | 19,8 | 9,94 | 54,67 | 12,3 | 21 | 59 | 25 | 6 | 27 | 7 |
| 54 | S9XE13 | Uncharacterized protein *(C. ferus)* | 82,6 | 12,16 | 32,28 | 71,26 | 5 | 10 | 15 | 5 | 17 | 22 |
| 55 | S9Y8C6 | Phosphoglucomutase 1 isoform 3-like protein *(C. ferus)* | 68,3 | 11,37 | 18,27 | 42,46 | 9 | 11 | 22 | 5 | 6 | 11 |
| 56 | S9Y3S5 | Olfactory receptor *(C. ferus)* | 108,3 | 19,83 | 37,9 | 43,59 | 4 | 6 | 7 | 5 | 9 | 11 |
| 57 | S9XT33 | Lipopolysaccharide-binding protein *(C. ferus)* | 47,5 | 11,05 | 9,43 | 16,89 | 12 | 10 | 17 | 5 | 4 | 7 |
| 58 | S9YL21 | Apolipoprotein A-I *(C. ferus)* | 22,5 | 19,05 | 68,11 | 82,98 | 27 | 49 | 57 | 5 | 12 | 14 |
| 59 | S9Y5X2 | Cell death activator CIDE-A-like protein *(C. ferus)* | 41,2 | 16,09 | 22,41 | 15,11 | 11 | 14 | 10 | 4 | 11 | 4 |
| 60 | S9XC74 | Osteopontin isoform OPN-c *(C. ferus)* | 34,6 | 8,45 | 12,77 | 34,67 | 8 | 11 | 23 | 4 | 10 | 24 |
| 61 | T0NLV9 | Epoxide hydrolase 1 *(C. ferus)* | 54,3 | 7,1 | 11,53 | 12,54 | 9 | 14 | 10 | 4 | 6 | 4 |
| 62 | S9WKD1 | Ribonuclease 4 *(C. ferus)* | 26,8 | 11,56 | 25,5 | 29,63 | 12 | 22 | 23 | 3 | 7 | 9 |
| 63 | S9WDV3 | Fibrinogen gamma chain isoform gamma-B *(C. ferus)* | 50,5 | 7,22 | 33,1 | 76,88 | 6 | 49 | 37 | 3 | 12 | 33 |
| 64 | S9XLJ3 | Brain-specific serine protease 4-like protein *(C. ferus)* | 44,6 | 7,52 | 8,26 | 9,3 | 10 | 10 | 12 | 3 | 3 | 4 |
| 65 | S9WY98 | Sodium/glucose cotransporter 1 *(C. ferus)* | 78,3 | 9,01 | 5,72 | 14,27 | 4 | 3 | 5 | 3 | 2 | 5 |
| 66 | S9WX48 | Alpha-1-acid glycoprotein *(C. ferus)* | 22,9 | 6,65 | 7,25 | 29,92 | 14 | 8 | 44 | 3 | 2 | 17 |
| 67 | W5P9V5 | Uncharacterized protein *(O. aries)* | 85,3 | 7,14 | 7,74 | 8,54 | 3 | 3 | 3 | 3 | 3 | 3 |
| 68 | T0NLF0 | Vitronectin *(C. ferus)* | 56,2 | 11,18 | 7,8 | 25,19 | 7 | 4 | 10 | 3 | 2 | 4 |
| 69 | Q0IIG8 | Ras-related protein Rab-18 *(B. taurus)* | 22,9 | 3,49 | 15,33 | 21,19 | 10 | 28 | 24 | 2 | 5 | 4 |
| 70 | S9YNY9 | Nucleobindin-1 *(C. ferus)* | 53 | 4,54 | 48,27 | 20,28 | 5 | 41 | 21 | 2 | 16 | 9 |

Molecular masses ($M_r$) of proteins are expressed in kDa, E-value in log, coverage in %. Spectra indicates the number of spectra permitting the identification of proteins. Major proteins identified in excised gel bands after SDS-PAGE are given in bold type.

*abundance of each protein was estimated from spectral count. The number of spectra of C. bactrianus (B) classified the table.

## 2.3.4 Camel milk protein profiling by LC-ESI-MS

Thirty individual milk samples, including *C. bactrianus* (n=10)*, C. dromedarius* (n=10), and hybrids (n=10) taken from the 58 milk samples analyzed in SDS-PAGE were submitted to LC-ESI-MS analysis. Milk proteins separated by RP-HPLC were identified based on their molecular mass, arising from ESI-MS. Putative genetic variants and post-translational (glycosylation and phosphorylation) isoforms were determined by deconvoluting multiple charged ion spectra in a real mass scale. Knowing their primary structures, it is possible to determine molecular masses of non post-translationally modified proteins, and then we can precisely know the mass of phosphorylation isoforms resulting from the addition of phosphate groups (±79.98 Da). Likewise, masses of isoforms arising from cryptic splice site usage, usually leading to the loss of the first codon (CAG) of an exon specifying a glutaminyl residue (-128 Da), are easily deduced. A camel mass reference database was thus created for the main milk proteins by combining the data available from *C. dromedarius*, *C. bactrianus, C. ferus, and Lama glama* milk protein sequences published in UniProtKB (ExPASy SIB Bioinformatics Resource Portal) and the National Centre for Biotechnology Information (NCBI).

To illustrate the efficiency of such an approach, a typical protein profile obtained with a milk from a hybrid camel sampled in Kyzylorda region is given in Figure 2.4. The analysis of molecular isoforms, identified from mass data, are reported in Table 2.3, in which experimental and theoretical molecular masses of camel milk proteins are given and confronted. The mass accuracy has allowed distinguishing about 50 protein molecules corresponding to isoforms belonging to nine protein families, eluted from the reverse-phase column as 15 peaks.

In peak I, the two molecular masses (21,157 Da and 21,184 Da) found were associated with glycoforms of κ-CN. The molecular mass of 21,157 Da corresponds to mono-phosphorylated variant A of κ-CN with tri-saccharides ((GaN-Ga-SA2) x 3 or (GaN-Ga) + (GaN-Ga-SA3) x 2, or (GaN-Ga-SA) + (GaN-Ga-SA2) + (GaN-Ga-SA3)). The molecular mass of 21,184 Da was expected to be non-phosphorylated variant B of κ-CN with penta-saccharides ((GaN-Ga) x 3 + (GaN-Ga-SA2) x 2, or (GaN-Ga) + (GaN-Ga-SA) x 4, or (GaN-Ga) x 2 + (GaN-Ga-SA) x 2 + (GaN-Ga-SA2), or (GaN-Ga) x 3 + (GaN-Ga-SA) + (GaN-Ga-SA3)). Peak II contained molecules of which the molecular masses (18,210 Da and 18,236 Da) were identified as non-phosphorylated variant B of κ-CN along with the A variant modified at its N-terminal residue to form a pyro-glutamic acid (pyro-E), which is formed spontaneously by cyclization of the N-terminal E residue. The two molecular masses: 12,564 Da and 12,644 Da, detected in peak III, were assigned to the WAP peptide chain without or with one P group,

respectively. Peaks IV, V, and VI were shown to contain $\alpha_{s1}$-CN. The molecular mass of 23,878 Da observed in peak IV was interpreted as being a short isoform (201-residues) of $\alpha_{s1}$-CN variant A with 4P groups, arising from exons 13' and 16 skipping events in the mature mRNA during the course of primary transcripts splicing, resulting in deleted sequences (residues E112-Q117 and E155-E162). Despite identification of only one splicing isoform with 4P groups (23,878 Da) in this milk sample, isoforms with 3P and 5P, along with cryptic splice site usage were identified in several other milk samples. Peak V consisted of three relative groups of three masses with sequential increments (s.i.) of 80 Da: 24,547 Da - 24,707 Da, 24,675 Da - 24,835 Da, and 24,689 Da - 24,849 Da. The mass difference (128 Da) between the first and the second group (Table 2.3.) corresponds to the loss of glutaminyl residue 83 (ΔQ83), encoded by the first codon (CAG) of exon 11. As reported previously (Kappeler et al., 1998), 24,755 Da was identified as the short isoform (207-residues) of the $\alpha_{s1}$-CN variant A originating from exon 16 skipping during the course of the primary transcript processing. The mass difference (14 Da) between the second (24,675 Da) and the third (24,689 Da) group is due to the aa substitution E30D reported by Shuiep et al., (2013) characterizing the C variant. Thus, it is concluded that the third mass group gathers $\alpha_{s1}$-CN short isoforms (207-residues) of variant C, with 5P, 6P and 7P, respectively, described in *C. dromedarius.* While cryptic splice site isoforms (ΔQ83) of variant C, with different phosphorylation levels, were not found in the milk sample shown at Figure 2.4, they were successfully found in several milk samples. Whereas, $\alpha_{s1}$-CN short isoform was systematically present in all camel milk samples with 5, 6 and 7P (Table 2.3.), by contrast, $\alpha_{s1}$-CN short isoforms of variant C occurred in some milk samples with 4P (24,611 Da) and up to 9P (24,929 Da). Herein, $\alpha_{s1}$-CN short isoforms of variants A and C carrying 6P groups are isoforms with the highest mass signal intensity values 50,634 *vs.* 47,392, respectively.

Peak VI was more complex to interpret. Masses found in this peak belonged to four different molecular mass groups: 14,430 Da (ascribed to α-LAC), 22,939-23,099 Da (s.i. of 80 Da), 25,646 Da and 25,693-25,773 Da (s.i. of 80 Da), and 25,787 Da. Masses around 23 kDa (22,939-23,099 Da), with a mass increment of two P groups (160 Da), were not referenced to any protein in our database. These findings strongly suggest the existence of an additional uncharacterized phosphorylated protein, namely UP1, which remains to be identified. The third mass group, 25,646 Da and 25,693-25,773 Da, corresponds to a mixture of two long isoforms (214 and 215 aa residues, respectively) of $\alpha_{s1}$-CN variant C with 5P and 6P (25,693-25,773 Da) which differs from variant A by an aa substitution (E30D) in the mature protein (Erhardt et al., 2016). The mass of 25,646 Da corresponds to a 214 aa residues isoform of $\alpha_{s1}$-CN variant C

(∆Q83), with 6P. The last molecular mass (25,787 Da) found in this peak was related to the mature variant A of $\alpha_{s1}$-CN bearing 6P groups, which is by far much less abundant than the short $\alpha_{s1}$-CN A-6P isoform (intensity of the mass signals: 3,472 *vs.* 50,634).

The four subsequent peaks (VII, VIII, IX, and X) all contained $\alpha_{s2}$-CN molecules, with phosphorylation levels ranging between 7P (21,825 Da, peak VII) and 12P (22,226 Da, peak X). Observed molecular masses of 21,825-21,984 Da were in perfect concordance with those predicted for $\alpha_{s2}$-CN displaying 7P and 9P, whereas $\alpha_{s2}$-CN with 8P (21,906 Da) was the most frequent isoform. In addition, the mass of 23,179 Da in peak VII probably corresponds to the UP1 found in fraction VI with one more P group. Masses ranging between 21,986 and 22,226 Da (s.i. of 80 Da) found in peaks VIII, IX, and X were related to $\alpha_{s2}$-CN variant A with 9P to 12P. These results suggest three more potential phosphorylation sites than reported by Kappeler et al., (1998) who mentioned a maximum of 9 S residues phosphorylated in camel $\alpha_{s2}$-CN. More recently, Felfoul et al., (2017) detected two $\alpha_{s2}$-CN isoforms with 10 and 11P groups in camel milk. Interestingly, peak X contains a second uncharacterized protein (UP2) with a molecular mass of 23,046 Da, not referring to any mass in our database for camel milk proteins. Such a mass was found in all camel milk samples analyzed so far (n=30). This suggests the possible existence of a further phosphoprotein in camel milk, very likely a CN, since two putative related isoforms with two (23,206 Da) and three (23,286 Da) additional P groups were detected in peak XI, in which the most abundant mass found (19,143 Da) was attributed to PGRP.

In the hybrid from Kyzylorda region (Table 2.3.), masses found in peak XII ranged between 66,481 and 67,342 Da. The most abundant masses 66,481 Da and 66,512 Da might be related to CSA of which the theoretical mass (peptide sequence predicted from the *C. dromedarius* genome, NCBI Accession number XP_010981066.1) is 66,477 Da. The mass differences of 4 Da and 35 Da could be attributed to putative genetic polymorphisms. The molecular weight reported by Felfoul et al., (2017) from fresh camel milk was estimated as 66,600 Da. However, one cannot exclude that such masses could correspond to LPO depending on cleavage sites of the propeptide, when comparing with bovine LPO and human myeloperoxidase (Dull et al., 1990).

Molecular masses of 24,793 - 24,953 Da (s.i. of 80 Da) found in peak XIII, were ascribed to β-CN variant A with 2P, 3P and 4P, first described in the *C. bactrianus*. Molecular masses of 24,891-24,970 Da, which differ from β-CN A-3P and 4P by a 18 Da, correspond to β-CN variant B, first described in *C. dromedarius*. The mass difference of 18 Da between variants A and B is due to the M186I substitution. Isoforms of β-CN with 4P predominate whatever the milk sample and the genetic variant were, with equivalent intensity values of the

mass signal for variants A and B, exemplified by a heterozygous hybrid camel: 84,494 *vs*. 87,973, respectively. In addition, the molecular mass of 24,842 Da, observed in peak XIII, corresponds to a splicing variant of β-CN B-4P. Such an isoform, which was so far considered as typical to the dromedary camel, was also found in hybrids and Bactrian camels. It is due to a cryptic splice site usage leading to the loss of the first codon (CAG) of exon 6, encoding residue Q29 in the protein.

Surprisingly, in the next peak (XIV), molecular masses around 24,000 Da (23,878 Da to 24,024 Da) were observed. Given the elution time and the mass range, these masses were very likely relative to the β-CN fraction, especially since a 18 Da mass differential existing between the pair of molecular masses (24,006 Da and 24,024 Da), is consistent with the occurrence of β-CN variants A and B, in both species. The important mass reduction, - 946 Da, relatively to the full-length β-CN, is hypothesized to be due to the cleavage by plasmin of the first seven N-terminal residues (1REKEEFK7) of the mature protein, given that this heptapeptide accounts for 947 Da. Furthermore, molecular masses equivalent to 23,878 Da and 23,895 Da are supposed to originate in the cryptic splice site usage (ΔQ29), previously mentioned. Finally, in the last peak (XV) mass values 12,357 Da and 12,376 Da again with the mass difference in 18 Da were observed. These masses correspond very likely to camel $\gamma_2$-CN A and B (12,357 Da *vs.* 12,376 Da, respectively), which are degradation products of β-CN (Beg et al., 1986).

This extensive analysis shows that mass accuracy provided by LC-ESI-MS was effective to allow protein identification of most of the protein isoforms by comparison of masses observed experimentally to theoretical molecular masses, and sufficiently powerful to recognize post-translational modifications (PTM) such as phosphorylation of CN, as well as genetic variants and long and short isoforms due to splicing inaccuracies.

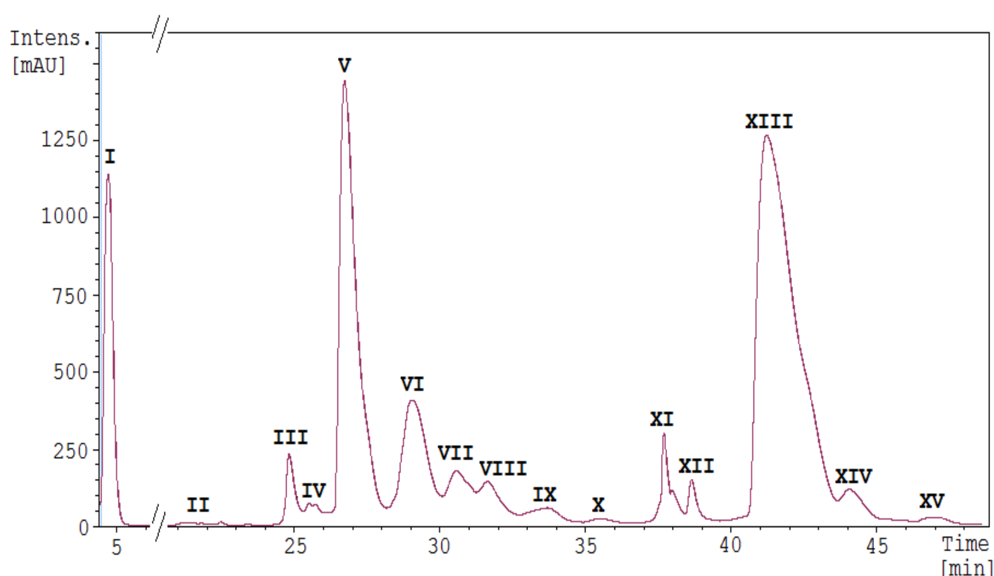


**Figure 2.4.** LC-ESI-MS profile of clarified crossed camel milk of Kyzylorda region. Nine major milk protein fractions were identified in the following order: peak I and II contained glycosylated ant natural isoforms of κ-CN; peak III: WAP; peaks IV, V: $\alpha_{s1}$-CN; peak VI: α-LAC, $\alpha_{s1}$-CN and UP1; peak VII: $\alpha_{s2}$-CN and UP1; peaks VIII, IX, and X $\alpha_{s2}$-CN along with UP2 in peak X; peak XI: PGRP and UP2; peak XII: CSA/LPO; peaks XIII and XIV: β-CN, and peak XV: $\gamma_2$-CN

**Table 2.3.** Identification of Camel milk protein (hybrid from Kyzylorda region) from observed molecular masses using LC-ESI-MS

| Peak | Ret.Time, min | Observed $M_r$, Da | Theoretical $M_r$, Da | Protein description | UniProt/ NCBI GenBank Accession number | Intensity |
|---|---|---|---|---|---|---|
| I | 4.50 | 21,157 | 21,158 | κ-CN A, 1P, (GaN-Ga-SA2)x3*, pyro-E | | 1,361 |
| | | **21,184** | **21,182** | **κ-CN B, 0P, (GaN-Ga)x3 + (GaN-Ga-SA2)x2**, pyro-E** | | **5,810** |
| II | 18.61 | 18,210 | 18,210 | κ-CN B, 0P ? | L0P304 | 161 |
| | | 18,236 | 18,235 | κ-CN A, 0P, pyro-E | P79139 | 72 |
| III | 24.32 | 12,564 | 12,564 | WAP, 0P | P09837 | 1,756 |
| | | 12,644 | 12,644 | WAP, 1P | | 1,575 |
| IV | 24.97 | 23,878 | 23,878 | $\alpha_{s1}$-CN A - short isoform (Δex 16 and Δex 13'), 4P | | 242 |
| V | 26.23 | 24,547 | 24,547 | $\alpha_{s1}$-CN C -short isoform (Δex 16), 5P, splice variant (ΔQ83) | | 4,885 |
| | | **24,627** | **24,627** | **$\alpha_{s1}$-CN C - short isoform (Δex 16), 6P, splice variant (ΔQ83** | | **21,606** |
| | | 24,707 | 24,707 | $\alpha_{s1}$-CN C - short isoform (Δex 16), 7P, splice variant (ΔQ83) | | 6,990 |
| | | 24,675 | 24,675 | $\alpha_{s1}$-CN C - short isoform (Δex 16), 5P | | 9,441 |
| | | **24,755** | **24,755** | **$\alpha_{s1}$-CN C - short isoform** (Δex 16)**, 6P** | **K7DXB9** | **47,392** |
| | | 24,835 | 24,835 | $\alpha_{s1}$-CN C - short isoform (Δex 16), 7P | | 7,046 |
| | | 24,689 | 24,689 | $\alpha_{s1}$-CN A - short isoform (Δex 16), 5P | | 9,748 |
| | | **24,768** | **24,769** | **$\alpha_{s1}$-CN A - short isoform** (Δex 16)**, 6P** | **O97943-2** | **50,634** |
| | | 24,849 | 24,849 | $\alpha_{s1}$-CN A - short isoform (Δex 16), 7P | | 6,909 |
| VI | 28.53 | **14,430** | **14,430** | **α-LAC** | **P00710** | **17,797** |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | 22,939 | n/a | Uncharacterized protein 1 (UP1) | n/a*** | 2,701 |
| | | 23,020 | n/a | UP1+80Da | n/a | 2,489 |
| | | 23,099 | n/a | UP1+160Da | n/a | 1,079 |
| | | 25,646 | 25,645 | $\alpha_{s1}$-CN C, 6P, splice variant (ΔQ83) | | 3,501 |
| | | 25,693 | 25,693 | $\alpha_{S1}$-CN C, 5P | | 564 |
| | | **25,773** | **25,773** | **$\alpha_{s1}$-CN C, 6P** | | **7,880** |
| | | **25,787** | **25,787** | **$\alpha_{s1}$-CN A, 6P** | **O97943-1** | **3,472** |
| VII | 30.05 | 21,825 | 21,826 | $\alpha_{s2}$-CN, 7P | | 552 |
| | | **21,906** | **21,906** | **$\alpha_{s2}$-CN, 8P** | **O9794** | **5,242** |
| | | 21,984 | 21,986 | $\alpha_{s2}$-CN, 9P | | 403 |
| | | 23,178 | n/a | UP1+240Da | n/a | 1,256 |
| VIII | 31.11 | 21,986 | 21,986 | $\alpha_{s2}$-CN, 9P | O97944 | 356 |
| | | **22,066** | **22,066** | **$\alpha_{s2}$-CN, 10P** | | **4,790** |
| IX | 33.18 | 22,066 | 22,066 | $\alpha_{s2}$-CN, 10P | | 148 |
| | | **22,145** | **22,146** | **$\alpha_{s2}$-CN, 11P** | | **1,964** |
| X | 35.05 | 22,226 | 22,226 | $\alpha_{s2}$-CN, 12P | | 894 |
| | | 23,046 | n/a | Uncharacterized protein 2 (UP2) | n/a | 231 |
| X | 37.16 | **19,143** | **19,143** | **PGRP** | **Q9GK12** | **7,207** |
| | | 23,206 | n/a | UP2+160Da | n/a | 1,592 |
| | | 23,286 | n/a | UP2+240Da | n/a | 735 |
| XII | 38.09 | 66,481 | 66,477 | CSA ? | XP_010981066.1 | 1,096 |
| | | | 66,491 | LPO ? | Q9GJW6 | |
| | | 66,512 | n/a | CSA ? LPO? | | 2,663 |
| | | 67,342 | n/a | CSA ? LPO? | | 1,010 |
| XIII | 40.67 | 24,746 | 24,745 | β-CN A, 3P, splice variant (ΔQ29) | | 2,073 |
| | | 24,793 | 24,792 | β-CN A, 2P | | 5,469 |
| | | 24,825 | 24,825 | β-CN A, 4P, splice variant (ΔQ29) | | 9,586 |
| | | 24,873 | 24,872 | β-CN A, 3P | | 10,177 |
| | | **24,953** | **24,953** | **β-CN A, 4P** | **A0A077SL35** | **84,494** |
| | | 24,842 | 24,842 | β-CN B, 4P, splice variant (ΔQ29) | | 10,029 |
| | | 24,891 | 24,890 | β-CN B, 3P | | 10,365 |
| | | **24,970** | **24,971** | **β-CN B, 4P** | **Q9TVD0** | **87,973** |
| XIV | 43.71 | 23,878 | 23,878 | β-CN A-short isoform (Δ946 Da), 4P, splice variant (ΔQ29) | | 707 |
| | | 23,963 | 23,958 | β-CN A-short isoform (Δ946 Da), 5P, splice variant (ΔQ29) | | 244 |
| | | 23,929 | 23,926 | β-CN A-short isoform (Δ946 Da), 3P | | 438 |
| | | **24,006** | **24,006** | **β-CN A-short isoform (Δ946 Da), 4P** | | **9,026** |
| | | 23,895 | 23,896 | β-CN B-short isoform (Δ946 Da), 4P, splice variant (ΔQ29) | | 625 |
| | | **24,024** | **24,024** | **β-CN B-short isoform (Δ946 Da), 4P** | | **5,545** |
| XV | 47.02 | 12, 357 | 12,358 | γ2-CN A, 0P | | 1,473 |
| | | 12,376 | 12,376 | γ2-CN B, 0P | | 1,065 |

Major proteins within each peak are in bold. Proteins and isoforms previously described are on grey background

*(GaN-Ga-SA2) x 3, or (GaN-Ga) + (GaN-Ga-SA3) x 2, or (GaN-Ga-SA) + (GaN-Ga-SA2) + (GaN-Ga-SA3)

**(GaN-Ga) x 3 + (GaN-Ga-SA2) x 2, or (GaN-Ga) + (GaN-Ga-SA) x 4, or (GaN-Ga) x 2 + (GaN-Ga-SA) x 2 + (GaN-Ga-SA2), or (GaN-Ga) x 3 + (GaN-Ga-SA) + (GaN-Ga-SA3)

***n/a - not applicable

### 2.3.5 Multiple spliced variants of CSN1S1

To confirm the occurrence of *CSN1S1* multiple splice variants, we took advantage of the possibility to extract RNA from milk fat globules to sequence PCR fragments of cDNA encoding $\alpha_{s1}$-CN. Three different *CSN1S1* transcripts were found in each species and both genetic variants A and C. The nucleotide sequence of the most frequent variant transcript was shown to be deleted of exon 16, encoding the octapeptide EQAYFHLE. Besides, we also observed an isoform displaying the same sequence in which the first codon of exon 11 was lacking. Finally, a full-length transcript including exon 16 and the first codon of exon 11 was also detected, at a lower concentration.

## 2.4 Discussion

Given the growing interest in camel milk, due to the health potential of its bioactive components (Al haj & Al Kanhal, 2010) and frequently reported high anti-microbial activity (El-Agamy, 2009), over the past 20 years and even more during the last decade, the milk protein fraction of Camelids, from all around the world has been extensively investigated (Alhaider et al., 2013; El-Agamy et al., 2009; Ereifej et al., 2011; Erhardt et al., 2016; Felfoul et al., 2017; Hinz et al., 2012; Kappeler et al., 1999; Konuspayeva et al., 2007; Merin et al., 2001; Ochirkhuyag et al., 1997; Salmen et al., 2012; Shuiep et al., 2013; Wangoh et al., 1998; Yang et al., 2013; Youcef et al., 2009). All these studies have explored, with more or less efficient approaches, the composition of the major milk proteins. However, the molecular diversity of these major proteins had not yet been studied. Then, our main objective was i) to provide, if not a comprehensive, at least an in-depth description of the protein fraction of camel milk; ii) to go further into an extensive analysis of the molecular diversity of major milk proteins from Camelids (*C. dromedarius*, *C. bactrianus,* and hybrids) sampled from different regions in Kazakhstan. For these purposes, different proteomic tools and methodological approaches were applied. For short, up to 391 protein species were identified in cumulating LC-MS/MS analyses of 8 individual *Camelus* milk, and the extensive characterization of CN and WP polymorphisms, using LC-ESI-MS, revealed a minimum of 50 molecular species.

## 2.4.1 Interspecies in-depth proteomic analysis of camel milk proteins

To our knowledge, the number of proteins identified in this study was relatively higher compared to the numbers reported in previous studies on the camel proteome (Alhaider et al., 2013; Yang et al., 2013). The largest camel milk proteome determined so far comprised about 238 proteins including some known camel proteins and heavy-chain immunoglobulins (Alhaider et al., 2013). In the abovementioned study carried out on *C. dromedarius*, proteins were identified from 2D SDS-PAGE with subsequent matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry analysis. However, it should be mentioned that several of the 238 proteins identified matched with the same protein in different species. Hence, at most *ca.* 140 proteins may be considered as unique. By comparison, in the present study a total of 391 unique protein species were determined from LC-MS/MS analyses of *C. bactrianus* (n=3), *C. dromedarius* (n=3), and hybrids (n=2), sampled from three different regions (Atyrau, Shymkent and Kyzylorda). Proteins such as flavin monoamine oxidase, perilipin 2, neutrophil gelatinase-associated lipocalin-like protein, brain-specific serine protease 4-like protein and others, which were not determined previously, were successfully detected. Conversely, about 30 proteins identified by Alhaider and co-workers (2013) were not found in our study.

However, as for other mammals, CN represent the major protein fraction of camel milk (80%), among which β-CN is the most abundant (Pauciullo et al., 2014). Quantitative analyses performed by Kappeler et al., (2003) on camel milk CN have demonstrated significant higher amounts of β-CN (15 g/L *vs.* 10 g/L) compared to the homologous bovine β-CN and significant lower amounts of κ-CN (0.8 g/L *vs.* 3.5 g/L). Regarding relative proportions, as previously reported (Kappeler et al., 1998), $\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-CN contribute to about 22%, 9.5%, 65%, and 3.5% of total CN, respectively. Taking into account the 30 milk samples analyzed in LC-ESI-MS, relative proportions of individual CN, estimated from the mass signal intensity of each CN family (summing the mass signal of its phosphorylation and splicing isoforms) relatively to the sum of mass signal intensities of all CN families (considering that ionizing properties of caseins and their isoforms are comparable), were 37% $\alpha_{s1}$-CN, 6.1% $\alpha_{s2}$-CN, 53.1% β-CN, and 3.8% κ-CN. These values varied considerably compared to those reported previously by Kappeler et al., (1998) essentially as far as $\alpha_{s1}$-CN and β-CN are concerned. Whereas $\alpha_{s1}$-CN accounts for 36.1% for *C. bactrianus,* it reaches 37.4% and 37.6% in *C. dromedarius* and

hybrids, respectively (Table 2.4.). Percentage of $\alpha_{s1}$-CN calculated in our study was 15% higher than the value reported by Kappeler et al., (1987) Such an increase is compensated in part by a decrease of 12% of β-CN. The small amount of κ-CN observed is probably underestimated, since most of the highly glycosylated isoforms were not detected. However, this is in agreement with the fact that the size distribution of CN micelles is inversely related to κ-CN content (Bijl et al., 2014; Ostersen et al., 1997), since camel CN micelles are the largest, ranging in size between 280-550 nm (Bornaz et al., 2009).

Even though, there are 2 potential phosphorylation sites in κ-CN (S141 and S159) conserved and phosphorylated in sheep and goats (Martin et al., 2013) only isoforms with a single or no P group in the first chromatographic peak comprising glycosylated isoforms with 3 or 5 carbohydrate motifs were detected. Five glycosylated isoforms of camel κ-CN ranging in size between *ca.* 24 and 25.9 kDa were found in camel milk using 2D SDS-PAGE (Hinz et al., 2012).

In addition, $\gamma_2$-CN, a C-terminal product resulting from a highly specific proteolysis of β-CN by the natural milk protease (plasmin) was successfully found in the milk samples analyzed. Previously published data suggested that the proportion of γ-CN in total CN fraction is highest at the beginning and the end of lactation, and in very low yielding animals (Ostersen et al., 1997). The molecular masses observed in this study (12,357 Da and 12,376 Da) were lower from those previously observed by Kappeler et al., (1998): 13.9 kDa, 15.7 kDa and 15.75 kDa.

Immune-related proteins such as GlyCAM1, MFGE8 and LTF were detected in camel milk. GlyCAM1, also named lactophorin or PP3 is a cysteine free protein, which belongs to the family of GlyCAM-type molecules (Beg et al., 1987). Two splicing variants A and B were distinguished in camel milk (Kappeler et al., 1999). Variant A encoding 137 aa residues has a $M_r$ of 15.7 kDa, while variant B encoding 122 aa residues has a $M_r$ of 13.8 kDa. The primary structure of Variant A reveals 54% identity with a protein isolated from bovine milk (Sørensen & Petersen, 1993). Until late, it has been claimed that camel GlyCAM1 is neither glycosylated nor phosphorylated as bovine GlyCAM1. However, Girardet et al., (2000) suggested the probable existence of one O-glycosylation site (16TDT18) in variant A of which the apparent $M_r$ was estimated as 22.5 kDa from SDS-PAGE. Using the same approach, two bands were found, in which we identified GlyCAM1 from LC-MS/MS analysis 22 kDa and 10 kDa, corresponding probably to the glycosylated and putatively phosphorylated isoform of GlyCAM1 observed by Girardet et al., (2000), and to a product of proteolysis, respectively. Surprisingly, no molecular masses corresponding to camel GlyCAM1 A and B were identified

by LC-ESI-MS analysis. Likewise, LC-ESI-MS did not permit to detect LTF, even though, SDS-PAGE and LC-MS/MS data confirm its presence in analyzed camel milk samples. On the other hand, molecular masses ranging between 74,338 Da-79,621 Da could be attributed to camel LTF of which the theoretical mass reported by Kappeler et al., (1999) for the mature protein (689 aa residues long) without PTM is 75,250 Da. Therefore, the mass difference observed is very likely attributable to PTM. In addition, Konuspayeva et al., (2005) reported that the level of LTF is affected by seasonal variations.

Elsewhere, MFGM-enriched proteins such as XO, BTN, fatty acid synthase, actin, ras-related protein Rab-18, ADP-ribosylation factor 1, tyrosine-protein kinase, GTP-binding protein SAR1b were identified in Kazakh camel milk samples in accordance with previous results obtained with *C. dromedarius* (Saadaoui et al., 2013) and *C. bactrianus* (Yang et al., 2013) milk samples. Surprisingly, whereas BTN was present in all milk samples, it seems to be absent in *C. bactrianus* from Atyrau region. This could be due to the way the band in the electrophoresis gel was cut, since BTN was found in the other seven samples analyzed. Regarding proteins originating from blood, such as serpin A3-1, apolipoprotein A-1, α-1-antitrypsin like protein, α-1-acid glycoprotein, β-2-microglobulin, complement C3-like protein, they were also found in Kazakh camel milks, in agreement with findings of Yang et al., (2013) reported for Bactrian camels from China. By contrast, as mentioned in the Results section, no trace of CSA was found in Kazakh milk samples from LC-MS/MS analyses, whereas its presence is suspected from LC-ESI-MS.

A heat shock protein (HSPA6 also called HSP70B') occurred at rank 23 amongst the first third of the most represented proteins in Kazakh camel milks (Table 2.2.). Expression of heat shock proteins, including HSP70 is increased during heat stress and involved in defense against dehydration or thermal stress in arid environments (Rhoads et al., 2013; Sharma et al., 2013). The entire sequence of this protein has been deduced from the nucleotide sequence of a full-length cDNA in *C. dromedarius* (Elrobh et al., 2011). Comprising 643 aa residues, the camel protein, of which the $M_r$ is 70,543 Da in agreement with the molecular mass estimated from SDS-PAGE, shares a high similarity (94% identity) with cow and pig HSP70.

Against all expectations, peptides with sequence similarity with bovine $\beta$-lactoglobulin, the major allergen in bovine milk, were identified in the 8 camel milk samples (Bactrian, dromedary and hybrids) from Kazakhstan, analyzed by LC-MS/MS. The coverage percentage ranged between 30 and 60% in individual milk samples, and reached 71% cumulating all the peptides found. Five peptides related to bovine $\beta$-lactoglobulin were also detected by Alhaider et al., (2013) in camel milk from Saudi Arabia and the United States. Youcef et al., (2009)

revealed a weak cross reaction between dromedary WPs and IgG anti bovine $\beta$-lactoglobulin. Such findings disagree with the usually admitted notion that $\beta$-lactoglobulin is absent in camel milk (Hinz et al., 2012; Restani et al., 1999). Even though we cannot exclude a possible contamination by bovine milk (unlikely with the 8 camel milk samples analyzed by LC-MS/MS) or the presence in camel milk of a Progesterone Associated Endometrial Protein (PAEP) displaying strong similarities with $\beta$-lactoglobulin. However, significant similarities between human PAEP and the peptides having allowed the identification of $\beta$-lactoglobulin in *C. bactrianus* milk, were not found.

**Table 2.4.** Relative proportion of each CN expressed in %, estimated from the mass signal intensity of each CN family relatively to the sum of mass signal intensities of all CN families in the three camel species

| | **κ-CN** | | **$\alpha_{s1}$-CN** | | **$\alpha_{s2}$-CN** | | **β-CN** | |
|---|---|---|---|---|---|---|---|---|
| | m | σ | m | σ | m | σ | m | σ |
| **Bactrian** | 3,09 | 1,89 | 36,09 | 2,33 | 7,13 | 1,49 | 53,68 | 2,08 |
| **Dromedary** | 3,63 | 2,13 | 37,39 | 3,89 | 5,79 | 0,98 | 53,19 | 3,46 |
| **Hybrid** | 4,77 | 3,01 | 37,57 | 3,03 | 5,25 | 1,56 | 52,41 | 4,18 |

m = mean; σ = standard deviation

## 2.4.2 Molecular diversity of camel caseins: genetic polymorphism and alternative splicing

Regarding camel $\alpha_{s1}$-CN, the situation is particularly confusing. Kappeler et al., (1998) first described two cDNA (short and long), encoding two protein isoforms of 207 and 215 aa, named A and B variants. The A variant corresponds to the short isoform (207 aa), in which the octapeptide 155EQAYFHLE162 encoded by exon 16 was missing, whereas this octapeptide is present in the 215 aa-long isoforms. In our study, two isoforms long and short showing a 1,018 Da mass difference were found, in which the short isoform was the major component (*ca.* 90%) of total camel $\alpha_{s1}$-CN. Such an alternative splicing event has been first reported in goats (Leroux et al., 1992), sheep (Chianese et al., 1996; P. Ferranti et al., 1995) and later in lama (Pauciullo & Erhardt, 2015). In addition, we observed the existence of two distinct genetic variants called A and C, arising from the E30D aa substitution, as previously reported by Shuiep et al., (2013). Since, variants A and B described by Kappeler et al., (1998) displayed a E aa residue in position 30 of the mature peptide chain, it becomes obvious that Kappeler's A and B variants derived in fact from a single allele, of which the primary transcript is subject to exon 16 skipping during

the splicing process. In other words, the B variant is nothing other than a splicing variant of a single allele that we propose to call *CSN1S1*A*.

Recently, Erhardt et al., (2016) reported in *C. dromedarius* from different regions of Sudan, the existence of a further variant, called D, clearly displaying a different IEF behavior. Excluding this D variant, which was not precisely characterized, there are $\alpha_{s1}$-CN long and short non-allelic isoforms arising from alternative splicing of a single primary transcript and only two perfectly characterized genetic variants A and C resulting from a single G>T nucleotide substitution in exon 4 and leading to E30D aa substitution. This molecular diversity is becoming more complex due to different phosphorylation levels ranging between 5-8P groups (see thereafter) and due to isoforms arising from cryptic splice site usage (Boumahrou et al., 2011; Ferranti et al., 1999; Leroux et al., 1992), leading to the loss of a Q residue corresponding to the first codon of exon 11. Results from cDNA sequencing substantiate this.

Electrophoretic and LC-MS analyses as well as cDNA sequencing confirmed that β-CN occurs as two genetic variants A and B, with the aa substitution M186I (yielding a -18 Da mass difference). The most frequent form of β-CN had 4P groups, one P group more than reported for Somali, Turkana and Pakistani camels by Kappeler et al., (1998). Surprisingly, in Kazakh populations, a second series of β-CN components with lower molecular masses (mass difference: -946 Da), relatively to the full-length β-CN were found. This phenomenon, observed with both genetic variants, might be due to the cleavage by plasmin of the first seven N-terminal residues (REKEEFK) of the mature protein. A mass difference of 947 Da was observed between the native full-length protein with 4P (24,953 Da and 24,971 Da for A and B variants, respectively) and the plasmin cleavage product at the same phosphorylation level (24,006 Da and 24,024 Da for A and B variants, respectively). The occurrence of a K residue in position 7 of the mature β-CN does not occur in any other species, of which the N-terminal sequence is known (Martin et al., 2013). However, our results strongly suggest that the peptide bond 7K-T8 is sensible to plasmin that is, like trypsin, a serine protease. Indeed, REKEEFK was present amongst tryptic peptides identified in LC-MS/MS analysis.

There is another even less probable possibility, involving the deletion of exon 5 that encodes 8 aa residues (ESITHINK for a mass of 923 Da), since a similar event was previously characterized from mare (Miranda et al., 2004) and donkey (Cunsolo et al., 2017) milks. However, sequencing of camel β-CN cDNA has not revealed any deletion in the mRNA encoding this protein (results not shown), consistently with Kappeler et al., (1998) who only reported a full-length sequence for β-CN, conversely to $\alpha_{s1}$-CN. Since in our study we were not able to provide any further confirmation of the presence of shorter mRNA of camel β-CN in

which exon 5 is spliced out, we give preference to the cleavage by plasmin of the first seven N-terminal residues of β-CN rather than an alternative splicing process.

Surprisingly, two so far uncharacterized proteins (UP1 and UP2) with molecular masses around 23,000 Da and different phosphorylation levels were observed, suggesting they are possibly proteins related to CN. However, to prove this hypothesis further research for in depth characterization of these proteins is necessary.

### 2.4.3 Post-translational modifications of milk proteins: phosphorylation of caseins

Among the various approaches developed in proteomics, electrospray ionization (ESI) mass spectrometry (MS) is eminently suitable for studying PTM, including phosphorylation and glycosylation, since the technique provides molecular mass determination of native proteins. Phosphorylation of proteins is one of the most frequent PTM in eukaryotic cells. It has become a common knowledge that phosphorylation of CN occurs at S or T aa residues in tripeptide sequences S/T-X-A where X represents any aa residue and A is an acidic residue (Mercier, 1981). This consensus sequence is recognized by FAM20C, a Golgi CN-kinase, which phosphorylates secreted phosphoproteins, including both CN and members of the small integrin-binding ligand N-linked glycoproteins (SIBLING) protein family, which modulate biomineralization (Ishikawa et al., 2012). Each phosphorylation event adds 79.98 Da to the molecular mass of the peptide chain (Larsen et al., 2006). (Larsen et al., 2006)It was predicted with high confidence 8 probably phosphorylated S residues in $\alpha_{s1}$-CN (S18, S68, S70, S71, S72, S73, S193, and S202), 9 potential phosphorylated S residues in $\alpha_{s2}$-CN (S8, S9, S10, S32, S53, S108, S110, S113, and S121), 4 S residues in β-CN (S15, S17, S18, and S19), and 2 S residues in κ-CN (S141 and S159). However, up to 9P residues per $\alpha_{s1}$-CN molecule were observed whatever the genetic variant is. Theoretically, given the S/T-X-A consensus rule, there are 4 T residues that could be phosphorylated (T55, T80, T153, and T196), leading to a maximum of 12 P groups per molecule. Therefore, we can put forward that at least one of the four T residues is phosphorylated in the $\alpha_{s1}$-CN-9P.

With 11 potentially phosphorylated aa residues matching the S/T-X-A motif (Figure 2.5), camel $\alpha_{s2}$-CN displays the highest phosphorylation level, in agreement with Felfoul et al., (2017), who reported recently 11P groups. To reach such a phosphorylation level, besides the nine SerP, two putative ThrP (T118 and T132) have to be phosphorylated. In all the Kazakh milk samples analyzed in LC-ESI-MS we found $\alpha_{s2}$-CN with 12 P groups, as the molecular

mass of 22,226 Da observed corresponds to the mass of the peptide backbone (21,266 Da) increased by 960 Da, a mass increment which coincides with 12 P groups. That means that at least another S/T residue that does not match with the canonic sequence recognized by the mammary kinase(s), is potentially phosphorylated. According to Allende et al., (1995) the sequence S/T-X-X-A follow-through with the minimum requirements for phosphorylation by the CN-kinase II (CK2). It is critical to highlight in this regard that E or D in this site can be replaced by SerP or ThrP. Two T residues, namely T39 and T129 in the camel $\alpha_{s2}$-CN fully meet the requirements of the above-mentioned motif (Figure 2.5) and could be phosphorylated. Such an event is the only hypothesis to reach 12P for camel $\alpha_{s2}$-CN. Since these two kinases are very likely secreted, the idea that phosphorylation at T39/T129 may occur in the extracellular environment cannot be excluded. This warrants further investigation. Fam20C, which is very likely the major secretory pathway protein kinase (Tagliabracci et al., 2015), might be responsible for the phosphorylation of S and T residues within S/T-X-A motif, whereas a CK2-type kinase might be responsible for phosphorylation of T residue within an S/T-X-X-A motif. This is in agreement with the hypothesis put forward by Bijl et al., (2014) and Fang et al., (2016), who suggest from phenotypic correlations and hierarchical clustering the existence of at least 2 regulatory systems for phosphorylation of $\alpha_s$-CN. Elsewhere, bovine milk osteopontin which is a multiphosphorylated glycoprotein also found in bone, was shown to contain 27 SerP and one ThrP (Sørensen et al., 1995). Twenty five SerP and one ThrP were located in S/T-X-E/S(P)/D motifs, whereas two SerP were found in the sequence S-X-X-E/S(P).

```
        10         20         30         40         50
KHEMDQGSSS EESINVSQQK FKQVKKVAIH PSKEDICSTF CEEAVRNIKE
        60         70         80         90        100
VESAEVPTEN KISQFYQKWK FLQYLQALHQ GQIVMNPWDQ GKTRAYPFIP
       110        120        130        140        150
TVNTEQLSIS EESTEVPTEE STEVFTKKTE LTEEEKDHQK FLNKIYQYYQ
       160        170
TFLWPEYLKT VYQYQKTMTP WNHIKRYF
```

**Figure 2.5.** Amino acid sequence of mature camel $\alpha_{s2}$-CN with potential phosphorylation sites. Seryl and Threonyl residues matching the S/T-X-A motif are in red and blue, respectively, and underlined. Threonyl residues matching the S/T-X-X-A motif are in green and underlined

## 2.5 Conclusions

In this study, six main findings combining proven proteomic and molecular biology approaches are provided. The first one is an enhancing of our knowledge of camel milk protein composition. The second one is deciphering the extreme complexity of camel CN fraction due to PTM (phosphorylation) and splicing events (exon skipping and cryptic splice site usage). The third finding is the detection of two unknown proteins, UP1 and UP2 that remain to be characterized. In addition, we provide results substantiating the possible existence of a camel β-lactoglobulin. However, this result requires further investigation, currently in progress in the laboratory. Afterwards, we report for the first time the presence of $\alpha_{s2}$-CN-12P, and short isoforms of β-CN probably arising from proteolysis by plasmin, the natural protease of milk. The ultimate finding is the demonstration that genetic variants, which hitherto seemed specific to a species (β-CN A for Bactrian and β-CN B for dromedary), are in fact present in both *dromedarius* and *bactrianus*.


## Acknowledgements

The study was carried out within the Bolashak International Scholarship of the first author, funded by the JSC «Center for International Programs» (Kazakhstan). The research was partly supported by a grant from the Ministry of Education and Science of the Republic of Kazakhstan under name "Proteomic investigation of camel milk" #1729/GF4, which is duly appreciated. The authors thank all Kazakhstani camel milk farms for rendering help in sample collection, as well as PAPPSO and @BRIDGe teams at INRA (Jouy-en-Josas, France) for providing necessary facilities and technical support.


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# Chapter 3

# Alternative splicing a fortuitous or a scheduled event to expand molecular diversity of camel CSN1S2 and increase its bioactive potentiality

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*Scientific Reports (Submitted)*

# Abstract

In a previous study on camel milk from Kazakhstan, we reported the occurrence of two unknown proteins (UP1 and UP2) with different levels of phosphorylation. We showed that UP1 and UP2 (called here i1 and i2, respectively) were isoforms of camel $\alpha_{s2}$-CN arising from alternative splicing events. The first described isoform (called here i0) was 193 amino-acid long with 9 phosphate groups (21,986 Da). UP1 (i1), a rather frequent (35%) isoform displaying a higher molecular mass (+1,033 Da), is present at four phosphorylation levels (8P to 11P). Using cDNA-sequencing, i1 was shown to be a variant arising from the splicing-in of an in-frame 27-nucleotide sequence, of which the presence at the genome level, flanked by canonic motifs defining an exon 13 encoding the nonapeptide ENSKKTVDM, was confirmed. UP2 (i2), which appeared to be present with 8P to 12P, was shown to include an additional decapeptide (VKAYQIIPNL) revealed by LC-MS/MS, encoded by a 3'-extension of exon 16. If it is well established that milk proteins represent a reservoir of biologically active peptides, capable of modulating different functions, one can therefore expect that the molecular diversity generated by differential splicing mechanisms can increase its content. Thus, multiple C-terminal peptide sequences of camel $\alpha_{s2}$-CN, in which peptides with anti-bacterial activity are encrypted, may have some influence on the biological properties of camel milk.

**Key words:** camel milk, protein, casein, polymorphism, post-translational modification

# 3.1 Introduction

Many recent studies have demonstrated that health-promoting properties are assigned to camel milk, which is consumed fresh or fermented and traditionally used for the treatment of tuberculosis, gastroenteritis, and allergy, in many countries (Mati et al., 2017). Milk proteins as well as bioactive peptides encrypted in milk protein sequences display a variety of potential activities: anti-microbial, anti-oxidative, anti-hypertensive, anti-inflammatory, immuno-modulating and antithrombotic activities (Marcone et al., 2017; Mohanty et al., 2016). These bioactive peptides may play a beneficial role in human health once they are released *in vivo* during gastrointestinal digestion or during bacterial fermentation (Mati et al., 2017).

Besides caseins that account for *ca*. 80% of camel milk proteins, camel whey is characterized by the presence of protective proteins such as lactoferrin, IgGs, lysozyme, lactophorin and a high content of peptidoglycan recognition protein (PGRP) (Kappeler et al., 2004). In addition, as reported for pig, rodents and lagomorpha (rabbit), camel milk contains a whey acidic protein (WAP) with superficial similarities with the insulin family of peptides and with some other peptides of biological importance (Beg et al., 1986). The physiological function of the WAP protein which occur not only in mammals (including marsupials and monotremes, even though absent from ruminant milks) but also in birds, reptiles, amphibians and fish (Smith, 2011), is still unknown. Several possible roles have been suggested so far. WAP domains are widely distributed and highly conserved, serving in diverse physiological processes such as proteinase inhibition, bacterial killing or inhibition of calcium transport (Iwamori et al., 2010).

Regarding peptides derived from camel milk proteins and having potential health-promoting activities, investigations mainly focus on caseins as the source of potential bioactive peptides. Data available mostly focus on *in vitro* antioxidant, antihypertensive and antimicrobial activities (Mati et al., 2017). However, very few peptides with a potential or effective bioactivity have been identified to date.

Recently, combining different proteomic approaches, the complexity of camel milk proteins was resolved to provide a detailed characterization of fifty protein molecules belonging to the 9 main milk protein families, including caseins: $\kappa$-, $\alpha_{s2}$-, $\alpha_{s1}$- and $\beta$-CN and two unknown proteins (UP1 and UP2), exhibiting molecular masses around 23,000 Da. Since UP1 and UP2 co-eluted in RP-HPLC with $\alpha_s$-CNs and displayed different phosphorylation levels, it was tempting to consider at first that these proteins could originate in CNs. However, with a molecular mass ranging between 22,939 and 23,179 Da for UP1, *ca*. 1,000-1,300 Da higher

than the main isoform of $\alpha_{s2}$-CN (21,906 Da, with 8P) and between 23,046 Da and 23,366 Da for UP2, *ca*. 2,500-2,800 Da lower than the main isoform of $\alpha_{s1}$-CN (25,773 Da, with 6P), the hypothesis of an additional casein in camel milk cannot be ruled out.

Genes encoding CNs are tightly linked on the same chromosome, BTA6 in cattle, CHI6 in goats (Hayes et al., 1993; Threadgill & Womack, 1990) and HSA4 in humans (Menon et al., 1992). The evolution of the CN gene cluster (Figure 3.1) is postulated to have occurred by a combination of successive intra- and inter-genic exon duplications (Groenen et al., 1993; Martin et al., 2013; Rijnkels et al., 2003). In some mammals, including horse, donkey, rodents and rabbit, there are two $\alpha_{s2}$-CN encoding genes differentiating in size (CSN1S2A and CSN1S2B), which may have arisen by a relatively recent gene-duplication event in rabbit (Cosenza et al., 2010; Dawson et al., 1993). However, the existence of a second $\alpha_{s2}$-CN encoding gene in camel has not been reported so far and is highly unlikely, given the distance existing in the camel genome between *CSN2* and *ODAM* (*ca.* 100 kb), comparatively to *Equus caballus* (*ca*. 240 kb) or human (*ca*. 200 kb).

Alternative splicing is a process by which multiple mRNA isoforms are generated. It is a powerful means to increase protein diversity. Such a process which is another possibility to increase the number of molecular species has been frequently reported to occur, as far as caseins are concerned, especially $\alpha_s$-CN (Leroux et al., 1992), without really knowing whether it is a fortuitous or a scheduled event to expand molecular diversity and functionality of milk proteins.

To substantiate the hypothesis according to which UP1 and UP2 originate in CNs and more precisely from $\alpha_s$-CN, studies were undertaken, at the protein and mRNA as well as at the genome levels. We thus showed that these two proteins are polypeptide chains derived from $\alpha_{s2}$-CN, not described up to now. They result from translation of mRNAs yielded during the processing of primary transcripts encoding $\alpha_{s2}$-CN and differing from the camel $\alpha_{s2}$-CN mRNA previously described with the splicing-in of an additional exon, encoding the nonapeptide ENSKKTVDT and with the integration of a 30-nucleotide intronic extension downstream from exon 16, encoding the decapeptide VKAYQIIPNL, respectively.

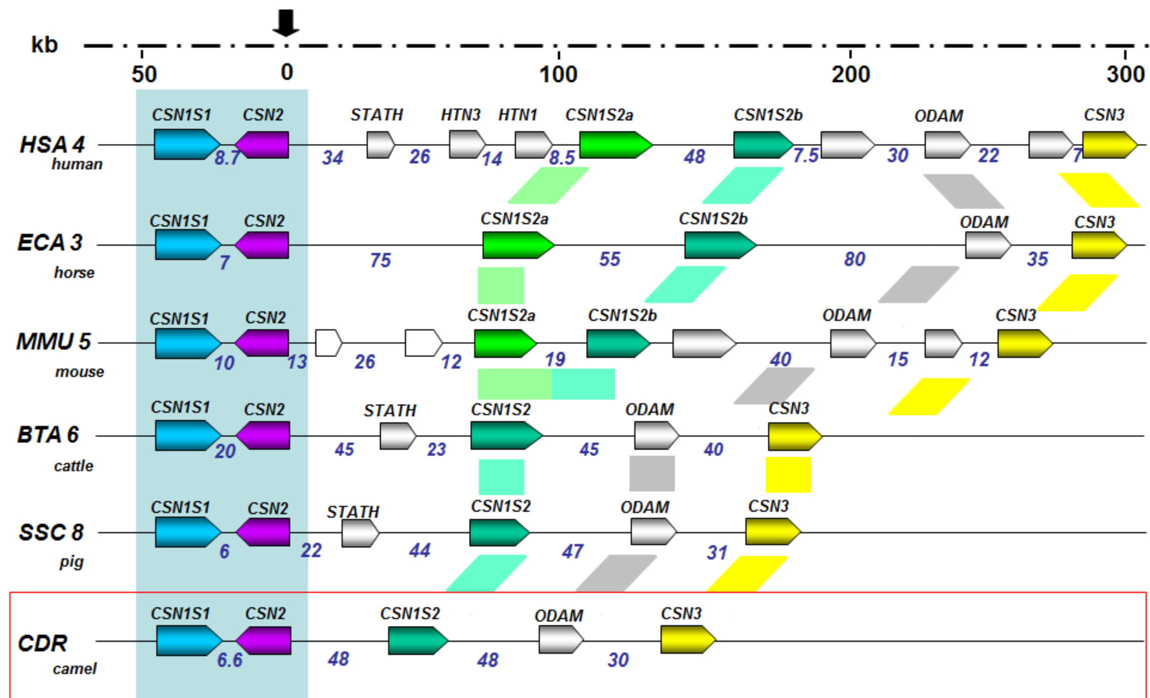


**Figure 3.1.** Evolution of the casein locus organization. Casein locus organization of human (*Homo sapiens*), horse (*Equus caballus*), mouse (*Mus musculus*), cattle (*Bos taurus*), pig (*Sus scrofa*) and camel (*Camelus dromedarius*) genomes (adapted from Martin et al., (2013) with additional genomic information from the NCBI) is compared. Genes are given by colored arrow boxes, showing the orientation of transcription. Putative genes based on similarity are indicated by empty boxes. Intergenic region sizes are given in kb.

# 3.2 Materials and Methods

## 3.2.1 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 with the exception of the rules of the afore-mentioned European Community regulations on animal experimentation, which were strictly followed.

## 3.2.2 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 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 2,500 *g* for 30 min at 4ºC (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.

## 3.2.3 Selection of Milk Samples for Analysis

In total, 58 milk samples were selected for SDS-PAGE analysis, based on lactation stages and number of parities (from 2 to 14) 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. Due to the lack of sufficient information, dromedary milk samples from AL region were excluded from subsequent analyses. Then, 8 milk samples (*C. bactrianus*, n=3, *C. dromedarius*, n=3, and hybrids, n=2) taken from the 58 milks analyzed by SDS-PAGE and from three different regions (SH, KZ, and ZKO), exhibiting the most representative electrophoretic profiles, were analyzed by LC-MS/MS (LTQ-Orbitrap Discovery, Thermo Finnigan) after a tryptic digestion of excised gel bands. Additionally, 30 milk samples (10 of each species), taken from the 58 milks analyzed by SDS-PAGE, were analyzed by LC-ESI-MS (Bruker Daltonics).

## 3.2.4 Milk Fat Globule Collection and RNA Extraction

Total RNA was extracted from MFG stored at -80°C using TRIzol® and TRIzol® LS solutions (Invitrogen, Life Technologies), respectively, following the original manufacturer's protocol with slight modifications, essentially as described by Brenaut et al., (2012).

## 3.2.5 Single-Strand cDNA Synthesis and PCR Amplification

First-strand cDNA was synthesized from 5 to 10 ng of total RNA primed with oligo(dT)20 and random primers (3:1, vol/vol) using Superscript III reverse transcriptase

(Invitrogen Life Technologies Inc., Carlsbad, CA) according to the manufacturer's instructions. One microliter of 2 U/µL RNase H (Invitrogen Life Technologies) was then added and the reaction mix was incubated for 20 min at 37°C to remove RNA from heteroduplexes. The single-strand cDNA thus obtained was stored at -20°C. cDNA samples covering the entire coding regions of caseins were amplified by PCR. PCR was performed in an automated thermocycler GeneAmp® PCR System 2,400 (Perkin-Elmer, Norwalk, USA) with GoTaq® G2 Flexi DNA Polymerase Kit (Promega Corporation, USA). Reactions were carried out with 0.2 mL thin-walled PCR tubes with flat cap strips (Thermo Scientific, UK) in 50 µL volumes containing 5X Green or Colorless GoTag® Flexi Buffer, $MgCl_2$ Solution 25 mM, PCR Nucleotide Mix 10 mM each, GoTag® G2 Flexi DNA Polymerase (5 u/µL), each oligonucleotide primer 10 mM, template DNA and nuclease-free water up to the final volume. Primer pairs, purchased from Eurofins (Eurofins genomics, Germany), were designed using published *Camelus* nucleic acid sequence (NCBI, LOC105090951). The forward primer 5'-TCATTTTTACCTGCCTTTTGGCTGT (25) -3' starting from nucleotide 71 is located in exon 2 of $\alpha_{s2}$-CN mRNA, whereas the reverse primer 5'-CGATTTTCCAGTTGAGCCATA (21) -3' terminating at nucleotide 692 of $\alpha_{s2}$-CN mRNA is located in exon 18. Thus, the amplified fragment covers a region of 622 nucleotides, including the sequence coding the mature protein, with genomic reference to the published sequence (NCBI, NM_001303561.1). Five (*C. bactrianus* (n=2), *C. dromedarius* (n=1), and hybrids (n=2)) of the 30 camel milk samples analyzed in LC-MS were selected for amplification of $\alpha_{s2}$-CN cDNA by RT-PCR and sequencing. Sequencing of PCR fragments was performed with primers used for PCR and sequenced from both strands according to the Sanger method by Eurofins (Eurofins genomics, Germany).

### 3.2.6 Identification of proteins by LC-MS/MS Analysis

In order to identify main milk proteins - mono dimensional electrophoresis (1D SDS-PAGE), followed by trypsin digestion and LC-MS/MS analysis - was used. After a long migration (10 cm) of proteins in such an 1D SDS-PAGE, the 16 main electrophoretic bands (1.5 $mm^3$) were cut on each gel lane, and analyzed essentially as described by Saadaoui et al., (2014).

### 3.2.7 LC-ESI-MS

Fractionation of camel milk proteins and determination of their molecular masses were performed by coupling RP-HPLC to ESI-MS (micrOTOFTM II focus ESI-TOF mass spectrometer; Bruker Daltonics). In total 20 µL of skimmed milk samples were first clarified by the addition of 230 µL of clarification solution 0.1 M bis-Tris buffer pH 8.0, containing 8 M urea, 1.3% trisodium citrate, and 0.3% DTT. Clarified milk samples (25 µL) were directly injected onto a Biodiscovery C5 reverse phase column (300 Å pore size, 3µm, 150x2.1mm; Supelco, France) and analyzed essentially as described by Ryskaliyeva et al., (2018).

## 3.3 Results and Discussion

### 3.3.1 What gene(s) UP1 and UP2 are arising from?

The mass accuracy has allowed distinguishing about fifty protein molecules belonging to isoforms of 9 protein families from LC-MS analyses. The chromatogram displays the presence of 15 major milk protein fractions labelled from I to XV, with retention times from 4.50 to 48.71 min, respectively. A typical protein profile obtained with milk from dromedary sampled in Shymkent region is given in Figure 3.2. The major milk proteins eluted in the following order: κ-CN A and B (peaks I and II), WAP (peak III), short and long isoforms of $\alpha_{s1}$-CN A and C along with splice variants (peaks IV, V, and VI), α-LAC (peak VI), $\alpha_{s2}$-CN (peaks VII to X), PGRP (peak XI), LPO/CSA (peak XII), long and short isoforms of β-CN A and B along with splice variants (peaks XIII and XIV) and γ2-CN A and B (peak XV). Additionally, the presence of two unknown proteins UP1 and UP2 in peaks VI to XI, where mainly $\alpha_{s1}$-CN, $\alpha_{s2}$-CN and PGRP are eluted, was previously reported by Ryskaliyeva et al., (2018). Regarding UP1, molecular masses ranged between 22,939 and 23,179 Da, whereas UP2 masses ranged between 23,046 Da and 23,286 Da (Table 3.1), with successive increments of 80 Da (mass of one phosphate group). In several samples we also found a mass corresponding to UP2 with one more phosphate group (23,366 Da). Eluting range of these two proteins was between 28.53-37.16 min, within the elution times of $\alpha_{s1}$- and $\alpha_{s2}$-CN, which confirms our first hypothesis: their probable belongings to $\alpha_s$-CN. However, UP1 and UP2 masses exceeded the observed mass of the major isoform of $\alpha_{s2}$-CN with 8P (21,906 Da) by 1,033 Da and 1,300 Da, respectively, and were lighter than the C variant of $\alpha_{s1}$-CN-6P (25,773 Da) by 2,834 Da and

2,567 Da, respectively. Even though it was not possible to exclude a splicing event leading to the inclusion of an additional exon sequence in the $\alpha_{s2}$-CN mRNA, the most probable hypothesis was the occurrence of exon-skipping event(s) affecting $\alpha_{s1}$-CN mRNA and leading to the loss of a peptide sequence accounting for a reduction of at least 2,567 Da. A possible scenario was skipping of exon 3 on the short isoform of $\alpha_{s1}$-CN C already impacted by a cryptic splice site usage (ΔCAG encoding Q83). The molecular mass of the protein proceeding from such messenger (23,205 Da) corresponded quite to the mass of UP2 +160 Da (23,206 Da). However, sequencing cDNA encoding $\alpha_{s1}$-CN isoforms failed to reveal the existence of a messenger in which exon 3 was lacking. Therefore, the alternative possibility, in other words the $\alpha_{s2}$-CN avenue, had to be explored.

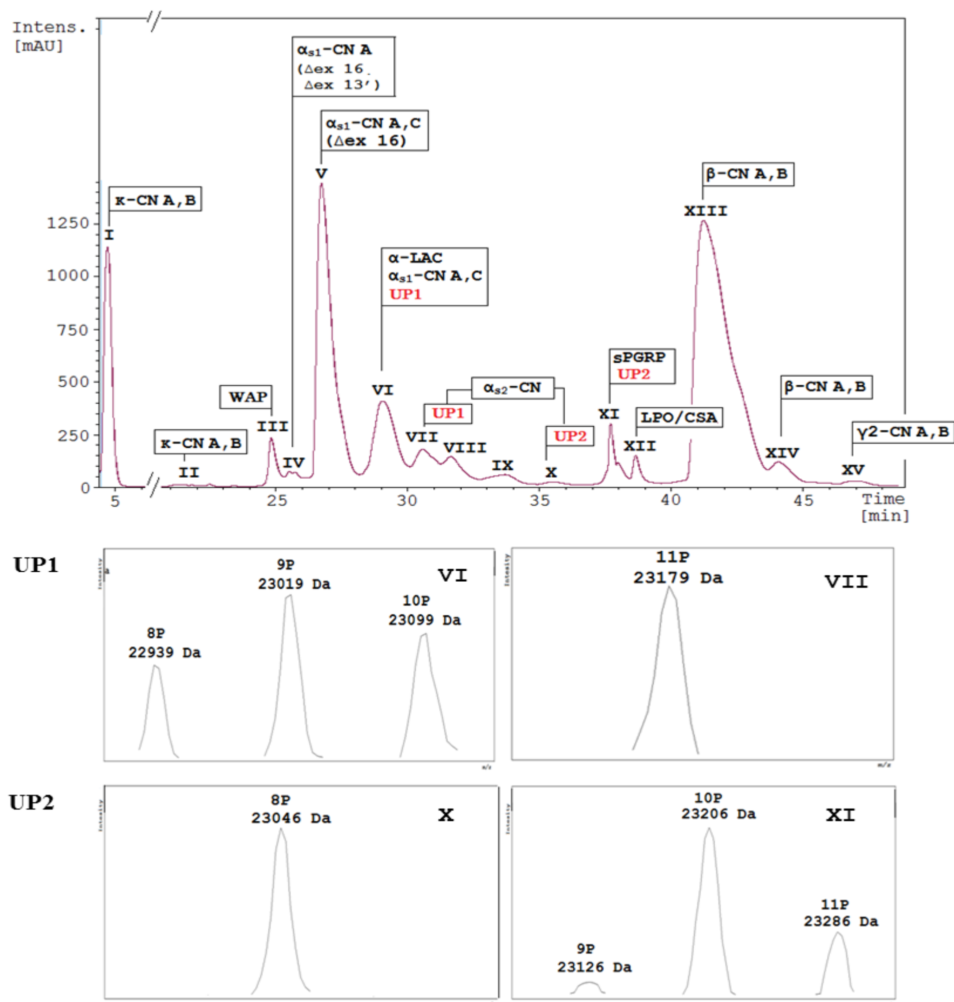


**Figure 3.2.** LC-ESI-MS profile of dromedary milk proteins. Deconvolution of multicharged ion spectra with emphasis on phosphorylation degrees (P) of two unknown proteins (UP1 and UP2) which are related to chromatographic peaks VI and VII, X and XI respectively.

**Table 3.1.** Analysis of molecular masses contained in peaks V-XI of dromedary milk sample from Shymkent region

| Peak | Ret.Time, min | Observed $M_r$, Da | Theoretical $M_r$, Da | Protein description | UniProt accession | Intensity |
|---|---|---|---|---|---|---|
| V | 26,31 | 24,547 | 24,547 | $\alpha_{s1}$-CN C -short isoform (Δex 16), 5P, splice variant (ΔQ83) | | 3,954 |
| | | 24,561 | 24,561 | $\alpha_{s1}$-CN A - short isoform (Δex 16), 5P, splice variant (ΔQ83) | | 4,385 |
| | | 24,627 | 24,627 | $\alpha_{s1}$-CN C - short isoform (Δex 16), 6P, splice variant (ΔQ83) | | 16,348 |
| | | 24,640 | 24,641 | $\alpha_{s1}$-CN A - short isoform (Δex 16), 6P, splice variant (ΔQ83) | | 17,422 |
| | | 24,675 | 24,675 | $\alpha_{s1}$-CN C - short isoform (Δex 16), 5P | | 7,758 |
| | | 24,689 | 24,689 | $\alpha_{s1}$-CN A - short isoform (Δex 16), 5P | | 8,004 |
| | | 24,722 | 24,721 | $\alpha_{s1}$-CN A - short isoform (Δex 16), 7P, splice variant (ΔQ83) | | 4,453 |
| | | **24,755** | **24,755** | **$\alpha_{s1}$-CN C - short isoform (Δex 16), 6P** | **K7DXB9** | **34,653** |
| | | **24,768** | **24,769** | **$\alpha_{s1}$-CN A - short isoform (Δex 16), 6P** | **O97943-2** | **37,452** |
| | | 24,835 | 24,835 | $\alpha_{s1}$-CN C - short isoform (Δex 16), 7P | | 5,026 |
| | | 24,849 | 24,849 | $\alpha_{s1}$-CN A - short isoform (Δex 16), 7P | | 4,851 |
| VI | 28.80 | **14,430** | **14,430** | **α-LAC** | **P00710** | **12,948** |
| | | **22,939** | **n/a*** | **UP1** | **n/a** | **2,676** |
| | | 23,019 | n/a | UP1, +80 Da | | 2,408 |
| | | 23,099 | n/a | UP1, +160 Da | | 958 |
| | | 25,645 | 25,645 | $\alpha_{s1}$-CN C, 6P, splice variant (ΔQ83) | | 1,736 |
| | | 25,659 | 25,659 | $\alpha_{s1}$-CN A, 6P, splice variant (ΔQ83) | | 1,057 |
| | | 25,693 | 25,693 | $\alpha_{s1}$-CN C, 5P | | 916 |
| | | **25,772** | **25,773** | **$\alpha_{s1}$-CN C, 6P** | | **5,014** |
| | | 25,787 | 25,787 | $\alpha_{s1}$-CN A, 6P | O97943-1 | 1,509 |
| VII | 30.07 | 21,826 | 21,825 | $\alpha_{s2}$-CN, 7P | | 709 |
| | | **21,906** | **21,905** | **$\alpha_{s2}$-CN, 8P** | **O97944** | **4,222** |
| | | 21,985 | 21,986 | $\alpha_{s2}$-CN, 9P | | 289 |
| | | 23,179 | n/a | UP1, +240 Da | | 1,430 |
| VIII | 31.26 | 21,986 | 21,985 | $\alpha_{s2}$-CN, 9P | O97944 | 866 |
| | | 22,066 | 22,065 | $\alpha_{s2}$-CN, 10P | | 3,682 |
| IX | 33.04 | 22,066 | 22,065 | $\alpha_{s2}$-CN, 10P | | 120 |
| | | 22,146 | 22,145 | $\alpha_{s2}$-CN, 11P | | 1,408 |
| X | 34.85 | 22,226 | 22,225 | $\alpha_{s2}$-CN, 12P | | 806 |
| | | 23,046 | n/a | UP2 | n/a | 295 |
| XI | 37.15 | **19,143** | **19,143** | **PGRP** | **Q9GK12** | **3,659** |
| | | 23,126 | n/a | UP2, +80 Da | | 150 |
| | | **23,206** | **n/a** | **UP2, +160 Da** | | **1,162** |
| | | 23,286 | n/a | UP2, +240 Da | | 940 |

n/a - not applicable

## 3.3.2 UP1 and UP2: new camel $\alpha_{s2}$-CN splicing variants

Amplification of camel $\alpha_{s2}$-CN cDNA revealed the presence of several PCR products differing in size between *ca.* 600 bp and 700 bp. Sequencing of PCR fragments generated two different nucleotide sequences, first identical from the forward primer to nucleotide 359, and then overlapped and shifted by 27 nucleotides (Figure 3.3). The main sequence corresponded to the 193-aa $\alpha_{s2}$-CN reported by Kappeler et al., (1998). The second sequence, which was with weaker signals, showed the insertion of the following sequence: GAA AAT TCA AAA AAG ACT GTT GAT ATG, between exons 12’ and 14. Thus, this insertion introduced an additional peptide sequence (ENSKKTVDM), identical to the aa sequence encoded by exon 13 in the bovine *CSN1S2* gene (Figure 3.4). The level of exon 13 conservation in both species appeared to be extremely high. This exon was also present in the lama gene with the same sequence except the last codon (ACG => ATG), leading to T => M substitution (Pauciullo & Erhardt, 2015). Except in mouse and rabbit, the same exon is present in the *CSN1S2* gene of most animal species. The insertion of this sequence leads to the increasing of the molecular mass of $\alpha_{s2}$-CN by 1,033 Da, exactly the mass difference observed between $\alpha_{s2}$-CN-8P and UP1.

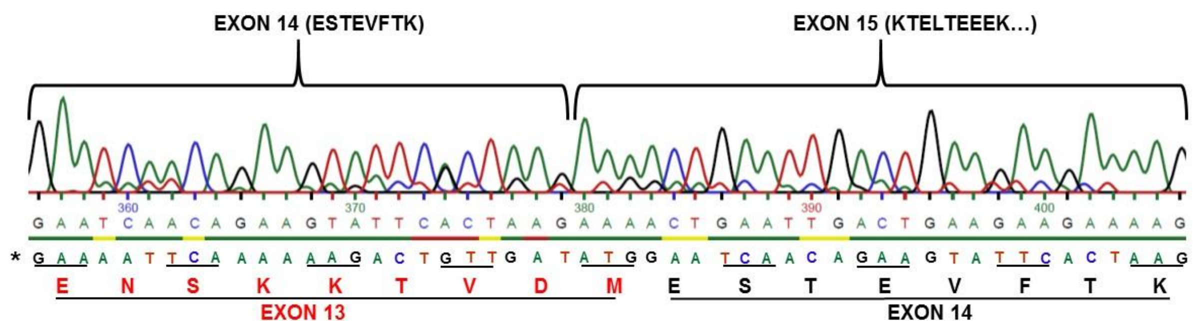


**Figure 3.3.** Sequence of *C. dromedarius* $\alpha_{s2}$-CN cDNA spanning exons 14 and 15 (main sequence). A secondary sequence (*) identified by manual reading of overlapping weak signals is given below the main sequence, evidentiating the existence of transcripts, in which exon 13 is included. The corresponding aa sequence is given beneath.

**Figure 3.4.** Multiple alignment of $\alpha_{s2}$-CN protein sequences from different mammalian species. *Bos taurus* (bovine in blue, M16644), *C. dromedarius* (camel in green, O97944), *Equus asinus* (donkey in orange, B7VGF9), and *Sus scrofa* (porcine in purple, X54975) protein sequences are compared. Sequences are split into blocks of amino acid residues to visualize the exon modular structure of the protein as deduced from known splice junctions of the bovine gene (Koczan et al., 1991). Exon numbering (top of blocks) is that of the bovine gene. Additional exons are numbered in single quotes. Italics indicate the signal peptides, of which the cleavage site is pointed out by the vertical black arrow. Colored boxes within the blocks depict constitutively out-spliced or missing exons. The dash indicates missing aa residue. Common aa residues are bolded.

A deep and comprehensive analysis of the nucleotide region from 417140 to 415110 in the dromedary camel *CSN1S2* gene sequence (whole genome shotgun sequence of *C. dromedarius* breed Arabia) available in Genbank (gi|742343530|ref|NW_011591251.1|), overlaying exon 12’ (ESTEVPTE) to exon 14 (ESTEVFTK) allowed identifying a 27-nucleotide sequence corresponding to exon 13 (Figure 3.5). This sequence is flanked with consensus sequences of intron end (polypyrimidine tract followed by XAG) and beginning (GTG/AAG). This exon is recognized, by choice as such, by the splicing machinery, and therefore alternatively skipped or considered during the course of camel $\alpha_{s2}$-CN pre-mRNA processing. The short transcript (without exon 13) encodes the 193 aa residues described by Kappeler et al., (1998) and the long transcript in which exon 13 is included codes for UP1 (202 aa).

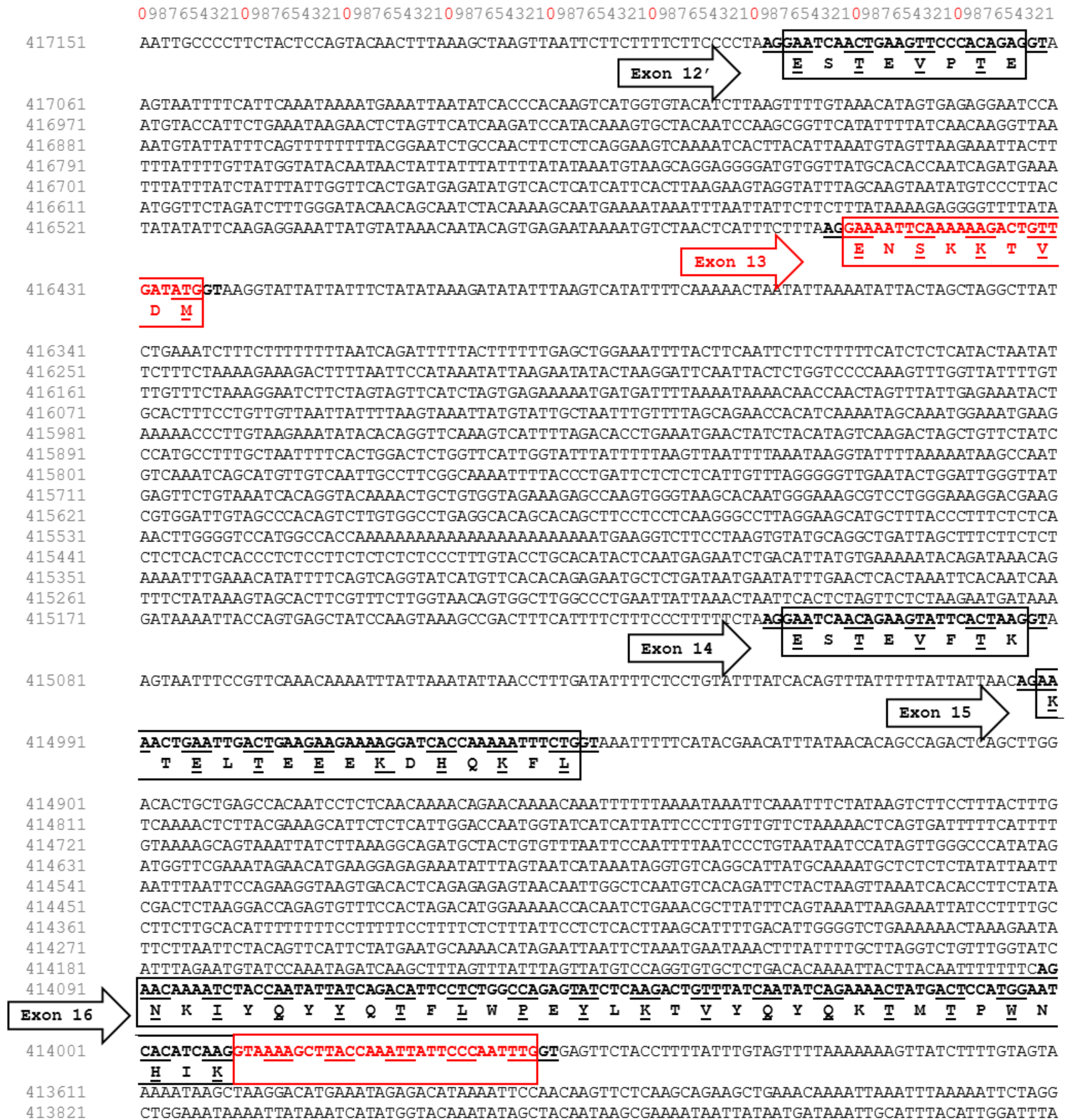


**Figure 3.5.** Nucleotide sequence view from 417151 to 413731 of *C. dromedarius* breed Arabia unplaced genomic scaffold of CSN1S2 (LOC105090951). Unplaced Scaffold Reference PRJNA234474_Ca_dromedarius_V1.0 Primary Assembly (Kappeler et al., 1998). Already known exons 12’, 14, 15 and 16 are given in black, and additional exon 13 and extension of 30 additional nucleotides of exon 16 are in red. Exon subdivisions are boxed with amino acid sequences beneath. Intron donor and acceptor splice sites are underlined.

To confirm such an additional exon 13 hypothesis, detection of $\alpha_{s2}$-CN peptides after trypsin action was performed using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). A tryptic peptide composed of 12 aa residues TVDMESTEVFTK (Figure 3.6), when compared with the *Bubalus bubalis* $\alpha_{s2}$-CN sequence (GenBank: APR74542.1) was attributed to two coherent arranged sequences (ENSKKTVDM and ESTEVFTK) encoded by exons 13 and 14, respectively. The presence of a TVDM peptide sequence confirmed the existence of transcripts having included exon 13 during the course of pre-mRNA processing. Therefore, the existence of an exon 13 alternatively spliced in camel *CSN1S2* gene was successfully confirmed both at the protein (LC-MS and LC-MS/MS and sequencing) and at the genomic levels. The same cDNA sequences encoding $\alpha_{s2}$-CN with a 27-nucleotide additional sequence (exon 13) were found in all individual samples analyzed, including *C. bactrianus, C. dromedarius*, and hybrids.

Concerning the second unknown protein detected (UP2), showing in LC-ESI-MS molecular masses comprised between 23,046 Da and 23,286 Da with n and n+3 phosphate groups, the mass difference observed was 1,140 Da, relatively to the 8P-11P $\alpha_{s2}$-CN protein reported by Kappeler and co-workers (1998). LC-MS/MS analysis revealed the occurrence of a 8-aa long peptide (YQIIPNLR) matching with the C-terminal sequence of *Sus scrofa* $\alpha_{s2}$-CN (NP_001004030.1), strongly suggesting that mRNA described by Kappeler et al., (1998) was in fact the result of a cryptic splice site usage occurring in the antepenultimate exon of the camel *CSN1S2* gene

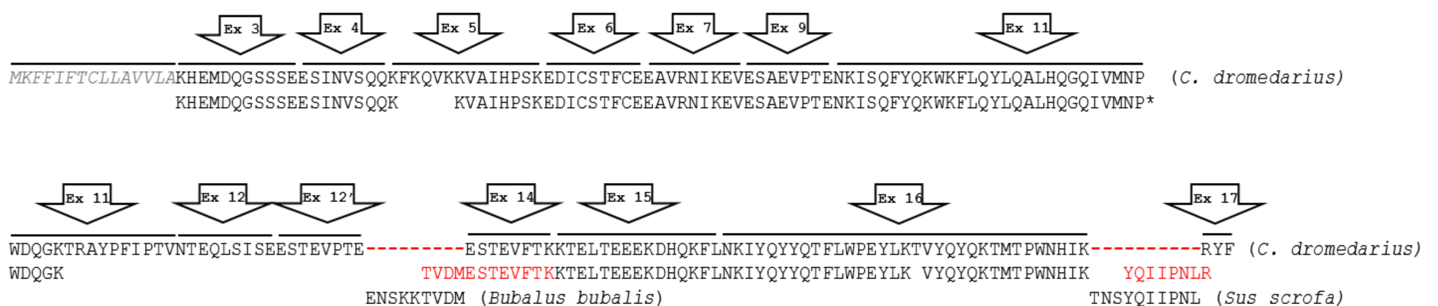


**Figure 3.6.** Total coverage of peptide sequences of camel $\alpha_{s2}$-CN derived after a tryptic digestion of electrophoretic bands excised from 1D SDS-PAGE. $\alpha_{s2}$-CN sequence (097944) is given in the first line, while the coverage of $\alpha_{s2}$-CN sequence from peptides identified by LC-MS/MS analysis is given below. Peptides indicating the presence of exon 13 and extension of exon 16 are in red. Peptides of interest belonging to *Sus scrofa* (P39036) and *Bubalus bubalis* (B6VPY3 and E9NZN2) are indicated in the third line. The signal peptide is in italics and in grey. The red dash indicates aa residues missing in the reference sequence (Kappeler et al., 1998). Exons (vertical arrows) are over lined.

Examination of intron sequence downstream exon 16 (Figure 3.5) highlighted a 30-nucleotide segment: GTA AAA GCT TAC CAA ATT ATT CCC AAT TTG encoding 10 aa residues (VKAYQIIPNL). The intron donor splice site following the previously considered ending sequence of exon 16 CACATCAAG│GTAAA was recognized by the spliceosome machinery to generate the protein described by Kappeler et al., (1998). Alternatively, the intron donor splice site may occur as well as at CCC AAT TTG│GTGAG sequence, which also qualifies all requirements of splicing recognition signal (Figure 3.5). As a result, this alternative splicing event is responsible for the occurrence of two peptide chains, the first one made of 193 aa residues (21,906 Da with 8P), and the second one 10 aa residues longer (23,046 Da with 8P). Interestingly, the 10 aa residues peptide (VKAYQIIPNL) included in the C-terminal part of the camel protein due to this alternative splicing was highly similar with the porcine (TNSYQIIPNL) and donkey (TNSYQIIPVL) $\alpha_{s2}$-CN sequence. Recently a shorter $\alpha_{s2}$-CN isoform, in which a deletion of the heptapeptide YQIIPVL, was reported in donkey milk (Cunsolo et al., 2017; Saletti et al., 2012).

## 3.3.3 Interspecies comparison of the gene encoding $\alpha_{s2}$-CN and primary transcripts maturation

Comparative analysis of camel *CSN1S2* gene organization with orthologous bovine and pig genes is illustrated in Figure 3.7. The first camel $\alpha_{s2}$-CN sequence published by Kappeler et al., (1998) is lacking three peptide sequences encoded in cattle by exons 8 (EYSIGSSSE), 10 (EVKITVDDKHYQKAL), and 13 (ENSKKTVDM) composed of 27, 45 and 27 nucleotides, respectively. By contrast, exon 12' that encodes a peptide of 8 aa residues (ESTEVPTE), which is present in camel and lama genes, is missing in the bovine counterpart, while it was present in porcine genome, coding for EPVSSSQE. We failed in finding a putative exon 12' in intron 12 of the bovine gene and, likewise, in finding a putative exon 8 in intron 7 of the camel gene. Present both in bovine and pig *CSN1S2* genes, exon 10 is also present in intron 9 of the camel gene, 9 nucleotides longer than in the other species (Figure 3.7), and bounded upstream and downstream by canonic intron consensus sequences. However, despite it seems to be perfectly eligible for splicing, we did not find any transcript nucleotide sequence, nor tryptic peptides at the protein level signing its presence in multiple mRNA encoding $\alpha_{s2}$-CN. By contrast, exon 13 was effectively present in some camel *CSN1S2* transcripts, as well as the peptide sequence it is coding for in isoform i1 of $\alpha_{s2}$-CN, as demonstrated in the present study. Finally, the camel *CSN1S2* gene is made of at least 17 exons, whereas its bovine and porcine counterparts are

made of 18 and 19 exons, respectively. Given there is a further exonic sequence (exon 7’) in Equidaes, we can hypothesize that the *CSN1S2* gene comprises at least 20 exons with different combinatory splicing schemes across species. Interestingly, within the bovine intron 7, as well as in camel and pig the sequence corresponding to horse and donkey exon 7’ is partially deleted.

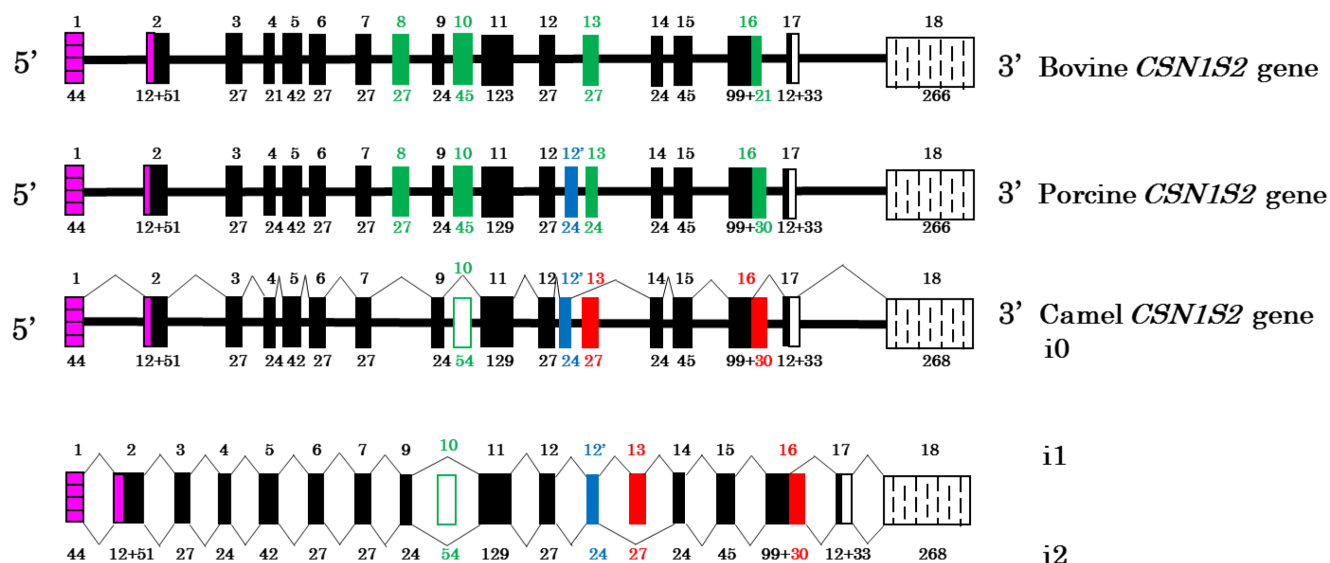


**Figure 3.7**. Gene structural organization of bovine, porcine and camel *CSN1S2* transcription units and splicing patterns for camel (i0, i1 and i2). Solid bars represent introns, and exons are depicted by blocks: 5’ UTR and noncoding sequence are given in pink, leader peptide and coding frame are in black, exons absent in camel protein are in green, exons absent in bovine protein are in blue, exons found in our study are in red, and 3’ UTR in white. Exons and exon sequences present in bovine and porcine *CSN1S2* but which were absent in camel until now are highlighted in green, while exons present in camel and porcine are in blue. Exon 13 and the extension of exon 16 identified in this study are in red. Exon numbering (referring to bovine) and sizes (in bp) are indicated in the top, and in the bottom of structures, respectively.

Genomic and mRNA analyses carried out previously demonstrated that deletions of aa residues in CNs across species occurred essentially by exon skipping during the processing of the primary transcripts. This event, leading to a shortening of the peptide chain length, is caused by weaknesses in the consensus sequences, either at the 5’ and/or 3’ splice junctions or at the branch point, or both (Martin et al., 2013). Therefore, alternative splicing has to be regarded as a frequent event, mainly in $\alpha_s$-CN encoding genes, for which the coding region is divided into many short exons. Usage of cryptic splice sites is also responsible for the occurrence of multiple transcripts and finally for generating a protein molecular diversity. For example, the peptide sequence (VKAYQIIPNL) encoded by the “extension” of 30 nucleotides at the 3’ end of exon 16, not previously detected in camel nor in lama $\alpha_{s2}$-CN, was shown here to be alternatively

included in camel. The combination of both splicing events such as exon skipping and cryptic splice site usage generates more transcript isoforms in the same species and is responsible for the differences across species in the aa sequences of $\alpha_{s2}$-CN. However, regarding $\alpha_{s2}$-CN messengers in camel we were not able to detect any transcript in which exon 13 and extension of exon 16 were present.

### 3.3.4 Phosphorylation level enhances camel $\alpha_{s2}$-CN isoform complexity

The non-phosphorylated peptide chain of the mature $\alpha_{s2}$-CN protein, which comprises 178 aa residues, yields a molecular weight of 21,266 Da (Kappeler et al., 1998). Comparing with other Ca-sensitive CNs, $\alpha_{s2}$-CN is the most phosphorylated with 12 potential phosphorylation sites and is likely therefore the major transporter of Ca-phosphate.

Structural characterization of $\alpha_{s2}$-CN fraction and relevant mRNA analyses have provided the demonstration that camel $\alpha_{s2}$-CN is present in milk as a mixture of at least 15 isoforms derived from three peptide chains comprising 178 (i0), 187 (i1, UP1) and 188 (i2, UP2) aa residues originating from alternative splicing phenomena (Figure 3.8). Indeed, splicing variants displayed different phosphorylation levels for each of them ranging between 7 and 12 P groups. Surprisingly, even though additional peptide sequence does not provide further phosphorylation sites, the predominant phosphorylation level of each peptide isoform was not the same: 8P for i0, 8P for i1, and 10P for i2. Addition of 10 aa residues in C-terminal part of i2 might induce conformational changes in the protein facilitating the modification of definite phosphorylable sites. Multiple non-allelic variants produced from different mRNAs have been shown to occur in all Kazakh individuals analyzed, apparently indicating a stabilized mechanism for the production of protein isoforms of different lengths, structures and biological activities.

With 11 potentially phosphorylated aa residues matching the S/T-X-A motif, camel $\alpha_{s2}$-CN displays the highest phosphorylation level, as mentioned by Ryskaliyeva et al. (2018). To reach such a phosphorylation level, besides the nine SerP, two putative ThrP (T118 and T132) have to be phosphorylated. In all the Kazakh milk samples analyzed in LC-ESI-MS we found i0 $\alpha_{s2}$-CN with 12 P groups, as the molecular mass of 22,226 Da observed corresponds to the mass of the peptide backbone (21,266 Da) increased by 960 Da, a mass increment which coincides with 12 P groups. That means that at least another S/T residue that does not match with the canonic sequence recognized by the mammary kinase(s), is potentially phosphorylated.

According to Allende et al., (1995) the sequence S/T-X-X-A follow-through with the minimum requirements for phosphorylation by the CN-kinase II (CK2). It is critical to highlight in this regard that E or D in this site can be replaced by SerP or ThrP. Two T residues, namely T39 and T129 in the camel $\alpha_{s2}$-CN fully meet the requirements of the above-mentioned motif and could be phosphorylated. Such an event is the only hypothesis to reach 12P for camel $\alpha_{s2}$-CN. Since these two kinases are very likely secreted, the idea that phosphorylation at T39/T129 may occur in the extracellular environment cannot be excluded. This warrants further investigation. Fam20C, which is very likely the major secretory pathway protein kinase (Tagliabracci et al., 2015), might be responsible for the phosphorylation of S and T residues within S/T-X-A motif, whereas a CK2-type kinase might be responsible for phosphorylation of T residue within an S/T-X-X-A motif. This is in agreement with the hypothesis put forward by Bijl et al., (2014) and Fang et al., (2016), who suggest from phenotypic correlations and hierarchical clustering the existence of at least 2 regulatory systems for phosphorylation of $\alpha_s$-CN.

```
                                 140       150       160       170       180       190       200      207
Bovine     SE--------ENSKKTVDMESTEVFTKKTKLTEEEKNRLNFLKKISQRYQKFALPQYLKTVYQHQKAMKPWIQPKTKV---IPYVRYL

Camel i0   SEESTEVPTE---------ESTEVFTKKTELTEEEKDHQKFLNKIYQYYQTFLWPEYLKTVYQYQKTMTPWNHIK----------RYF
Camel i1   SEESTEVPTEENSKKTVDMESTEVFTKKTELTEEEKDHQKFLNKIYQYYQTFLWPEYLKTVYQYQKTMTPWNHIK----------RYF
Camel i2   SEESTEVPTE---------ESTEVFTKKTELTEEEKDHQKFLNKIYQYYQTFLWPEYLKTVYQYQKTMTPWNHIKVKAYQIIPNLRYF
Camel i3   SEESTEVPTEENSKKTVDMESTEVFTKKTELTEEEKDHQKFLNKIYQYYQTFLWPEYLKTVYQYQKTMTPWNHIKVKAYQIIPNLRYF
```

**Figure 3.8.** Comparison of C-terminal sequences of camel $\alpha_{s2}$-CN isoforms with $\alpha_{s2}$-CN bovine counterpart. Sequences of the three C-terminal isoforms (i0, i1 and i2) identified are given, as well as a fourth putative isoform (i3) comprising sequences encoded by exon 13 and the extension of exon 16. Bioactive peptides encrypted in bovine $\alpha_{s2}$-CN f(150-188) with antibacterial activities reported by Zucht *et al*. (1995) are highlighted in yellow, while two antibacterial domains f(164-179) and f(183-207) described by Recio & Visser (1999) are indicated in red. Amino acid residues increasing significantly antibacterial potency are in green.

### 3.3.5 Camel milk $\alpha_{s2}$-CN a source of potentially biologically active peptides

Interest in the utilization of $\alpha_{s2}$-CN in dairy products and nutrition has been renewed by studies on its biological activities via potentially biologically active peptides (Farrell et al., 2009). The production of novel potentially bioactive peptides by $\alpha_{s2}$-CN splicing isoforms identified in camel milk cannot be excluded. At present, according to the splicing patterns, including or not extension of exon 16, two peptide sequences (KTMTPWNHIKRYF and

KTMTPWNHIKVKAYQIIPNLRYF) occur at the C-terminus of the molecule (Figure 3.8), giving rise to different peptides after digestion by proteolytic enzymes, including trypsin, from the digestive tract. Previous studies have demonstrated the antibacterial properties of bovine peptides derived from the same domain of the molecule (Farrell et al., 2009).

The first antibacterial peptide isolated from $\alpha_{s2}$-CN inhibiting the growth of *Escherichia coli* and *Staphylococcus carnosus*, was called Casocidin-I (Zucht et al., 1995). The antibacterial domain of the active peptide is located at the C-terminus of the mature protein f(150-188). In addition, two different antibacterial domains f(164-179) and f(183-207) at C-terminus of $\alpha_{s2}$-CN were further found in peptic digests (Recio & Visser, 1999). McCann et al., (2005) identified 5 antibacterial peptides from chymosin digests of $\alpha_{s2}$-CN; all were from the C-terminal end with f(164–207) and f(172–207) being most active against *Listeria innocua*. More recently, it was demonstrated the importance of specific amino acids (P and R residues) at the C-terminus of the bovine milk-derived $\alpha_{s2}$-CN f(183-207) peptide to its antibacterial activity against the food-borne pathogens *Listeria monocytogenes* and *Cronbacter sakazakii* (Alvarez-Ordóñez et al., 2013). These residues are also present in the camel sequence. From these studies it appears clearly that the C-terminal part of $\alpha_{s2}$-CN was predicted to yield peptides with defensin-like activity, which may aid the immune system in fighting bacteria (Farrell et al., 2009). Therefore, sequence modifications occurring in f(193-207), which is affected by a splicing event in the camel $\alpha_{s2}$-CN pre-mRNA, would likely impact antibacterial properties of $\alpha_{s2}$-CN C-terminal peptides, since several aa residues of this region were shown to be essential regarding this activity (Recio & Visser, 1999; Zucht et al., 1995).

# 3.6 Conclusions

The data reported here allowed identifying UP1 and UP2, two unknown proteins detected in our previous study, as splicing isoforms of $\alpha_{s2}$-CN. $\alpha_{s2}$-CN in camel milks from *C. bactrianus* and *C. dromedarius* (as well in hybrids) was shown to be a mixture of 15 or so isoforms differing in polypeptide chain length and phosphorylation levels. Isoform i0, which was initially reported in the literature, was the main isoform of $\alpha_{s2}$-CN. Isoforms i1 and i2 were splicing isoforms of $\alpha_{s2}$-CN arising from alternative processing of primary transcript and differing from i0 with the insertion of exon 13 (ENSKKTVDT) in i1 and an extension of exon 16 (VKAYQIIPNL) in i2. Such splicing events generate molecular sequence diversity that may influence biological properties of peptides encrypted in caseins from camel.

# Acknowledgments

The study was carried out within the Bolashak International Scholarship of the first author, funded by the JSC «Center for International Programs» (Kazakhstan). The research was partly supported by a grant from the Ministry of Education and Science of the Republic of Kazakhstan under name "Proteomic investigation of camel milk" #1729/GF4, which is duly appreciated. The authors thank all Kazakhstani camel milk farms for rendering help in sample collection, as well as PAPPSO and @BRIDGe teams at INRA (Jouy-en-Josas, France) for providing necessary facilities and technical support.

# Author Contributions

AR carried out the study, collected milk samples, performed the experiments, and interpreted the data. CH performed LC-MS/MS analysis and analyzed the data. GM performed LC-ESI-MS analysis and analyzed the data. BF and GK provided funding. PM conceived and supervised the research, interpreted the data. The manuscript was written by AR, revised and approved by PM. All authors reviewed and contributed to the final manuscript.

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# Chapter 4

# Characterization of multiple protein isoforms arising from the usage of a cryptic splice site in camel WAP precursors to mRNA in which a GC-AG intron occurs

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*Manuscript in preparation*

# Abstract

Camel milk samples from *Camelus dromedarius, Camelus bactrianus* and crossed hybrids reared in Kazakhstan were analyzed for molecular diversity of their protein fraction in which many splicing variants and post-translational modifications were recently reported. Using LC-ESI-MS analysis, we identified in *Camelus bactrianus* a whey protein with different phosphorylation levels that co-eluted in RP-HPLC with WAP, exhibiting a molecular masse (12,596 Da) 32 Da higher than the original WAP (12,564 Da). To determine the origin of such a polymorphism, nucleotide sequences of four unrelated individual camels (one Bactrian, two dromedary and one hybrid) were compared across the complete coding sequence of WAP cDNA. We identified a transition G/A, leading to a codon change (GTG/ATG) in the nucleotide sequence of the Bactrian cDNA, which modifies a single amino acid residue at position 12 of the mature protein (V12M). This single-point mutation (V: 99 Da vs. M: 131 Da) accounts precisely for the mass difference which is in perfect agreement with the mass difference found between WAP variants. In addition, we report from LC-MS/MS analyses, the existence of a splicing variant of camel WAP precursors to mRNA, arising from an alternative usage of the canonical splice site recognized as such in the other mammalian species expressing WAP in their milk. However, the WAP isoform first described as displaying an additional sequence of 4 amino acid residues (56VSSP59) in the peptide segment connecting the two 4-DSC domains is predominantly present in camelids milk. This major isoform results from the usage of an unlikely intron cryptic splice site, extending camel exon 3 on its 5' side by 12-nucleotides encoding the tetrapeptide VSSP in which a potentially phosphorylable Serine residue occurs. Another interesting feature of the camel WAP gene, is the GC-AG type of intron 3, that we confirmed by sequencing a genomic DNA fragment encompassing exon 3 to exon 4, the GC donor site showing a compensatory effect in terms of a dramatic increase in consensus at the acceptor exon position.

**Key words:** camel, milk, whey acidic protein, splicing, genetic polymorphism

# 4.1 Introduction

Camel milk has an overall composition very similar to that of bovine milk, especially as far as macro nutrients (protein, fat and lactose) are concerned. Camel milk is characterized by a high content of vitamin C (average content ranging between 50 and 250 mg/L), and endowed with a unique composition of protein components (Alhaider et al., 2013; Hinz, et al., 2012; Ryskaliyeva et al., 2018). Its protein content (35-50 g/L) is rather high (Konuspayeva et al., 2009), with *ca.* 80% are caseins and 20% whey proteins that are soluble at pH 4.6 whereas caseins precipitate close to this pH. The casein fraction comprises 4 caseins ($\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-casein) encoded by four autosomal genes (*CSN1S1, CSN1S2, CSN2* and *CSN3*, respectively) mapped on chromosome 6 in cattle and goat (Hayes et al., 1993; Threadgill & Womack, 1990). This fraction is rather complex with many splicing variants and post-translational modifications (Ryskaliyeva et al., 2018). The whey proteins of camel milk mainly consist of α-lactalbumin (α-LA), glycosylation-dependent cell adhesion molecule 1 (GlyCAM1) or lactophorin which is closely related to the bovine proteose peptone component 3 (PP3), the innate immunity Peptido Glycan Recognition Protein (PGRP) and the Whey Acidic Protein (WAP). Concentration of WAP reaches 157 $mg.L^{-1}$ in camel milk, whereas it is a hundred times higher (15 $g.L^{-1}$) in rabbit milk (Grabowski et al., 1991).

Whey Acidic Protein (WAP) is a major whey protein identified in the milk of several species from eutherians as well as marsupial and monotremes (Sharp et al., 2007). It was first shown to be secreted in rodent milks (Hennighausen & Sippel, 1982), and a whey protein, rich in half-cysteine residues (n=16), showing strong similarities with rodents WAPs was characterized two years later in camel milk (Beg et al., 1986). Then, the WAP has been identified in rabbit (Devinoy et al., 1988), porcine (Simpson et al., 1998), wallaby (Topcic et al., 2009), brushtail possum (Demmer et al., 2001) and more recently in canine (Seki et al., 2012) milks. Whey acidic protein (WAP) is expressed in the mammary gland under an extracellular matrix and lactogenic hormones regulation (Lin et al., 1995). WAP gene expression is induced by prolactin, inhibited by progesterone, and strongly amplified by glucocorticoids (Devinoy et al., 1994).

The overall organization of the eutherian WAP genes is highly conserved through evolution among species (Martin et al., 2013; Sharp et al., 2007). It is composed of 4 exons: E1, E3, E4 and E6 (Fig 4.1). While the size of each exon remains rather conserved between species, intron size varies considerably. The first exon encodes the 5'-UTR, N-terminal signal

peptide of 19 aa residues, and first 8-10 aa residues of the mature eutherian proteins. Exons 3 and 4 encode two cysteine-rich domains (DI and DIIA) known as four-disulfide cores (4-DSC) in eutherian species. A third domain (DIII) encoded by exon 2 (missing in eutherian genes) is found in Monotrema and Marsupial species. Exon 6 encodes the last 5-9 aa residues and the 3'-UTR while exon 5 (DIIB) is only used in Monotrema and Marsupial species. However, WAP is not found in all eutherian milks. The functionality of the gene encoding WAP has been lost in ruminants and primates due to a frameshift mutation (Hajjoubi et al., 2006). Consequently, there is no WAP in the milk of ruminants and primates.

The presence of 4-DSC domains in cysteine-rich proteins led to their classification as the WAP gene family. Proteins containing WAP domains with a characteristic 4-DSC occur not only in mammals but also in birds, reptiles, amphibians and fish (Smith, 2011). Each domain comprises eight C residues with a core of six spatially conserved and equally linked, while the remaining two are positioned at variable distances amino terminal from the core (Simpson & Nicholas, 2002).

The sequence conservation of 4-DSC motifs across species is significant, and it seems likely that the region may be involved in the biological function of the molecule. WAPs share structural similarity with serine protease inhibitors containing WAP motif domains characterized by a four-disulfide core (4-DSC) (Hennighausen & Sippel, 1982). Possible physiological functions of WAP have been proposed, based on its similarity to protease inhibitor (Grabowski et al., 1991). Using *in vitro* and *in vivo* systems, Nukumi et al., (2007) suggested that WAP plays an important role in regulating the proliferation of mammary epithelial cells by preventing elastase-type serine proteases from carrying out extracellular matrix laminin degradation. In addition, a bacteriostatic activity of rat WAP against *Staphylococcus aureus* was reported (Iwamori et al., 2010)

The present study was undertaken first to precise the origin of a WAP polymorphism detected in the Bactrian species by LC-ESI-MS. In addition, we provide here information to explain why an additional sequence of 4 amino acid residues occurs in the camel WAP, comparatively to the other 5 mammalian species in which the WAP gene is expressed. Finally, results reported here clarify discrepancies and erroneous data found in sequence databases and literature. We also report that in camel, the gene encoding WAP comprises a rare GC-AG intron-type which represents less than 1% of annotated donor sites.

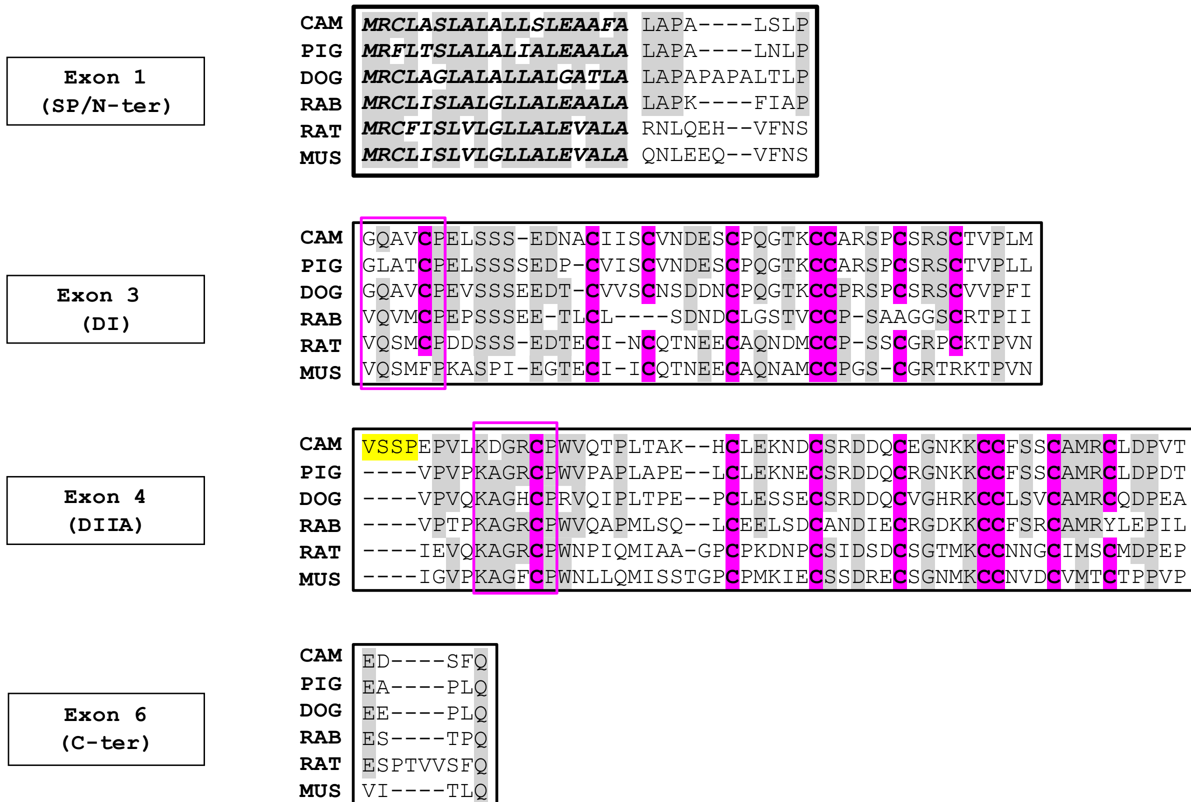


**Fig 4.1**. Multiple sequences alignment of WAP among Eutherian species including camel (NCBI, 105095719), pig (O46655), dog (GenBank AAEX02035361, positions 25,184-23,606), rabbit (P09412), rat (G3V718), and mouse (Q7M748). Four exons: E1, E3, E4 and E6 (numbering of the putative ancestral gene, proposed by (Sharp et al., 2007), are given in black boxes. Exons 3 and 4 represent 4-DSC domains (DI and DIIA), while exon 1 and 6 indicate the signal peptide (SP) with the N-terminal part (N-ter) of the mature protein and the C-terminal part (C-ter) of the protein, respectively. WAP motifs are boxed in pink. Conserved Cysteine residues (C) in each 4-DSC domain are pink shaded. Residues identical in more than 3 animal species are grey shaded. Gaps are introduced to maximize similarities. Tetrapeptide VSSP, that is specific to camel WAP, is highlighted in yellow.

# 4.2 Materials and Methods

## 4.2.1 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 and blood sampling were 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 with the exception of the rules of afore-mentioned European Community regulations on animal experimentation, which were strictly followed.

### 4.2.2 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.

### 4.2.3 Selection of Milk Samples for Analysis

In total 58 milk samples were selected for SDS-PAGE analysis, based on lactation stages and number of parities (from 2 to 14) 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. Then, 8 (*C. bactrianus*, n=3, *C. dromedarius*, n=3, and hybrids, n=2) of the 58 remaining milk samples from three different regions exhibiting the most representative profiles were analyzed by LC-MS/MS (LTQ-Orbitrap Discovery, Thermo Finnigan) after a tryptic digestion of excised gel bands. Additionally, 30 milk samples (*C. bactrianus*, n=10; *C. dromedarius*, n=10; hybrids, n=10), taken from the 58 milks analyzed by SDS-PAGE, were analyzed by LC-ESI-MS (Bruker Daltonics). Meanwhile, 2 of these 30 camel milk samples (*C. bactrianus*, n=1; and hybrid, n=1) were selected for amplification of WAP cDNA by RT-PCR and cDNA sequencing.

### 4.2.4 Milk Fat Globule Collection - RNA Extraction and Single-Strand cDNA Synthesis

Total RNA was extracted from MFG fraction stored at -80°C using Trizol (Invitrogen) following the protocol from the manufacturer as described by Brenaut et al., (2012). Then, first-strand cDNA was synthesized from 5 to 10 ng of total RNA primed with oligo(dT)20 and

random primers (3:1, vol/vol) using Superscript III reverse transcriptase (Invitrogen Life Technologies Inc., Carlsbad, CA) according to the manufacturer's instructions. One microliter of 2 U/µL RNase H (Invitrogen Life Technologies) was then added and the reaction mix was incubated for 20 min at 37°C to remove RNA from the heteroduplexes. Single-strand cDNA thus obtained was stored at -20°C.

## 4.2.5 Genomic DNA Isolation

The genomic DNA was isolated from fresh blood of *C. dromedarius* collected in EDTA using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, USA). Briefly, for 3 ml sample volume, 9 ml of Cell Lysis Solution was added and centrifuged at 2,000 x *g* for 10 min at room temperature (RT), after incubating the mixture for 10 min at RT. The supernatant was removed and to the resuspended white pellet containing red and white blood cells, 3 ml of Nuclei Lysis Solution was added. Then, 1 ml of Protein Precipitation Solution was added to the nuclear lysate, and centrifuged at 2,000 x *g* for 10 min at RT. The supernatant was transferred to a tube containing 3 ml isopropanol and centrifuged at 2,000 x *g* for 1 min at RT. After decanting the supernatant, one sample volume of 70% ethanol was added to the DNA and centrifuged at 2,000 x *g* for 1 min at RT. The ethanol was aspirated using a drawn Pasteur pipette and the pellet was air-dried for 10-15 min. DNA was rehydrated by adding 250 µl of DNA Rehydration Solution and incubated at 65°C for 1 hour and stored at 2-8°C.

## 4.2.6 PCR Amplification

cDNA and DNA samples were amplified using primer pairs, purchased from Eurofins (Eurofins genomics, Germany), which were designed using published *Camelus* gene sequence (NCBI, LOC105095719) as showed in Table 4.1. PCR was performed in an automated thermocycler GeneAmp® PCR System 2,400 (Perkin-Elmer, Norwalk, USA) with GoTaq® G2 Flexi DNA Polymerase Kit (Promega Corporation, USA). Reactions were carried out with 0.2 ml thin-walled PCR tubes with flat cap strips (Thermo Scientific, UK) in 50 µL volumes containing 5X Green or Colorless GoTag® Flexi Buffer, $MgCl_2$ Solution 25 mM, PCR Nucleotide Mix 10 mM each, GoTag® G2 Flexi DNA Polymerase (5 u/µL), each oligonucleotide primer 10 mM, template DNA and nuclease-free water up to the final volume. Sequencing of PCR fragments was performed using primer pairs used for PCR and sequenced from both strands according to the Sanger method by Eurofins.

**Table 4.1.** Primers used to amplify the cDNA and gDNA target of the WAP gene

| | Position | Primer | Sequence 5'->3' | aa residue | Tm, ºC |
|---|---|---|---|---|---|
| cDNA | 5'-flanking region | Forward | ATCTGTCACCTGCCTGCCACCTG | 23 | 66 |
| | 3'-flanking region | Reverse | TGAAGCTGAGTGGGTTTTTATTAGC | 25 | 60 |
| gDNA | intron 2 | Forward | CAGCTGAGGCTGGCCCGCCTC | 21 | 70 |
| | intron 3 | Reverse | GCTAGTCTGACACCCTCTCTCTA | 23 | 62 |

## 4.2.7 1D Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Both major and low-abundance proteins resolved by SDS-PAGE were identified after excision by mass analysis of the tryptic hydrolysate. The method used in the study was based on that from Laemmli (Laemmli, 1970).

## 4.2.8 Identification of proteins by LC-MS/MS Analysis

In order to identify main milk proteins - mono dimensional electrophoresis (1D SDS-PAGE), followed by trypsin digestion and LC-MS/MS analysis - was used. After a long migration (10 cm) of proteins in such an 1D SDS-PAGE, the 16 main electrophoretic bands (1.5 $mm^3$) were cut on each gel lane, and analyzed essentially as described by (Saadaoui et al., 2014).

## 4.2.9 LC-ESI-MS

Fractionation of camel milk proteins and determination of their molecular masses were performed by coupling RP-HPLC to ESI-MS (micrOTOFTM II focus ESI-TOF mass spectrometer; Bruker Daltonics). In total 20 µL of skimmed milk samples were first clarified by the addition of 230 µL of clarification solution 0.1 M bis-Tris buffer pH 8.0, containing 8 M urea, 1.3% trisodium citrate, and 0.3% DTT. Clarified milk samples (25 µL) were directly injected onto a Biodiscovery C5 reverse phase column (300 Å pore size, 3µm, 150x2.1mm; Supelco, France) and analyzed essentially as described by Ryskaliyeva et al., (2018).

## 4.3 Results and discussion

### 4.3.1 Identification and characterization of a new WAP genetic variant in Bactrian camel milk

Two molecular masses, 12,596 Da and 12,676 Da differing by 80 Da (one phosphate group), were found in the same peak (peak III, eluting time: 24.3 min) as WAP (Fig 4.2, Table 4.2), with a mass difference of 32 Da comparatively to the molecular mass of the cognate WAP (12,564 Da and 12, 644 Da). Such a difference strongly suggested the existence of a genetic variant of WAP, which have not been described so far in camels.

To reinforce this hypothesis, RNA from milk fat globule was extracted; reverse transcribed and cDNA-encoding WAP was amplified and sequenced. Nucleotide sequences of two unrelated individuals were compared across the complete coding sequence of the camel WAP cDNA, in both directions. PCR yielded a fragment of the expected length for a complete mRNA open reading frame of 408 bp, demonstrating that the primary transcript was correctly spliced. However, examining the nucleotide sequence manually, a transition G/A may be easily noticed, leading to the fourth codon change (GTG/ATG) of exon 2, confirmed by the reverse complement sequence. This single base substitution corresponds to the V/M amino acid substitution in position 12 of the mature protein (Fig 4.3), in agreement with the mass difference of 32 Da (V12M, 99 Da => 131 Da), found between WAP variants detected in LC-ESI-MS. We propose to name the camel WAP (V12) described by Beg et al., (1986) as variant A and the newly identified variant (M12) as variant B. Consequently, molecular masses observed by LC-ESI-MS (12, 596 Da, 12,676 Da) precisely correspond to phosphorylation isoforms of WAP variant B carrying 0 or 1 phosphate group. This B variant which was only found in one (Bactrian) of the 30 camel milk samples analyzed in LC-ESI-MS, at the heterozygous state, appears therefore to be rare in the Kazakh population.

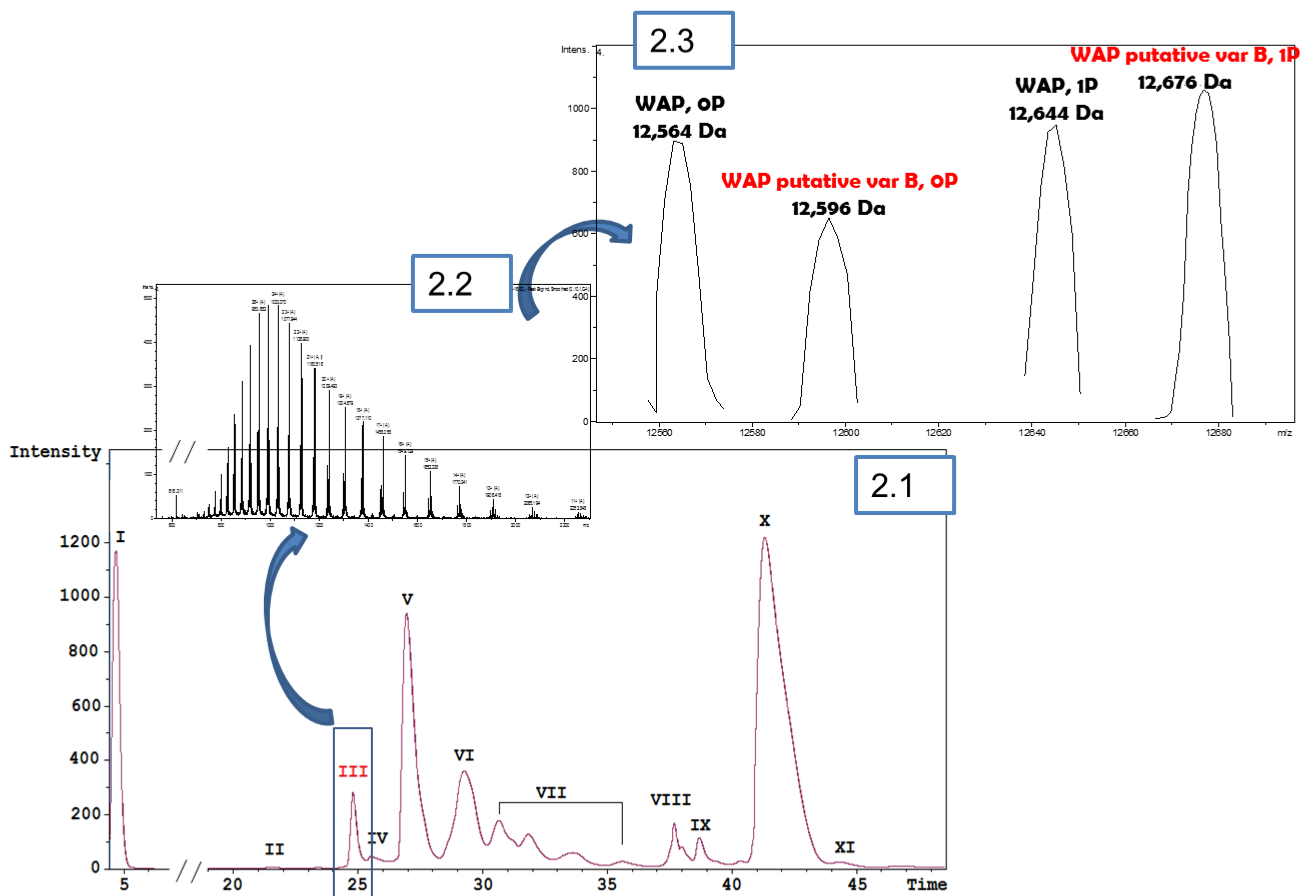


**Fig 4.2. Milk protein profiling by LC-ESI-MS of a clarified Bactrian camel milk from the Shymkent region**. Eleven major milk protein fractions were identified from RP-HPLC profile (2.1) in the following order: glycosylated κ-CN A and B (I), non-glycosylated κ-CN A and B (II), WAP (III), shorter (Δex16 and 13') + short (Δex16) isoforms of $\alpha_{s1}$-CN A and C (IV and V), α-LAC + $\alpha_{s1}$-CN A and C + $\alpha_{s2}$-CNΔex16 (VI), $\alpha_{s2}$-CN* (VII), PGRP + $\alpha_{s2}$-CN (VIII), LPO/CSA (IX), β-CN A and B (X) and $\gamma_2$-CN A and B (XI). Spectrum of the multicharged-ions from compounds of fraction III (2.2). After deconvolution (2.3) the spectrum shows the presence of cognate camel WAP-0P (12,546 Da) and 1P (12,644 Da) indicated in black, and molecular masses corresponding to the new WAP variant (named B) without (12,596 Da) and with (12,676 Da) one phosphate group, indicated in red.

**Table 4.2. Identification of WAP from molecular mass determination using LC-ESI-MS of clarified Bactrian milk from Shymkent region**

| Peak | Ret.Time, (min) | Observed $M_r$ (Da) | Theoretical $M_r$ (Da) | Protein description | UniProt accession | Intensity |
|---|---|---|---|---|---|---|
| III | 24.31 | 12,564 | 12,564 | WAP, 0P | P09837 | 896 |
| | | 12,596 | n/a | WAP variant B, 0P | n/a | 652 |
| | | **12,644** | | **WAP, 1P** | | **951** |
| | | **12,677** | | **WAP variant B +80Da** | | **1,059** |

*n/a - not applicable*

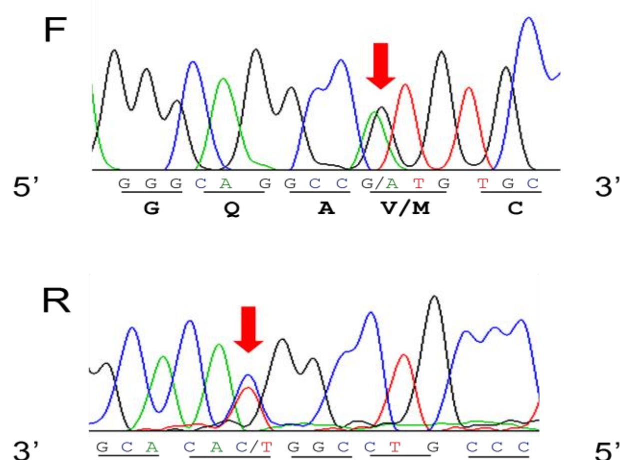


**Fig 4.3.** Partial sequence of WAP cDNA from *C. bactrianus* from Shymkent region. *Nucleotides arising by manual reading, considering possible G/A, and C/T transitions, in forward (F) and reverse (R) sequences, respectively. Nucleotides are clustered by three (codon) corresponding to aa residues (below codons in the forward sequence).

The complete camel WAP mRNA sequence (408 nucleotides open reading frame) encodes a N-terminal signal peptide of 19 aa residues and a mature protein of 117 aa residues, of which the molecular mass is 12,564 Da, as far as the 0P isoform is concerned. Camel WAP contains five potential phosphorylation sites (S-X-A code) per molecule (S17, S18, S19, S58, and S87), meanwhile rat WAP has only three potential phosphorylation sites. It was reported, that mouse WAP is apparently non-phosphorylated (Hennighausen & Sippel, 1982). From mass data, it is clear that only one site is phosphorylated. Given the extremely constrained and compact structure of the molecule with 8 S-S bridges, essential for folding and functionality of the protein, it is very likely that S58 which is located within the additional sequence connecting the two 4-DSC domains, is the only one seryl residue which is alternatively phosphorylated in camel. Indeed, WAP contains 16 cysteinyl (C) residues, all of which involved in disulfide bridges. C residues appear in unique arrangements, divided into two domains. Camel WAP consists of two 4-DSC domains, which are located between aa residues 9 and 55 (DI) and 64 and 111 (DIIA). Each domain begins with a six aa WAP motif (9GQAV**C**P14 and 64KDGR**C**P69), containing the first C residue of the 8 found in the domain.

### 4.3.2 A cryptic splice site usage during the splicing of precursors to WAP mRNA is responsible for the insertion of 4 amino acid residues in the camel WAP

Camel WAP shows the higher sequence identity at the aa level (76%) to porcine WAP and much lower aa sequence identities to the WAP from dog (65%), rabbit (51%), rat (40%) and mouse (39%). The comparison of camel WAP sequence with that of the other 5 eutherian species in which the WAP gene is expressed *(Sus scrofa, Canis familiaris, Oryctolagus cuniculus, Rattus norvegicus* and *Mus musculus)*, shows a 4 aa residues (56VSSP59) insertion in the camel polypeptide chain at the beginning of the second 4-DSC domain (Fig 4.1). From the *Camel dromedarius* gene sequence (GenBank 105095719) this appears to be the consequence of the usage of an unlikely intron cryptic splice site extending camel exon 3 on its 5' side by 12-nucleotides, whereas in the other 5 species the canonic 3' end of intron 2 is used (Fig 4.4). Indeed, there is two potential intron donor splice sites responding to all requirements of splicing recognition signal: CCCGGCCAG|TCTCTTCCCCAG|AGCCTGTCCTG. Paradoxically it is the weakest site that is preferentially used by the splicing machinery. We confirmed this sequence, by sequencing a 580-bp fragment amplified from genomic DNA encompassing integrally exon 4 and flanking intron sequences (Fig 4.4).

Alternative pre-mRNA splicing generates multiple protein isoforms from a single gene. Leading to a shorter or longer peptide chain, a non-allelic exon-skipping event may occur during the course of the pre-mRNA splicing. It is thought to be provoked by the weakness in the consensus sequences, either at the 5' and/or 3' splice junctions or at the branch point, or both (Martin et al., 2002). As far as camel WAP is concerned, even though cDNA sequencing allowed characterizing a single transcript we could not exclude the existence of a non-allelic short isoform of camel WAP, encoded by a shorter mRNA arising from the usage, as in the other species, of the canonic 3' splice site. This assumption was supported by the detection in LC-MS/MS (implemented to confirm the identification of proteins) of two tryptic peptides: SCTVPLMVSSPEPVLK and SCTVPLMEPVLK identifying camel WAP and differentiating in the presence or absence of the VSSP tetrapeptide (Table 4.3).

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        098765432109876543210987654321098765432109876543210987654321098765432109876543210987654321
286251                                                     ATCTGTCACCTGCCTGCCACCTGCCACCATGCGCTGTCTTGCCAGCC
                                                                                        M  R  C  L  A  S
286161  TGGCCCTGGCCCTGCTCTCCCTGGAGGCTGCCTTTGCACTGGCCCCAGCCCTCTCCTTGCCAGGTAAGCCCAGGAGGGGCATCCTGCCCT
        L  A  L  A  L  L  S  L  E  A  A  F  A  L  A  P  A  L  S  L  P
286071  CCCTCTGCTCCAGGTCCCCCCACCCCCCGAAATGCTGCCCAGGCCTCACAGTTTGGTGACCAGGACAAGAGAGGGAGCAGAACGAGGGAG
285981  GTGCACCCTTGGGGTATCAGTGACAACAGAAGGGTGGGGTGCTGCCCAGAAATGCCCATGCATGGGGGGCCAAGAGCTGCAGGGCCGAGG
285891  CCACCGTCCCGGGATGCAAAGTGACCGGGCAGCTAGCTGGGTGTGGGGGGATCCTGTCCTCGGCCCTTCGGGGTCTTAACTCCACCTCCT
285801  GCAGTGGGCCTGTGGCCTCATTTCCTGACTTTCACAGCTGACTCACAATTTCTGTCAGCCATGTCGAATGTCAGTGTTGGGGTGCTGGAG
285711  ACCGGACTCCCCAAGGTTCTTGTCCGCGCCCCACCTTGCTTTTTCCTGAGGCGCCCCAGGGGAGGTGGGATCCAGCCAGCCCCCCACCTC
285621  CCCGCTGGTCTCGTGCGCAGGGCAGGCCGTGTGCCCAGAGCTCAGCTCCTCCGAGGACAACGCCTGCATAATCTCCTGTGTCAACGATGA
                            G  Q  A  V  C  P  E  L  S  S  S  E  D  N  A  C  I  I  S  C  V  N  D  E
285531  GAGCTGTCCCCAAGGCACCAAGTGCTGCGCCAGGAGCCCCTGCAGCCGATCCTGCACGGTCCCCTCATGGGTAAGGCCACGTCTGGACA
          S  C  P  Q  G  T  K  C  C  A  R  S  P  C  S  R  S  C  T  V  P  L  M
285441  GAGGGAGGAACCAGCTTTGTCAGGGGTCCTCCTTGAGTCTGCACTCCACACCCCTCTCTCGGTGAGCCCCCCAGCAGCCCCATAAACTCG
285351  GCCCGGCCATGGCACGGGTTCACCCACCTGAGGCCCAGCTGAGGCTGGCCCGCCTCCTCCCAGCATCACCTGCTGGGCCCGCAGGCCACA
285261  GTCTCCCTCCCAGCCTGGGCCCTGAAGGCTCCAGGCCACCAAGGGGGAGGGAAGGGGGGACCAGCTGCGGGGCGGCCCCAGCAGGCCAGC
285171  GGGTGATGACGGCCCGGCCAGTCTCTTCCCCAGAGCCTGTCCTGAAGGATGGCCGCTGCCCCTGGGTGCAGACCCCGCTGACCGCAAAGC
                             V  S  S  P  E  P  V  L  K  D  G  R  C  P  W  V  Q  T  P  L  T  A  K
285081  ACTGTTTGGAGAAAAACGATTGCTCCAGGGACGACCAGTGTGAGGGCAACAAGAAATGCTGCTTCAGTTCGTGTGCCATGAGGTGTCTGG
        H  C  L  E  K  N  D  C  S  R  D  D  Q  C  E  G  N  K  K  C  C  F  S  S  C  A  M  R  C  L
284991  ACCCCGTCACAGGCAAGCGTCTCAGAGACCCTTCTCCTAGGCCACCTTCCCAGCCACCACCCTTCTTTCTCCCTTTGCCTGCACCGACCG
        D  P  V  T  G  K  R  L  R  D  P  S  P  R  P  P  S  Q  P  P  P  F  F  L  P  L  P  A  P  T
284901  TGGTGGCCTCTGCAAGGCTCTAAGAGCAATAAGATGCATGGAAGGGTGGGGGCTCAGGAGGCGGCGTGACCCGGGCCTGCGCCAGCTGCA
        V  V  A  S  A  R  L  *
284811  GGCCCCTGCGGGCGCTCAGTGTGGCCATTGGGTTAGAGAGAGGGTGTCAGACTAGCAAAAGGGGCTCAGAGAAACCCAGAGACTTGCCTG
284721  GTGGGACCTGGTCCGGCCGGGTGGGCCCACCTGCAAGTCTGTGCCTTTGCCACTTAAGCCTGGGGAGGAGCTCTGAATTAGGAGCCCAGA
284631  GACGTGGTTCCCTGATAACAGTCCCTACCACCTGTGACCTGGCCGGCCTGCCTGCGGGTGATGGTGAGGGCCCAGGAGGGGTGGGTGTGG
284541  AGGGCTTACTGGCCCACCTGCAGTGTCCCTCCCTCCCCTGACCCAACTGTGGCGGGGCTCCACCACCCCTGAGGCCTCCATGTCCCCTGC
284451  AGAGGACTCTTTTCAATGAGGGGCATCCCTGGGAGCTGCCAGGAGTGACCAGCCTGAGTCTGCTCAGCAAGAACCTTCTCTCTCGGATCC
           E  D  S  F  Q  *
284361  AGACAGCACAAGATGCCTCCTATCTGCTGCTAATAAAAACCCACTCAGCTTCATGTCTGTCTGTCTGTCTGCCCTCAGCACCTGCCAGGA
284271  GAGACCAAGCACACAGGCCTTGCCTTCCAAGCAGGCTTGGAAGCTCCTGCAAACAGGCGATTCTGCCTGGGGACACGTGTGACGAAGTAG
```

**Fig 4.4.** Complete sequence of *C. dromedarius* WAP gene available in GenBank (NCBI, 105095719). Primer pairs used for PCR and DNA sequencing are highlighted in green. Introns are indicated in italics. Intron donor and acceptor sites are bolded. The effective intron donor site (GCAAG) is highlighted in fuchsia. The reading frame in WAP gene is preserved through a 2-1 exon phase (nucleotides involved at the 5'- and 3'-ends are highlighted in yellow). Triplet codons encoding aa are given in blue and encoding specific to camel WAP tetrapeptide VSSP in red. The wrong protein sequence is indicated in grey and polyA site (AATAAA) is in purple. Stop codons are indicated with *: the correct one is in blue and the putative is in grey.

**Table 4.3. Sequences of WAP tryptic peptides identified by LC-MS/MS in the milk of a *C. bactrianus* from the Kyzylorda region**

| ID | UniProt accession | Species | Peptide sequence | aa residue | | M*r* | Charge | Spectra |
|---|---|---|---|---|---|---|---|---|
| | | | | Start | Stop | | | |
| 1 | P09837 | *C. dromedarius* | LAPALSLPGQAVCPELSSSEDNACIISCVNDESCPQGTK | 1 | 39 | 4,174.90 | 3 | 1 |
| 2 | P09837 | *C. dromedarius* | LSLPGQAVCPELSSSEDNACIISCVNDESCPQGTK | 5 | 39 | 3,822.69 | 3 | 1 |
| 3 | P09837 | *C. dromedarius* | IISCVNDESCPQGTK | 25 | 39 | 1,707.77 | 2 | 1 |
| 4 | P09837 | *C. dromedarius* | VNDESCPQGTK | 29 | 39 | 1,234.54 | 2 | 2 |
| 5 | P09837 | *C. dromedarius* | **SCTVPLMVSSPEPVLK** | 49 | 64 | 1,743.90 | 2 | 6 |
| 6 | P09837 | *C. dromedarius* | **SCTVPLMVSSPEPVLKDGR** | 49 | 67 | 2,072.05 | 2 | 3 |
| 7 | P09837 | *C. dromedarius* | **PLMVSSPEPVLKDGR** | 53 | 67 | 1,624.87 | 2 | 1 |
| 8 | P09837 | *C. dromedarius* | DGRCPWVQTPLTAK | 65 | 78 | 1,628.82 | 2 | 1 |
| 9 | P09837 | *C. dromedarius* | CPWVQTPLTAK | 68 | 78 | 1,300.67 | 2 | 11 |
| 10 | P09837 | *C. dromedarius* | HCLEKNDCSR | 79 | 88 | 1,318.56 | 2 | 2 |
| 11 | P09837 | *C. dromedarius* | HCLEKNDCSRDDQCEGNK | 79 | 96 | 2,264.91 | 3 | 1 |
| 12 | P09837 | *C. dromedarius* | HCLEKNDCSRDDQCEGNKK | 79 | 97 | 2,393.00 | 2 | 1 |
| 13 | P09837 | *C. dromedarius* | KCCFSSCAMR | 97 | 106 | 1,322.51 | 2 | 1 |
| 14 | P09837 | *C. dromedarius* | CCFSSCAMR | 98 | 106 | 1,161.39 | 2 | 1 |
| 15 | P09837 | *C. dromedarius* | CLDPVTEDSFQ | 107 | 117 | 1,310.56 | 2 | 13 |
| 16 | S9XKL5 | *C. ferus* | **SCTVPLMEPVLK** | 130 | 141 | 1,389.71 | 2 | 1 |

The table is classified by the start aa residues from the N-terminal sequence. Obtained data matches against UniprotKB taxonomy cetartiodactyla (SwissProt + Trembl) database. Molecular masses ($M_r$) of peptides are expressed in Da. Spectra indicates the number of spectra permitting the identification of peptides. Charge corresponds to the number of charges (z) of multi-charged ions precursors having given the MS/MS spectra. Peptide sequences including or not the tetrapeptide VSSP, which is highlighted in green, are in bold. Numbering of the *C. ferus* peptide sequence is from KB016488 Genomic DNA Translation.

## 4.3.3 Intron 3 of camel WAP gene is a GC-AG intron type

During the maturation process of pre-mRNA, introns are precisely removed by a large ribonucleoprotein complex: the spliceosome. This splicing step requires splice signals at the 5' and the 3' ends of the intron to be removed and a branch point (Burge et al., 1999). The vast majority of introns begin with the standard form dinucleotide GT at the 5' splice site and terminate with the dinucleotide AG at the 3' splice site, so-called GT-AG introns. This rule

hold in most cases, however some exceptions have been found (Wu & Krainer, 1999). For example, at the 5' terminus of a few introns, a dinucleotide GC can be occasionally found (Thanaraj & Clark, 2001). Based on the data sets derived from annotated gene structures, it has been reported, that GC donor sites account for less than 1% of annotated donor sites and possess a strong consensus sequence (Thanaraj & Clark, 2001). GC-AG introns are processed by the same splicing pathway (U2-type spliceosome) as conventional GT-AG introns (Aebi et al., 1987). GC-AG introns works in balance with alternative GT splice donor and uses alternative donor and acceptor splice sites, and lack a reasonable poly pyrimidine tract (Thanaraj & Clark, 2001). In humans, about 0.7% of GC-AG introns are involved in regulated splicing (Farrer et al., 2002). In *Caenorhabditis elegans,* experiments indicate that the conserved C at the +2 position of the tenth intron of the let-2 gene is essential for developmentally regulated alternative splicing (Farrer et al., 2002). In camel WAP gene, the C might allow the splice donor to function as a very weak splice site that works in balance with an alternative GT splice donor. In this respect, the only possibility would be the use of the GTCAC site, 7 nt upstream of the GCGAG. Such an assumption would have for consequence a modification of the 3' acceptor splice site of intron 3 to maintain a frame of reading in phase and to cause the loss of 3 aa residues: V and T (5' side of intron 3) and E (3' side of intron 3) in the camel WAP sequence. The C-terminal sequence of the protein described by Beg et al., (1986) terminates with the peptide sequence DPVTEDSFQ. The protein sequence deduced from our cDNA sequencing, in accordance with the molecular mass determined from LC-MS, terminates with the identical DPVTEDSFQ peptide sequence. The usage of the postulated alternative GT donor splice site or any else GT donor splice site cannot be excluded. However, we were unable to detect a DPDSFQ C-terminal or any other C-Terminal sequence, as well in LC-MS/MS as through cDNA sequencing. Surprisingly, in WAP gene, available in GenBank (NCBI, 105095719) exon 3 is prolonged with 99 nucleotides encoding 33 aa residues until the occurrence of a potential TAA stop codon, which would make exon 4 ineffective. From our results, in agreement with the protein structure reported by Beg et al., (1986), the use of this GC donor site is more than likely. Especially since it was reported that alternative GC-AG introns shows a compensatory effect in terms of a dramatic increase in consensus at the donor (AG-GCAAG) as well as at the polyYx(C/T)AG-G acceptor exon positions (Thanaraj & Clark, 2001).

## 4. 4 Conclusions

In this study, combining proven proteomic and molecular biology approaches, three main findings in respect to camel WAP are provided. The first is the identification of a new genetic variant (B), originating from a transition G => A, leading to a codon change (GTG/ATG) in the nucleotide sequence of a Bactrian cDNA, which modifies a single amino acid residue at position 12 of the mature protein (V12M). The second is the detection of two transcripts coding for camel WAP, of which one is arising from an improper and unusual processing of a unique pre-mRNA, due to a cryptic splice site usage. This phenomenon leads to the gain or loss of 4 amino acid residues (56VSSP59), of which one serine residue is potentially phosphorylable. Such a genetic polymorphism and splicing events generate a molecular sequence diversity that may account for biological properties of camel WAP. The physiochemical properties of the long and short isoforms might be quite different, and could contribute unique properties to camel milk. Finally, we report here the occurrence of a GC-AG intron-type (intron 3) in camel gene encoding WAP, showing a compensatory effect in terms of a dramatic increase in consensus at the acceptor exon position.

## Acknowledgments

The study was carried out within the Bolashak International Scholarship of the first author, funded by the JSC «Center for International Programs» (Kazakhstan). This work was supported in part by the grant for Scientific Research project named "Proteomic investigation of exosomes from milk of *Camelus bactrianus* and *Camelus dromedarius*" #AP05134760 from the Ministry of Education and Science of the Republic of Kazakhstan, which is duly appreciated. The authors thank all Kazakhstani camel milk farms for rendering help in sample collection, as well as PAPPSO and @BRIDGe teams at INRA (Jouy-en-Josas, France) for providing necessary facilities and technical support.

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# Chapter 5

# Comprehensive Proteomic Analysis of Camel Milk-derived Extracellular Vesicles

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*Manuscript in preparation*

# Abstract

Extracellular vesicles were recovered by optimized density gradient ultracentrifugation from milk of *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 $9.49 \times 10^8 - 4.18 \times 10^{10}$ 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.

**Key words:** milk, camel, exosomes, extracellular vesicles, MFGM, proteome, tetraspanins

# 5.1 Introduction

Milk is usually consider 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 (Tkach & Théry, 2016). 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 (Delcayre et al., 1996). 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 (Abels & Breakefield, 2016). Exosomes represent the smallest population among EVs ranging in size from 30 to 150 nm in diameter (Hromada et al., 2017). 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 (van der Pol et al., 2012). Found in all biofluids exosomes harbor different cargos as a function of cell type and physiologic state (Abels & Breakefield, 2016).

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 (Kelleher & Lonnerdal, 2001). Milk is therefore thought to play an important role in the development of the immune system of the offspring (Hanson, 2007). 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 non-coding 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 (Colombo et al., 2014; Simons & Raposo, 2009). 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 (Kabani & Melki, 2016).

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 (Kanada et al., 2015). 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 (Keller et al., 2006). Recently, cell culture studies showed that bovine milk-derived exosomes act as a carrier for chemotherapeutic/chemopreventive agents against lung tumor xenografts *in vivo* (Munagala et al., 2016). 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 (Reinhardt et al., 2013; van Herwijnen et al., 2016), there are only one dealing with exosomes derived from camel milk (Yassin et al., 2016). 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). 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 & 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).

## 5.2 Materials and methods

### 5.2.1 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 with the exception of the rules of afore-mentioned European Community regulations on animal experimentation, which were strictly followed.

### 5.2.2 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$^{o}$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.

### 5.2.3 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) Mass Spectrometer after a tryptic digestion of excised gel bands.

## 5.2.4 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). 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). 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). 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.

## 5.2.5 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).

## 5.2.6 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.

## 5.2.7 Proteomic analysis

To estimate the concentration of total EVs, the Coomassie Blue Protein Assay was used (Bradford, 1976). Absorbance at 595 nm was measured using the UV-Vis spectrophotometer (UVmini-1240, Shimadzu). The reference standard curve was done with 1 mg/mL commercial bovine serum albumin (BSA, Thermo Fischer Scientific).

In order to identify proteins, mono dimensional electrophoresis (1D SDS-PAGE) followed by trypsin digestion and LC-MS/MS analysis, was used. Ten µg of each individual skimmed camel milk sample were loaded onto 4-15% Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad, Marnes-la-Coquette, France) and subjected to electrophoresis. Samples were prepared with Laemmli Lysis-Buffer (Sigma-Aldrich) with β-mercapto ethanol and denatured at 100°C for 15 min. Separations were performed in a vertical electrophoresis apparatus (Bio-Rad, Marnes-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), meanwhile the alkylation of free cysteinyl residues with iodoacetamide (IAM, 50 mM, Sigma) at RT for 45 min in total obscurity. After gel pieces were washed twice, first, with 100 µL 50% ACN/50 mM NH4HCO3 and then with 50 µL 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) coupled to a QExactive (Thermo Fischer Scientific) 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 carbamido 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.

### 5.2.8 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 (Huang et al., 2009).

## 5.3 Results and Discussion

### 5.3.1 Isolation of camel milk-derived EVs

EVs are complex and delicate systems requiring optimized isolation and characterization adapted to each fluid type of origin (de la Torre Gomez et al., 2018), which may be achieved by a variety of methods, including ultracentrifugation, filtration,

immunoaffinity isolation and microfluidics techniques (Witwer et al., 2013). 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 (Zonneveld et al., 2014). 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 ultracentrifuged to allow for the separation and concentration of EVs. After ultracentrifugation, individual fractions were collected, and EVs enriched fractions (10 to 12) were pooled.

## 5.3.2 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 native morphology intact (Yamada et al., 2012). To visualize and characterize the morphology and size distribution of camel milk-derived EVs, TEM and NTA analyses was performed. In all 6 milk samples analyzed, we observed 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. 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 5.1). Thus, 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 (Yassin et al., 2016) and on milk of other species, such as bovine (Reinhardt et al., 2012), porcine (Chen

et al., 2016), horse (Sedykh et al., 2017) and human (Admyre et al. 2007), obtained characteristics for *Camelus* milk appear common to EVs across species. Thus, we can conclude that the method of differential ultracentrifugation with density gradient ultracentrifugation resulted in efficient and reliable isolation of camel milk-derived EVs.

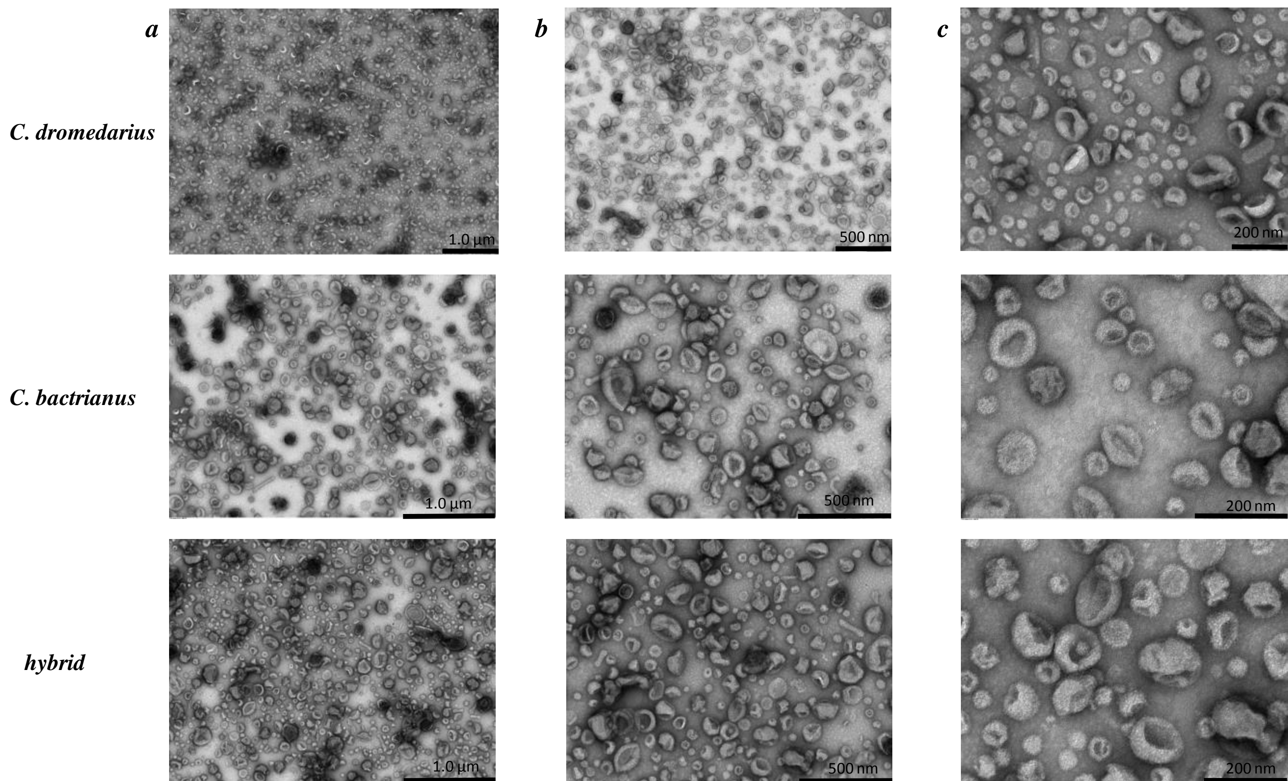


**Figure 5.1.** Representative electron micrographs of *C. dromedarius, C. bactrianus and hybrid* camel milk-derived EVs. Scale bar represents a) 1 µm, b) 500 nm, c) 200 nm.

## 5.3.3 In-depth proteomic analysis of camel milk-derived EVs

Apart from the morphology, specific protein composition enables to characterize EVs. To identify proteins present in camel milk-derived EVs extensive analyses involving trypsin digestion, LC-MS/MS (Q Exactive, Thermo Fisher Scientific) and database searches were performed. Recently, using a similar approach a total of 1,963 proteins were identified in human milk-derived EVs (van Herwijnen et al., 2016), and 2,107 unique proteins were described in bovine milk-derived EVs (Reinhardt et al., 2012). 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[1]). About 890 proteins were common between the three camel species as shown in Figure 5.2 (a), 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 (Ryskaliyeva et al., 2018). 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 (Michalski et al., 2011) in terms of sensitivity. Comparing the proteomes between camel milk and camel milk-derived EVs, identified 222 proteins as common (Figure 5.2 (b)), the list of which are provided as a supplementary data (S2[1]).

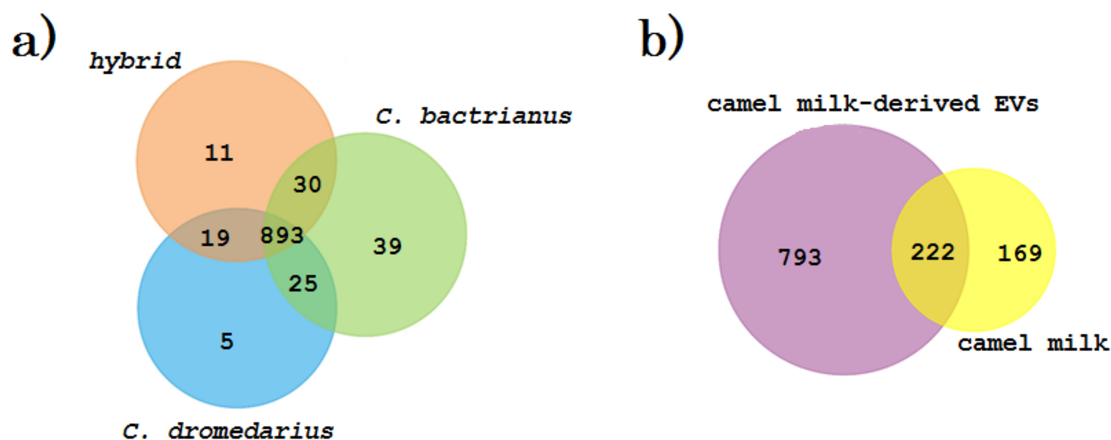


**Figure 5.2. a)** Venn diagram comparing proteins identified in *C. dromedarius, C. bactrianus* and hybrids milk-derived EVs. The diagram illustrates common and unique EV proteins between the three species **b)** Venn diagram comparing proteins identified in camel milk-derived EVs and proteins detected in camel milk reported in our previous study (Ryskaliyeva et al., 2018).

[1] S1 and S2 will be available on request once the manuscript published

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 (Lu et al., 2014). 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 5.3, 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" (van Herwijnen et al., 2016). 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 (McManaman & Neville, 2003). Thus, the MFGM can reflect dynamic changes within the MEC and may provide a "snapshot" of mammary gland biology, under particular 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 (Lu et al., 2014). In addition, some membrane proteins from intracellular organelles such as cytosol, mitochondrion and Golgi apparatus were highly expressed in camel milk-derived EVs.

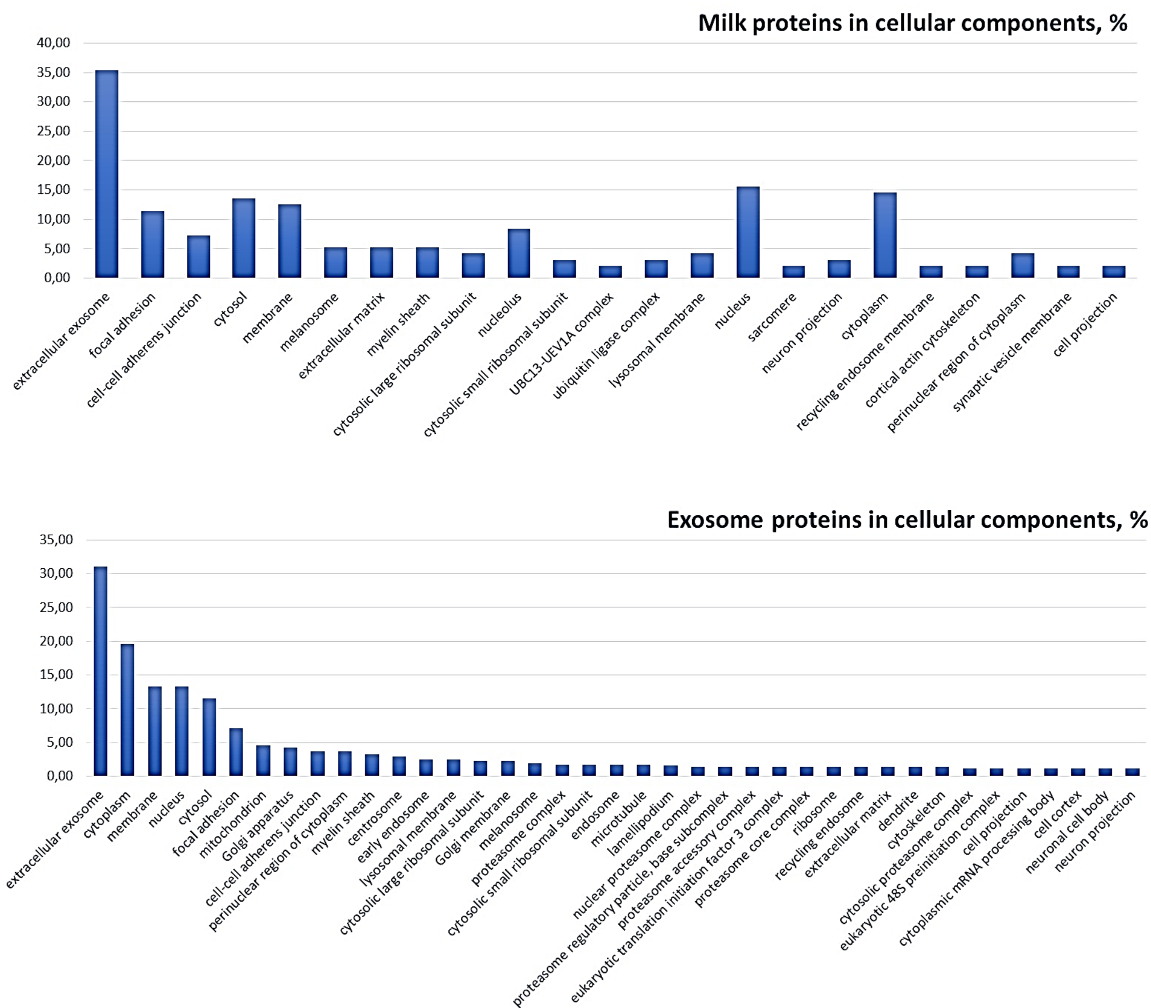


**Figure 5.3.** Functional annotations of camel milk and milk-derived EV proteins classified into cellular components using DAVID bioinformatics resources 6.8.

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 as shown in figure 5.4. 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 (Keller et al., 2006). 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 considered to be associated with GTP binding function (Figure 5.4), regulating membrane-vesicle trafficking

process. Proteins expressed in camel milk-derived EVs were categorized into 34 different KEGG pathways. As shown in Table 5.1, 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 (Keller et al., 2006).

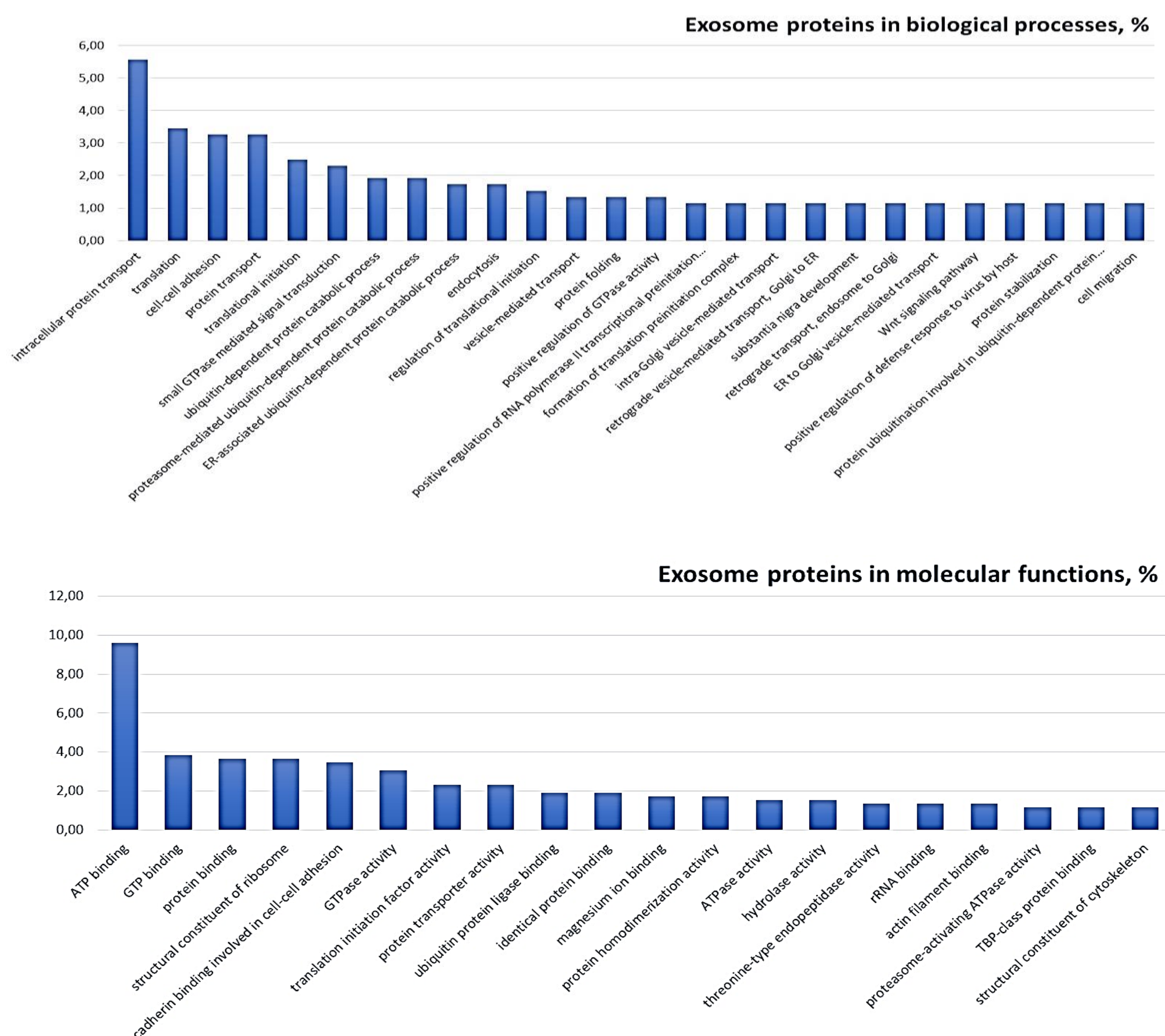


**Figure 5.4.** GO enrichment analysis of camel milk-derived EV proteins classified into biological processes and molecular functions using DAVID bioinformatics resources 6.8.

**Table 5.1.** 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 |
| 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 |

### 5.3.4 Exosomes are a rich source of potential milk biomarkers

Isolation of EVs from milk is complicated by the high lipid content of milk (Witwer et al., 2013). 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 (Lopez & Ménard, 2011), 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 (Reinhardt et al., 2012; Witwer et al., 2013). 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 anti-tumor immune responses, which

have become novel target pathways for cancer- and immunotherapy development (Cubillos-Ruiz & Conejo-Garcia, 2011; Kuhajda, 2000; Neutzner et al., 2007).

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 (Samuel et al., 2017; Yassin et al., 2016). 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 (Baietti et al., 2012). Moreover, HSP70 and HSP90 proteins implicated in innate immune responses and antigen presentation (Srivastava, 2002), 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 (Simons & Raposo, 2009). 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 (Théry et al., 2002). 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 (Raimondo et al., 2011). 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 (Rana & Zöller, 2011). Recently CD9, CD63, and CD81 tetraspanins have been defined as novel markers characterizing heterogeneous populations of EVs subtypes (Kowal et al. 2016), 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 reported, and the necessity of analyzing instead either CD81- or CD9-bearing EVs was reported (Kowal et al., 2016).

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 (Witwer et al., 2013). 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 (Vaswani et al., 2017), which may co-purify in vesicle preparations and confound analysis (Mathivanan, Ji, & Simpson, 2010; Witwer et al., 2013). Minimizing contamination in the isolation of exosomes is vital in providing reliable information upon which to base new paradigms (Vaswani et al., 2017). 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 (Yamada et al., 2012). 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 (Yassin et al., 2016), human (van Herwijnen et al., 2016) and bovine milk exosomes (Reinhardt et al., 2012).

## 5.4 Conclusions

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.

## Acknowledgments

The study was carried out within the Bolashak International Scholarship of the first author, funded by the JSC «Center for International Programs» (Kazakhstan). This work was supported in part by the grant for Scientific Research project named “Proteomic investigation of exosomes from milk of *Camelus bactrianus* and *Camelus dromedarius*” #AP05134760 from the Ministry of Education and Science of the Republic of Kazakhstan, which is duly appreciated. The authors thank all Kazakhstani camel milk farms for rendering help in sample collection, as well as PAPPSO, @BRIDGe, and MIMA2 platforms at INRA (Jouy-en-Josas, France) for providing necessary facilities and technical support. We are grateful to Christine Longin for assistance with TEM analysis.

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# Chapter 6

# General Discussion

The general objective of this thesis was to explore the protein fraction of camelid milks in order to identify original molecules (peptide, proteins) potentially responsible for the properties attributed to camel milk.

The analysis of the proteins of the milk fat globule membrane (MFGM) having been the subject of an in-depth study carried out previously (Saadaoui et al., 2013), we initially committed to analyze globally the composition of the milk protein fraction of camelids (proteomic approach), focusing mainly on caseins and the molecular diversity of caseins.

Regarding WPs, various options were possible, given the originality of camel milk in this regard. Detected in mammary secretions of porcine and camel (Kappeler et al., 2004), PGRP that binds to murein peptidoglycans (PGN) of Gram-positive bacteria, was a good candidate. This pattern receptor, involved in innate immunity, may kill Gram-positive bacteria by interfering with peptidoglycan biosynthesis. Lactophorin (GlyCAM1) which is highly expressed in camel milk was also an interesting WP since this phosphoglycoprotein, a component of the milk fat globule membrane, inhibits spontaneous lipolysis in milk by the lipoprotein lipase (Girardet et al., 1993). GlyCAM1 is also suspected to be a scaffold for carbohydrates that mediate functions such as epithelium protector in addition to cell adhesion (Dowbenko et al., 1993). Given the time we had, we made choices and we focused our efforts on the whey acidic protein (WAP) whose protease inhibitory properties are well established and which is an originality of camelids (only large mammals with the pig expressing this protein in milk).

Finally, we started to isolate extracellular vesicles from milk, which are known to carry genetic information (mRNA and microRNA) and proteins involved in the communication between cells and organisms, in order to characterize their proteome.

Variations observed in camel milk composition could be attributed to several environmental factors, such as geographical locations, seasonal variations, feeding conditions, and samples being taken from different breeds, in addition to other parameters including lactation stage, age and calving number (Al haj & Al Kanhal, 2010; Konuspayeva, Faye, et al., 2009). Therefore, for this study, we collected about 180 milk samples from two camel species (*C. bactrianus* and *C. dromedarius,* and their hybrids), at different lactation stages. Camels were grazed on four various natural pastures distant for more than 3,500 kms between the regions at extreme points of Kazakhstan: Almaty (AL) at the foot of Tien Shan Mountain, Shymkent (SH) along deserts Kyzylkum and Betpak-Dala, Kyzylorda (KZ) on the edge of the steppe, and Atyrau (ZKO) at the mouth of the Caspian Sea.

These milks were submitted to different proven analytical techniques and proteomic approaches (SDS-PAGE, LC-MS/MS and LC-ESI-MS). This study also aimed to evaluate possible differences between species (genetic variability).

## 6.1 Global analysis: complexity of the camel milk proteome

To get an overview of the protein complexity of camel milk, and on its potential impact on milk characteristics, we provided a complete profiling of the milk protein fraction of Bactrian and dromedary camels from Kazakhstan, including a detailed characterization of camel CN and whey proteins, including variants related to genetic polymorphisms, splicing defects, phosphorylation levels. In addition, we introduce a reference point for further investigation on milk protein polymorphisms in the camel species. The main attractive point in searching for milk protein polymorphisms is to understand the biological significance of the genetic variations, which can be highlighted by evolutionary studies (Caroli et al., 2009). It has been already established clearly that mutations responsible for polymorphisms in milk proteins, occurring at the genomic level either alone or in combinations, might influence milk protein composition at the quantitative as well as at the qualitative levels (Martin et al., 2002).

A detailed characterization of 50 protein molecules, relating to genetic variants and isoforms arising from post-translational modifications and alternative splicing events, belonging to nine protein families (κ-, $\alpha_{s1}$-, $\alpha_{s2}$-, β-; and γ-CN, WAP, α-LAC, PGRP, CSA/LPO), was achieved by LC-ESI-MS. Against all expectations, peptides with sequence similarity with bovine *β*-lactoglobulin, the major allergen in bovine milk, were identified in the 8 camel milk samples (Bactrian, dromedary and hybrids), analyzed by LC-MS/MS. The coverage percentage ranged between 30 and 60% in individual milk samples, and reached 71% cumulating all the peptides found. Five peptides related to bovine *β*-lactoglobulin were also detected by Alhaider et al., (2013) in camel milk from Saudi Arabia and the United States. Youcef et al., (2009) revealed a weak cross reaction between dromedary WPs and IgG anti bovine *β*-lactoglobulin. Such findings disagree with the usually admitted notion that *β*-lactoglobulin is absent in camel milk (Hinz et al., 2012; Restani et al., 1999). Even though we cannot exclude a possible contamination by bovine milk (however this seems unlikely with the 8 camel milk samples analyzed by LC-MS/MS) or the presence in camel milk of a Progesterone Associated Endometrial Protein (PAEP) displaying strong similarities with *β*-lactoglobulin. However, significant similarities between human PAEP and the peptides having allowed the

identification of $\beta$-lactoglobulin in *C. bactrianus* milk, were not found. So far, camel milk has never (or only rarely) been extensively studied with the cutting-edge proteomics.

**Table 6.1.** The nine major camel milk proteins including genetic and splicing variants

| Protein | Var. | AA sequences | MW, Da | AA residues |
|---|---|---|---|---|
| **$\alpha_{s1}$-CN** | A E30 | RPKYPLRYPEVFQNEPDSIEEVLNKRKILELAVVSPIQFRQENIDELKDTRNEP<br>TEDHIMEDTERKESGSSSSEEVVSSTTEQKDILKEDMPSQRYLEELHRLNKYKL<br>LQLEAIRDQKLIPRVKLSSHPYLEQLYRINEDNHPQLGEPVKVVTQEQAYFHLE<br>PFPQFFQLGASPYVAWYYPPQVMQYIAHPSSYDTPEGIASEDGGKTDVM<br>PQWW | 25,307 | 215 |
| | | RPKYPLRYPEVFQNEPDSIEEVLNKRKILELAVVSPIQFRQENIDELKDTRNEP<br>TEDHIMEDTERKESGSSSSEEVVSSTTEQKDILKEDMPSQRYLEELHRLNKYKL<br>LQLEAIRDQKLIPRVKLSSHPYLEQLYRINEDNHPQLGEPVKVVTQPFPQFFQL<br>GASPYVAWYYPPQVMQYIAHPSSYDTPEGIASEDGGKTDVMPQWW | 24,289 | 207 |
| | C D30 | RPKYPLRYPEVFQNEPDSIEEVLNKRKILDLAVVSPIQFRQENIDELKDTRNEP<br>TEDHIMEDTERKESGSSSSEEVVSSTTEQKDILKEDMPSQRYLEELHRLNKYKL<br>LQLEAIRDQKLIPRVKLSSHPYLEQLYRINEDNHPQLGEPVKVVTQEQAYFHLE<br>PFPQFFQLGASPYVAWYYPPQVMQYIAHPSSYDTPEGIASEDGGKTDVMPQWW | 25,293 | 215 |
| | | RPKYPLRYPEVFQNEPDSIEEVLNKRKILDLAVVSPIQFRQENIDELKDTRNEP<br>TEDHIMEDTERKESGSSSSEEVVSSTTEQKDILKEDMPSQRYLEELHRLNKYKL<br>LQLEAIRDQKLIPRVKLSSHPYLEQLYRINEDNHPQLGEPVKVVTQPFPQFFQL<br>GASPYVAWYYPPQVMQYIAHPSSYDTPEGIASEDGGKTDVMPQWW | 24,275 | 207 |
| **$\alpha_{s2}$-CN** | i0 | KHEMDQGSSSEESINVSQQKFKQVKKVAIHPSKEDICSTFCEEAVRNIKEVESA<br>EVPTENKISQFYQKWKFLQYLQALHQGQIVMNPWDQGKTRAYPFIPTVNTEQLS<br>ISEESTEVPTEESTEVFTKKTELTEEEKDHQKFLNKIYQYYQTFLWPEYLKTVY<br>QYQKTMTPWNHIKRYF | 21,266 | 178 |
| | i1 | KHEMDQGSSSEESINVSQQKFKQVKKVAIHPSKEDICSTFCEEAVRNIKEVESA<br>EVPTENKISQFYQKWKFLQYLQALHQGQIVMNPWDQGKTRAYPFIPTVNTEQLS<br>ISEESTEVPTEENSKKTVDTESTEVFTKKTELTEEEKDHQKFLNKIYQYYQTFL<br>WPEYLKTVYQYQKTMTPWNHIKRYF | 22,268 | 187 |
| | i2 | KHEMDQGSSSEESINVSQQKFKQVKKVAIHPSKEDICSTFCEEAVRNIKEVESA<br>EVPTENKISQFYQKWKFLQYLQALHQGQIVMNPWDQGKTRAYPFIPTVNTEQLS<br>ISEESTEVPTEESTEVFTKKTELTEEEKDHQKFLNKIYQYYQTFLWPEYLKTVY<br>QYQKTMTPWNHIKVKAYQIIPNLRYF | 22,406 | 188 |
| **β-CN** | A<br>I186 | REKEEFKTAGEALESISSSEESITHINKQKIEKFKIEEQQQTEDEQQDKIYTFP<br>QPQSLVYSHTEPIPYPILPQNFLPPLQPAVMVPFLQPKVMDVPKTKETIIPKRK<br>EMPLLQSPVVPFTESQSLTLTDLENLHLPLPLLQSLMYQIPQPVPQTPMIPPQS<br>LLSLSQFKVLPVPQQMVPYPQRAIPVQAVLPFQEPVPDPVRGLHPVPQPLVPVI<br>A | 24,632 | 217 |
| | | TAGEALESISSSEESITHINKQKIEKFKIEEQQQTEDEQQDKIYTFPQPQSLVY<br>SHTEPIPYPILPQNFLPPLQPAVMVPFLQPKVMDVPKTKETIIPKRKEMPLLQS<br>PVVPFTESQSLTLTDLENLHLPLPLLQSLMYQIPQPVPQTPMIPPQSLLSLSQF<br>KVLPVPQQMVPYPQRAIPVQAVLPFQEPVPDPVRGLHPVPQPLVPVIA | 23,685 | 210 |
| | | RKEMPLLQSPVVPFTESQSLTLTDLENLHLPLPLLQSLMYQIPQPVPQTPMIPP<br>QSLLSLSQFKVLPVPQQMVPYPQRAIPVQAVLPFQEPVPDPVRGLHPVPQPLVP<br>VIA | 12,357 | 111 |
| | | REKEEFKTAGEALESISSSEESITHINKQKIEKFKIEEQQQTEDEQQDKIYTFP<br>QPQSLVYSHTEPIPYPILPQNFLPPLQPAVMVPFLQPKVMDVPKTKETIIPKRK | 24,650 | 217 |

| | | | | |
|---|---|---|---|---|
| | B<br>M186 | EMPLLQSPVVPFTESQSLTLTDLENLHLPLPLLQSLMYQIPQPVPQTPMIPPQS<br>LLSLSQFKVLPVPQQMVPYPQRAMPVQAVLPFQEPVPDPVRGLHPVPQPLVPVI<br>A | | |
| | | TAGEALESISSSEESITHINKQKIEKFKIEEQQQTEDEQQDKIYTFPQPQSLVY<br>SHTEPIPYPILPQNFLPPLQPAVMVPFLQPKVMDVPKTKETIIPKRKEMPLLQS<br>PVVPFTESQSLTLTDLENLHLPLPLLQSLMYQIPQPVPQTPMIPPQSLLSLSQF<br>KVLPVPQQMVPYPQRAMPVQAVLPFQEPVPDPVRGLHPVPQPLVPVIA | 23,703 | 210 |
| | | RKEMPLLQSPVVPFTESQSLTLTDLENLHLPLPLLQSLMYQIPQPVPQTPMIPP<br>QSLLSLSQFKVLPVPQQMVPYPQRAMPVQAVLPFQEPVPDPVRGLHPVPQPLVP<br>VIA | 12,375 | 111 |
| **κ-CN** | A<br>F11 | EVQNQEQPTCFEKVERLLNEKTVKYFPIQFVQSRYPSYGINYYQHRLAVPINNQ<br>FIPYPNYAKPVAIRLHAQIPQCQALPNIDPPTVERRPRPRPSFIAIPPKKTQDK<br>TVNPAINTVATVEPPVIPTAEPAVNTVVIAEASSEFITTSTPETTTVQITSTEI | 18,254 | 162 |
| | B<br>C11 | EVQNQEQPTCCEKVERLLNEKTVKYFPIQFVQSRYPSYGINYYQHRLAVPINNQ<br>FIPYPNYAKPVAIRLHAQIPQCQALPNIDPPTVERRPRPRPSFIAIPPKKTQDK<br>TVNPAINTVATVEPPVIPTAEPAVNTVVIAEASSEFITTSTPETTTVQITSTEI | 18,210 | 162 |
| | C<br>C11,<br>I111,<br>V131 | EVQNQEQPTCCEKVERLLNEKTVKYFPIQFVQSRYPSYGINYYQHRLAVPINNQ<br>FIPYPNYAKPVAIRLHAQIPQCQALPNIDPPTVERRPRPRPSFIAIPPKKTQDK<br>TVIPAINTVATVEPPVIPTAEPVVNTVVIAEASSEFITTSTPETTTVQITSTEI | 18,236 | 162 |
| **α-LAC** | | KQFTKCKLSDELKDMNGHGGITLAEWICIIFHMSGYDTETVVSNNGNREYGLFQ<br>INNKIWCRDNENLQSRNICDISCDKFLDDDLTDDKMCAKKILDKEGIDYWLAHK<br>PLCSEKLEQWQCEKW | 14,430 | 123 |
| **GlyCAM1** | | SLNEPKDEIYMESQPTDTSAQVIMSNHQVSSEDLSMEPSISREDLVSKDDVVIK<br>SARRHQNQNPKLLHPVPQESSFRNTATQSEETKELTPGAATTLEGKLVELTHKI<br>IKNLENTMRETMDFLKSLFPHASEVVKPQ | 15,442 | 137 |
| **LTF** | | ASKKSVRWCTTSPAESSKCAQWQRRMKKVRGPSVTCVKKTSRFECIQAISTEKA<br>DAVTLDGGLVYDAGLDPYKLRPIAAEVYGTENNPQTHYYAVAIAKKGTNFQLNQ<br>LQGLKSCHTGLGRSAGWNIPMGLLRPFLDWTGPPEPLQKAVAKFFSASCVPCVD<br>GKEYPNLCQLCAGTGENKCACSSQEPYFGYSGAFKCLQDGAGDVAFVKDSTVFE<br>SLPAKADRDQYELLCPNNTRKPVDAFQECHLARVPSHAVVARSVNGKEDLIWKL<br>LVKAQEKFGRGKPSGFQLFGSPAGQKDLLFKDSALGLLRISSKIDSGLYLGSNY<br>ITAIRGLRETAAEVELRRAQVVWCAVGSDEQLKCQEWSRQSNQSVVCATASTTE<br>DCIALVLKGEADALSLDGGYIYIAGKCGLVPVLAESQQSPESSGLDCVHRPVKG<br>YLAVAVVRKANDKITWNSLRGKKSCHTAVDRTAGWNIPMGLLSKNTDSCRFDEF<br>LSQSCAPGSDPRSKLCALCAGNEEGQNKCVPNSSERYYGYTGAFRCLAENVGDV<br>AFVKDVTVLDNTDGKNTEQWAKDLKLGDFELLCLNGTRKPVTEAESCHLAVAPN<br>HAVVSRIDKVAHLEQVLLRQQAHFGRNGRDCPGKFCLFQSKTKNLLFNDNTECL<br>AKLQGKTTYEEYLGPQYVTAIAKLRRCSTSPLLEACAFLMR | 75,250 | 689 |
| **WAP** | A V12 | LAPALSLPGQAVCPELSSSEDNACIISCVNDESCPQGTKCCARSPCSRSCTVPL<br>MVSSPEPVLKDGRCPWVQTPLTAKHCLEKNDCSRDDQCEGNKKCCFSSCAMRCL<br>DPVTEDSFQ | 12,564 | 117 |
| | B<br>M12 | LAPALSLPGQAMCPELSSSEDNACIISCVNDESCPQGTKCCARSPCSRSCTVPL<br>MVSSPEPVLKDGRCPWVQTPLTAKHCLEKNDCSRDDQCEGNKKCCFSSCAMRCL<br>DPVTEDSFQ | 12,596 | 117 |
| **PGRP** | | REDPPACGSIVPRREWRALASECRERLTRPVRYVVVSHTAGSHCDTPASCAQQA<br>QNVQSYHVRNLGWCDVGYNFLIGEDGLVYEGRGWNIKGAHAGPTWNPISIGISF<br>MGNYMNRVPPPRALRAAQNLLACGVALGALRSNYEVKGHRDVQPTLSPGDRLYE<br>IIQTWSHYRA | 19,143 | 172 |

Amino acid sequences of mature proteins with potential phosphorylation sites. Potential phosphorylation sites are bolded: Seryl and Threonyl residues matching the S/T-X-A motif are in red and blue, respectively. Threonyl residues matching the S/T-X-X-A motif are in green. Aa residues generated by genetic polymorphism and alternative splicing are marked bold in red and highlighted in yellow.

## 6.2 Complexity of the "casein" fraction: the case of $\alpha_{s2}$-CN and potential impact in terms of function

Up to now, the composition of camel casein fraction appeared to be relatively well established. However, analyzing milk of camelids originating from Kazakhstan, both in *C. dromedarius*, *C. bactrianus* and their hybrids, differences, lead us to consider more subtle composition though having obvious consequences at the technological and nutritional levels. Indeed, a great diversity of molecular species, originating in genetic variants, post-translational modifications but also in the processing of primary transcripts (splicing variants), was highlighted. This situation is particularly conspicuous regarding $\alpha_{s2}$-CN for which three splicing variants were identified, including exon skipping and cryptic splice site usage. Camel $\alpha_{s2}$-CN was shown to be a mixture of three splicing isoforms differing in polypeptide chain length. Isoform i0, initially reported in the literature, was the main isoform of $\alpha_{s2}$-CN. Isoforms i1 and i2 were splicing isoforms of $\alpha_{s2}$-CN arising from alternative processing of primary transcript and differing from i0 with the insertion of exon 13 (ENSKKTVDT) in i1 and an extension of exon 16 (VKAYQIIPNL) in i2, with phosphorylation levels for each of them ranging between between 7 and 12 Phosphate groups.

With 11 potentially phosphorylated aa residues matching the S/T-X-A motif, camel $\alpha_{s2}$-CN displays the highest phosphorylation level of camel caseins. To reach such a phosphorylation level, besides the nine SerP, two putative ThrP (T118 and T132) have to be phosphorylated. In all the Kazakh milk samples analyzed in LC-ESI-MS we found $\alpha_{s2}$-CN with 12 P groups, as the molecular mass of 22,226 Da observed corresponds to the mass of the peptide backbone (21,266 Da) increased by 960 Da, a mass increment which coincides with 12 P groups. That means that at least another S/T residue that does not match with the canonic sequence (S/T-X-A) recognized by the mammary kinase(s), is potentially phosphorylated.

In the camel $\alpha_{S2}$-CN, two threonine residues (T39 and T129) are located in a motif S/T-X-X-E/D/pS, which is a recognition motif for CN-kinase II (CK2). Indeed, albeit the consensus sequences of CK2 and the genuine casein kinase isolated from the Golgi apparatus of the lactating mammary gland (G-CK) are definitely distinct, they could be similar and sometimes overlapping (Tibaldi et al., 2015). The hypothesis that there are two different phosphorylation systems (kinases) suggested by Bijl et al., (2014) and then by Fang et al., (2016) takes therefore a little bit more consistency. This warrants further investigation. Fam20C which seems to be the major secretory pathway protein kinase (Tagliabracci et al., 2015) is very likely responsible

for the phosphorylation of S and T residues within S/T-X-A motifs, whereas at least one T residue occurring in a S/T-X-X-A motif (T39 or T139) might be phosphorylated by aCK2-type kinase.

Such results provide useful novel information for the understanding of the evolution of the casein genes and their expression across Mammals. With the growing number of genes encoding milk proteins sequenced and displaying complex patterns of splicing, thus increasing the coding capacity of genes, the extreme protein isoform diversity generated from a single gene can no longer be considered as an epiphenomenon. Is it a fortuitous or a scheduled event to expand molecular diversity of milk caseins? Structural diversity and variability in expression level are both responsible for modifications in the organization and, consequently, changes in the physico-chemical properties of the casein micelle. A parsimonious vision of this issue addresses a major question: does this convey any biological significance? It has been established, that milk proteins represent a reservoir of biologically active peptides, capable of modulating different functions; the molecular diversity generated by differential splicing mechanisms can only increase their content. Thus, alternative splicing events produce novel potentially bioactive peptides. Important new insights are expected, in this field, in the near future.

## 6.3 WAP: originality of the protein and of the gene in the camel species

Camel WAP is a 117 aa residues protein (136 aa residues for the pre-protein) that shows the higher sequence identity at the aa level (76%) to porcine WAP (113 aa residues). It contains five potential phosphorylation sites per molecule (S17, S18, S19, S58, and S87), whereas the rat WAP (118 aa) and the rabbit WAP (108 aa) have only three and two potential phosphorylation sites, respectively. It was reported, that mouse WAP (115 aa) is apparently not phosphorylated (Hennighausen & Sippel, 1982). From mass data (LC-MS) it appears that only one serine can be phosphorylated. Given the extremely constrained and compact structure of the molecule with 8 S-S bridges, essential for folding and functionality of the protein, we hypothesized that S58 which is located within the additional sequence connecting the two 4-DSC domains, is the seryl residue which is alternatively (*ca.* 50%) phosphorylated in camel.
The comparison of camel WAP sequence with that of the other 5 eutherian species in which the WAP gene is expressed (pig, dog, rabbit, rat and mouse), displays an insertion of 4 aa residues (56VSSP59) which extend the sequence inter 4-DSC domains. From the *Camel dromedarius*

gene sequence (GenBank 105095719) this appears to be the consequence of the usage of an unlikely intron cryptic splice site extending camel exon 3 on its 5' side by 12-nucleotides, whereas in the other 5 species the canonic 3' end of intron 2 is used. There are actually two potential intron donor splice sites responding to all requirements of splicing recognition signal: CCCGGCCAG|TCTCTTCCCCAG|AGCCTGTCCTG, and paradoxically it is the weakest site, a polypyrimidic stretch interrupted by a GG doublet, which seems to be preferentially used by the splicing machinery. Indeed, the existence of a non-allelic short isoform of camel WAP, encoded by a shorter mRNA arising from the usage, as in the other species, of the canonic 3' splice site seems plausible. This assumption was supported by the occurrence of two tryptic peptides (SCTVPLMVSSPEPVLK and SCTVPLMEPVLK) identifying camel WAP in LC-MS/MS that differentiate by the presence or absence of the tetrapeptide 56VSSP59. Such a result confirms that the usage of a cryptic splice site during the splicing of precursors to WAP mRNA is responsible for the insertion of 4 amino acid residues between the two 4-DSC domains of the camel WAP.

However, this is not the only originality of this gene that exhibits in the camel species, extremely rare fact, an intron (intron 3) of the GC-AG type. The existence of variants to the standard (canonical) GT-AG introns is known but extremely rare (Burge et al., 1999; Burset, 2000). Burset and co-workers (2001) observed that GC splice sites account for 0.5% of annotate donor sites and that GC donor sites possess a strong consensus sequence. Since the maturation process is ensured by the same splicing machinery (U2-type spliceosome), whatever the intron is, GT-AG or GC-AG type, there is a mismatch between the donor site sequence and the U1 snRNA. To compensate for such a weakening base pairing, the consensus sequence at the GC donor site has strengthened. It should be noted that such a rare event led to an erroneous annotation of this gene in genome database, since automated algorithms based on consensus sequences are used to predict donor and acceptor sites of introns.

## 6.4 EVs: Beyond their role in the communication between cells, what possible effects on the consumer (newborn or adult humans)

Using an optimized isolation protocol, we obtained milk-derived EVs from camel milk samples that satisfied the typical requirements for "exosomal" morphology, size and protein content. Thus, we provide, to our knowledge, a first comprehensive proteome of camel milk-derived exosomes that appears, with *ca*. one thousand different proteins identified, wider than the camel milk proteome (391 functional groups of proteins). As previously mentioned, camel

milk-derived exosomes contain proteins, such as lactadherin/MFG-E8, Ras-related proteins or CD9 also present in MFG (Saadaoui et al., 2013). In addition, our results strongly suggest that EVs isolated from camel milks have different cellular origin, since besides EVs originating from mammary epithelial cells there are very likely EVs from immune cells.

Théry et al., (2002) established specific markers of the EVs (membrane and cytosolic proteins) and a specific subset of cellular proteins targeted specifically to exosomes, of which the function still remain unknown. This is particularly interesting in relation to their possible involvement in human diseases. Therefore, the knowledge of exosome proteomics can help not only in understanding their biological roles but also in supplying new biomarkers (Raimondo et al., 2011).

If we consider that milk-derived EVs also carry microRNAs, these vesicles have to be recognized as another important bioactive component of milk that might be involved in the transfer of immune components 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. Several publications suggest that EVs in foods, specifically bovine and human milk, carry a wide range of compounds with biological activities such as, lipids, proteins, noncoding RNAs (including microRNAs), and mRNAs. Exosomes seem to be a particularly important class of EVs since they protect labile cargos against degradation and provide a vehicle for cargo uptake through endocytosis of exosomes in virtually all tissues (Benmoussa et al., 2016; Gu et al., 2012; Izumi et al., 2012; Izumi et al., 2015; Raposo & Stoorvogel, 2013; Wolf et al., 2015; Zempleni et al., 2017).

Humans of all ages consume worldwide milk from various sources and since microRNAs are highly conserved across mammals, one can expect that microRNA from milk-derived EVs may mediate the beneficial effects of dairy milk consumption in rheumatoid arthritis (Arntz et al., 2015) or in immune functions (Melnik et al., 2014). This is probably one of the greatest challenges facing milk science in the immediate future: to provide the food industry and consumers with the basis for health-promoting properties before their inclusion as ingredients into functional foods (Martin et al., 2013).

## 6.5 What should be implemented now?

One of the actions that should now be implemented as a priority would be to profile the RNA content (mRNA and microRNA) of extracellular vesicles isolated from camelids milk (Bactrian and camel) so that such profiles can be compared, in particular in microRNAs, with

those produced from milks of other species including large and small ruminants. This requires the implementation of functional tests on animal models in vivo and/or ex vivo (cell cultures) to evaluate potential effects of the content of EVs from camelids milk and more generally milk on the consumers' physiology. As example, we can mention the case of a polyarthritis model in mice, used by Arntz et al., (2015) to highlight attenuation effects of bovine milk-derived EVs. We can still quote the work of Chen et al., (2016) who reported that porcine milk-derived exosomes can facilitate intestinal cell proliferation and intestinal tract development, thus giving a new insight for milk nutrition and newborn development and health.

Regarding the beneficial properties of camel milk, among them its antimicrobial activity, no doubt that it can be attributed to the high content of protective proteins such as PGRP-1 and possibly WAP. This protein first described as an anti-protease able to limit tissue damage during inflammation, displays in fact a variety of other functions, including direct antimicrobial activity. However, several studies (Alvarez-Ordóñez et al., 2013; Farrell et al., 2009; McCann et al., 2005; Recio & Visser, 1999; Zucht et al., 1995) have demonstrated the antibacterial properties of bovine peptides derived from the C-terminal part of $\alpha_{s2}$-CN. Given the possible extension of the repertoire of bioactive peptides of milk camelids in connection with splicing variants arising from camel $\alpha_{s2}$-CN precursors to mRNA, it would be interesting now to test biological activities and potentially the health value of peptides derived from camel $\alpha_{s2}$-CN.

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# Acknowledgments

# Curriculum vitae

Alma Ryskaliyeva was born on 30 August in Uralsk, Kazakhstan. In 2009, she obtained B.Sc. and, in 2011, Master degree in chemical technology of organic compounds from Al-Farabi Kazakh State National University, in Almaty, Kazakhstan. She did research on exploring the terpenoid content of *Mosses* growing in Kazakhstan with emphasis on medical properties. During her master, in 2010, she undertook an internship in Vorozhtsov Novosibirsk Institute of Organic Chemistry SB RAS, in Novosibirsk, Russia. In 2014, she was awarded a Bolashak International Scholarship established by President of the Republic of Kazakhstan and enrolled in the PhD project at AgroParisTech - Université Paris-Saclay - INRA. Her research focused on the exploring the fine composition of *Camelus* milk from Kazakhstan with emphasis on protective components, and the results are presented in this thesis.

# List of Publications

## Peer-reviewed publications

**Ryskaliyeva, A.,** Henry, C., Miranda, G., Faye, B., Konuspayeva, G., and Martin P. 2018. Alternative splicing a fortuitous or a scheduled event to expand molecular diversity of camel CSN1S2 and increase its bioactive potentiality. *Scientific Reports. (Submitted).*

**Ryskaliyeva, A.,** Henry, C., Miranda, G., Faye, B., Konuspayeva, G., and Martin P. 2018. Combining different proteomic approaches to resolve complexity of the milk protein fraction of dromedary, Bactrian camels and hybrids, from different regions of Kazakhstan. *PLOS ONE, 13(5).* https://doi.org/10.1371/journal.pone.0197026

## Manuscript in preparation

**Ryskaliyeva, A.,** Henry, C., Miranda, G., Faye, B., Konuspayeva, G., and Martin P. 2018. Characterization of multiple protein isoforms arising from the usage of a cryptic splice site in camel WAP precursors to mRNA in which a GC-AG intron occurs

**Ryskaliyeva, A.,** Krupova, Z., Henry, C., Miranda, G., Faye, B., Konuspayeva, G., and Martin P. 2018. Comprehensive Proteomic Analysis of Camel Milk-derived Extracellular Vesicles.

## Conference proceedings

**Ryskaliyeva, A.,** Miranda, G., Henry, C., Faye B., Konuspayeva G. and Martin P. 2017. Alternative splicing a fortuitous or a scheduled event to expand molecular diversity of milk proteins: Camel CSN1S2, a relevant model to try to provide some response elements. Student Travel Award Winner in: 14th International Symposium on Milk Genomics and Human Health 2017, 26-28 September, Quebec, Canada.

**Ryskaliyeva, A.,** Henry, C., Miranda, G., Faye, B., Konuspayeva, G., and Martin, P. 2016. Proteomic analysis of Camelus milks from Kazakhstan. In: The ICAR 2016 Satellite Meeting on Camelid Reproduction, 1st-3rd July, Tours, France.

# Individual and Training Supervision Plan

## Dissemination of Knowledge

### *International conferences*

| | |
|---|---|
| 14th International Symposium on Milk Genomics and Human Health, Quebec, Canada (oral presentation) | 2017 |
| The FSEV Annual Meeting on Extracellular Vesicles, Paris, France | 2017 |
| The ICAR Satellite Meeting on Camelid Reproduction, Tours, France (oral presentation) | 2016 |

### *Seminars and Workshops*

| | |
|---|---|
| Workshop Technique of International Symposium on Microgenomics, 31 May- 1 June, Jouy-en-Josas, France | 2016 |
| Annual meeting of the doctoral candidates of the Animal Genetics Division of INRA, Toulouse, France (oral presentation) | 2016 |
| Professionnal PhD networking forum of AgroParisTech & ABIES doctoral school | 2016 |
| Annual meeting of the doctoral candidates of the Animal Genetics Division of INRA, La Rochelle, France (poster presentation) | 2015 |
| Professionnal PhD networking forum of AgroParisTech & ABIES doctoral school | 2015 |
| My professional project in 180 seconds (oral presentation) | 2015 |

## Professional Skills Support Courses

| | |
|---|---|
| Introduction to Research Ethics and Scientific Integrity | 2018 |
| Write Right – Writing and structuring of the scientific articles, Paris, France | 2015 |
| Building Your Base, Paris, France | 2014 |
| French as a foreign language, Jouy-en-Josas, France | 2014-2016 |

Complied with the educational requirements set by the Graduate School of Agriculture, Food, Biology, Environment and Health of the Agricultural, Veterinary and Forest Institute of France