Casein composition and differential translational efficiency of casein transcripts in donkey's milk

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Abstract

The amount of the four caseins (α_{s1}, α_{s2}, β and κ-CN) in donkey milk was evaluated by Urea-PAGE analysis at pH 8.6, followed by immuno-detection with polyclonal antibodies, coupled to densitometric analysis. The results showed the percentage of each casein in decreasing order: β (54.28) > α_{s1} (35.59) > α_{s2} (7.19) > κ-CN (2.79). The mRNA quantification of donkey casein transcripts, carried out by RT-qPCR, showed that the average percentage of corresponding gene transcripts (*CSN2, CSN1S1*, *CSN1S2* I and *CSN3*) was 70.85, 6.28, 14.23 and 8.65, respectively. The observed translation efficiency, assessed as percentage of single milk casein fraction out of single percentage of transcript, was 0.76, 5.66, 0.50 and 0.32, respectively. The analysis of the sequences flanking the start codon, the codon usage frequencies and the coding sequence length might explain, at least in part, the differential transcriptional and translational rate observed among the casein transcripts.

Keywords: Donkey, casein, mRNA, quantification

In recent years donkey's milk (DM) has attracted an increasing interest in human nutrition, since it may represent the best natural substitute of cow's milk for children affected by milk protein allergy, a condition of increasing incidence (Businco et al. 2000; Monti et al. 2012; Cunsolo et al. 2017). Allergic manifestations to DM are rare and, to date, only one case of work-related DM allergy has been documented (Giorgis et al. 2018). DM may be considered a valid alternative to powdered milks, soybean milk replacement or other formulas employed in the diet therapy of these patients. The reason lies in the low casein content and in the ratio casein to whey protein that is closer to human milk than what observed in ruminant milk (Guo et al. 2007). Recently, the presence of all four casein fractions α_{s1}, β, α_{s2} and κ-CN was demonstrated in donkey's milk (Chianese et al. 2010), as well as in the horse (Ochirkhuyag et al. 2000) and pony (Miranda et al 2004). The proteomic approach has also allowed characterization of the casein compositional heterogeneity due to post-translational modifications, like phosphorylation (α_{s1}, α_{s2}

and β-CN), glycosylation (κ-CN) and non-allelic forms generated by RNA incorrect splicing (α_{s1} and β-CN) (Cunsolo et al. 2009a; Cunsolo et al. 2009b; Chianese et al. 2010). In particular, the complete primary structure of α_{s1}-casein (202 amino acids, Cunsolo et al. 2009a), β (226 amino acids, Cunsolo et al. 2009b) and α_{s2} (221 amino acids, Chianese et al. 2010) have been determined. Moreover, the complete sequences of the genes encoding for the β- (*CSN2*, EMBL No. FN598778), α_{s1}- (*CSN1S1*, EMBL No. FN386610) and κ-casein (*CSN3*, Hobor et al. 2008; FR822990) and the related promoter regions have been determined.

Similarly, two different donkey αs2 encoding genes (*CSN1S2* I and *CSN1S2* II) have been identified (Cosenza et al. 2010). The first, spanning over a fragment of 1016 nt, is constituted by 19 exons and it encodes for the protein of 221 amino acids (called α_{s2}-I) also characterized by Chianese et al. (2010); the second, constituted by 16 exons, probably originated by gene duplication, encodes for a predicted peptide (named α_{s2}-II) of 168 amino acids (Cosenza et al. 2010), not yet detected at proteomic level. Studies on the genetic polymorphism of DM are limited when compared to those carried out in the major dairy species, and it is only recently that researchers have paid particular attention to the proteomic and genomic characterization of proteins in DM. In particular, Criscione et al. (2009) have identified an individual DM sample lacking α_{s1}-casein, like in goats, known as the species expressing the highest genetic variability for this casein fraction (Cosenza et al. 2008). In addition, Chianese et al. (2010) have characterized a genetic variant of β-casein having a molecular weight value 28 mass units higher than the common β-CN phenotype. Finally, regarding the *CSN3* and *CSN1S2* I genes, the analysis of nucleotide sequences has allowed the identification of several silent and missense polymorphisms (Hobor et al. 2008; Cosenza et al. 2010). On the contrary, no studies have been carried out on the expression of casein genes in the donkeys, as well as on their translational efficiency, whereas cattle, sheep, goat (Bevilacqua et al. 2006), buffalo (Cosenza et al. 2011) and yak (Bai et al. 2013) data have been reported.

The hypothesis of our study was that in donkey, similarly to what is observed in ruminants, a significant difference in the translation efficiency characterises the genes encoding the four caseins. In

order to verify such hypothesis, we evaluated the expression of the four casein fractions in DM taking into account the phenotypic and genotypic aspects. The protein quantification of α_{s1}, α_{s2}, β and κ-CN was carried out by means of electrophoresis at alkaline pH and immunoblotting with polyclonal antibodies coupled to densitometry analysis. The quantitative determination of the four casein mRNAs was assessed by RT-qPCR and their translation efficiency was estimated through the percentage ratio of single milk casein fractions/single percentage of transcripts.

Materials and methods

Donkey milk sampling and casein extraction

Individual milk samples from 8 donkeys of Martina Franca breed were collected in the same farm (Aquila, Italy). Martina Franca are large-sized donkeys that originated in the Apulia region in the South-East of Italy. In the past, the Martina Franca donkey breed has been considered useful for the production of hybrids. Currently in Italy, the breed is used mainly in an amateur context, although different potential uses (recreational, pet therapy, meat and milk production) are developing. The maximum milk yield per milking corresponds to 700 grams (approximately 1.4 L) and regarding milk composition (g/100 g), the maximum values are 0.97 for fat, 1.67 for protein, 6.87 for lactose and 9.05 for SCC (x 1000 cells/mL) (D'Alessandro et al. 2009). All donkeys were free of clinical mastitis and were comparable for age (about 6 years old), lactation and parity order. Each casein sample was prepared by acid precipitation from skimmed milk, as described by Aschaffenburg & Drewry (1959).

Quantitative determination of the nitrogen fractions (TN, SN,CN, NPN) in donkey milk

The total nitrogen in DM was determined by Kjeldahl method according to the IDF Method (1993). A nitrogen protein conversion factor of 6.38 was used in all cases. All samples were analyzed in triplicate and results presented as means ± standard deviations.

Urea polyacrylamide gel electrophoresis (Urea-PAGE) at pH 8.6 and immunoblotting analysis

Urea- PAGE at pH 8.6 and the immunoblotting analysis were carried out according to the procedure described by Chianese et al. (2009), using polyclonal antibodies against bovine peptides α_{s1}-CN (187-199) and β-CN (195-199) and porcine κ and α_{s2}-CN. Each casein fraction were analyzed from the Coomassie blue stained gel pattern by scanning with an Ultroscan XL enhanced laser densitometer equipped with the software supplied by the manufacturer (Amersham Biosciences AB, Uppsala, Sweden). Chemicals, the distribution of nitrogenous components, sample preparation and conditions of the immunoelectrophoresis analysis were reported in supplementary materials.

RNA analysis

Total RNA was isolated from somatic cells present in the eight representative fresh milk samples using Nucleospin Blood and NucleoSpin® Extract Kits (Macherey-Nagel). The quantity, quality, purity and integrity of RNA, after DNase treatment, were estimated by means of Thermo Scientific NanoDrop 2000c and by electrophoresis on a denaturing agarose gel. Reverse-Transcription reaction mix, quantitative PCR amplification mix, thermal condition and primers sequnces are reported in online Supplementary Methods and supplementary table S1.

Results and discussion

Quantitative analysis of the nitrogen fractions (TN, SN, CN, NPN) in donkeys' milk

In the individual donkey milks analysed, the average protein content was 1.48% ± 0.2, ranging between 1.10% and 1.81% (Supplementary Table S2) consistent with data reported by Salimei et al. (2004) and Guo et al. (2007). In particular, the average content of caseins (34.61%) and whey proteins (49.80%), with a casein to whey proteins ratio of 0.69, showed remarkable differences in comparison with bovine and other ruminant milks but were within the range of donkey's milk variability, reported in literature (Salimei et al. 2004; Guo et al. 2007). The one exception was CN content being lower than that reported by Guo et al. (2007) for Chinese donkey milk. The high NPN content (15.55%) was very close to that of

125 human and mare's milk (Malacarne et al. 2002). The nutritional and biological significance of this milk
126 fraction is still far from being completely understood, but it seems to be related to the development of the
127 infant (Lonnerdal, 1994). It has been suggested that the high amount of whey protein (49.81%) in
128 donkey's milk, similar to mare's milk, may make it more favourable for human nutrition than cow's milk,
129 because of the relatively higher acute postprandial availability of essential amino acids.
130
131 *Qualitative and quantitative characterization of donkey's caseins by Urea-PAGE at pH 8.6,*
132 *immunoblotting and densitometry analysis*
133 The individual casein samples analysed by Urea-PAGE at pH 8.6 and shown in Fig. 1, were stained with
134 either Coomassie Brilliant Blue (CBB) or specific polyclonal antibodies against α_{s2}, α_{s1}, β and κ-CN to
135 identify each casein fraction in the electrophoretic pattern. In the Urea-PAGE profiles, at least three
136 components exhibiting the highest mobility toward the anode and migrating head α_{s1}-CN were detected
137 as α_{s2}-CN after immunoblotting; each component accounted for 10, 11 and 12 P/mole as previously
138 reported (Chianese et al. 2010). The α_{s1}-CN fraction showed a complex heterogeneity, after
139 immunostaining with specific antibodies, since five main components were identified as α_{s1}-CN,
140 exhibiting an intermediate anodic mobility between donkey β- and α_{s2}-CN. The compositional
141 heterogeneity of donkey α_{s1}-CN could be due to different phosphorylation degree of its components as
142 well as the presence of deleted forms (Cunsolo et al. 2009a), as in mare counterparts (Miranda et al. 2004;
143 Mateos et al. 2009) as well as in ruminants (Martin et al., 2003). After immunodetection the β-CN was
144 constituted of two/three main components, differing for the phosphorylation degree (5, 6 and 7 P/mole)
145 (Chianese et al. 2010), as found in mare's milk also (Girardet et al. 2006).
146 The electrophoretic profiles stained with CBB were quantitatively evaluated by densitometric
147 analysis. Taking into account the high intensity of electrophoretic bands, the donkey β-CN may be the
148 most abundant casein fraction. Finally, the CBB stained bands, characterised by a lower negative charge
149 than β-CN, were identified after immunoblotting as κ-CN, without overlapping with the other casein

fractions. It is known that κ-CN components exhibited a weak intensity to CBB, both owing to the poor susceptibility of this fraction to staining and low content in the casein micelle.

After densitometric analysis, β-CN was by far the most abundant casein fraction (54.28% ± 5.68), followed by α_{s1}-CN (35.59% ± 5.06), a composition certainly closer to that of human than cow's milk. This latter, in fact, is rich in α_{s1} and α_{s2}-caseins, that are lacking or present in traces in breastmilk. The allergenic advantage of non-bovine milks, such as goat's and now donkey's milk, might be attributed to this difference (Bevilacqua et al. 2001). The amounts of α_{s2}-CN (7.19% ± 2.55) and κ-CN (2.79% ± 0.85) were the lowest among casein fractions. However, it is well known that these latter casein fractions represent the minor components also in the horse (Miranda et al. 2004). In Table 1, the percentage and relative amounts of each casein fraction in donkey were reported in comparison with pony horse, goat, yak, cattle, buffalo and camel milk.

Compared with ruminants' milk, the relatively low level of caseins observed in DM coupled with the low protein content may be responsible for the soft curd produced in the stomach. For example, a similar condition was observed also in goat carriers of defective alleles. Goat milk lacking the α_{s1}-CN has poor coagulation properties in comparison with milk containing α_{s1}-CN, and it also decreases intestinal and systemic sensitization to β-lactoglobulin in guinea pigs (Bevilacqua et al. 2001).

Although with different values, the trend of the casein fraction content in donkey ($\beta > \alpha_{s1} > \alpha_{s2} > \kappa$) is similar to that observed for camel (Kappeler et al. 1998), but different from those observed for horse, yak and goat ($\beta > \alpha_{s1} > \kappa > \alpha_{s2}$) (Miranda et al. 2004; Bevilacqua et al. 2006; Bai et al. 2013), cattle ($\beta = \alpha_{s1} > \alpha_{s2} > \kappa$) (Miranda et al. 2004) and buffalo ($\beta > \alpha_{s2} > \alpha_{s1} > k$) (Cosenza et al. 2011).

These data confirm that the casein-type composition (as well as the protein/fat ratio) is different in most dairy animals, and the physicochemical properties of the milk depend on it, both contributing to the functionality of milk and playing an important role in cheese making (Roncada et al. 2012). It is well-known that the different proportion of casein fractions, besides genetic variants and post-translational

modifications of caseins family, directly affect the conformation and the sizes of the micelles in the milk from different dairy animals and, consequently the technological properties.

Transcripts quantification and translation efficiency

In order to quantify the mRNA transcribed from the casein genes of eight lactating donkeys, we used a RT-qPCR approach using the 18S rRNA as housekeeping gene and a standard curve for a complete quantification of transcripts. The obtained results show that the average percentage of donkey casein transcripts were 6.28, 70.85, 14.23 and 8.65 for *CSN1S1*, *CSN2*, *CSN1S2* I and *CSN3*, respectively (Table 2). These values are somewhat different from that observed for the transcripts of homologous genes in buffalo species from Cosenza et al. (2011), in yak (Bai et al. 2013) and in cattle, goat and sheep (Bevilacqua et al. 2006). In particular, for the latter four species each casein transcript represents nearly 20-30% of the whole casein transcript population, while the incidence rate of buffalo *CSN1S1*, *CSN1S2* transcripts are higher than those observed in the donkey (Table 2).

In order to evaluate the translation efficiency of the donkey gene casein transcripts, the ratio between the percentage of single milk casein fractions and the single percentage of transcripts produced in the milk somatic cells has been estimated.

The values obtained show a low translation efficiency for the *CSN1S2 I* (0.50), *CSN3* (0.32) and *CSN2* (0.76) transcripts, whereas much higher efficiency (5.66) was found for the *CSN1S1*. The trend of donkey casein translation efficiency is almost similar to that observed by Bai et al. (2013) for the yak (0.30, 0.6, 1.5 and 1.8 for *CSN1S2*, *CSN3*, *CSN2* and *CSN1S1*, respectively) and for cattle, goat and sheep by Bevilacqua et al. (2006). In particular, for the latter species β- and αs1- casein mRNA showed the highest translational efficiency, with ratio values 2.5- to 4-fold over the values recorded for αs2- and κ-casein transcripts (Bevilacqua et al. 2006). These results differ from those obtained in river buffalo, where *CSN3* (2.69), *CSN2* (2.39) and *CSN1S1* (1.31) are characterized by a higher translation efficiency, while *CSN1S2* showed the lowest value (0.25) (Cosenza et al. 2011).

The molecular mechanisms responsible for the observed differences in the individual transcript efficiency can be different. Each mRNA is represented by various sequence-derived and functional features related to translation. In order to investigate whether the mRNA sequences might be responsible for the observed differences, a comparison of nucleotide sequences with the Kozak consensus sequence (GCCA/GCCAUGG) was accomplished. Kozak consensus sequence is an element highly conserved in the eukaryotic genomes, which represents the most efficient context for the correct translation initiation (Kozak, 1994). In particular, more the sequence around the initiation codon is homologous to the Kozak sequence (i.e., "strong" consensus), higher should be the efficiency of mRNA translation (Kozak, 1984). The sequence comparison of the four casein transcripts in donkey (Table 3) showed for the *CSN2*, *CSN1S2* I and *CSN3* mRNAs the highest homology with the Kozak sequence. In particular, *CSN2* is characterized by four conservative nucleotides (-5, -3, -2 and -1) directly upstream of the initiation (nucleotide 'A' in AUG is numbered +1 and the number increases further downstream). Three of them (-3, -2 and -1) are consecutive residues, similar to *CSN1S2* I, while *CSN3* is characterized by a tandem conservative nucleotides (-2, -3 and -5, -6). On the contrary, *CSN1S1* showed the worst combination. Despite three nucleotides match with the consensus sequence, these are not consecutive (-5, -3 and -1) and, therefore, it can be considered as a "weak" context (Table 3).

These observations are, apparently, in contradiction with the values obtained for the efficiency of translation. However, it is worth noting that donkey *CSN2*, *CSN1S2* I and *CSN3* are each characterized by a single nucleotide substitution with respect to the canonic Kozak sequence, such as the G→T in position -6 for *CSN2*, G→A in position -6 and C→T in position -5 for *CSN1S2* I and C→G in position -1 for *CSN3* (Table 3). Different studies demonstrated that mutations in these positions of the Kozak consensus site decreased the efficiency of translation, thus confirming the hypothesized key role of the nucleotides -6, -5 and -1 in the optimization of the translation process (Afshar-Kharghan et al. 1999; Usuki & Maruyama, 2000; De Angioletti et al. 2004). For example, the G localized in position -6 with respect to the AUG, is present in 44% of the 699 vertebrate mRNA sequences analyzed (Kozak, 1987). This high conservation suggests that the G at position -6 is also important in the initiation of translation (De

225 Angioletti et al. 2004). An outstanding example exists in rabbit, where the substitution of the G at -6 with
226 a T in the β-globin 5'UTR reduced the efficiency of the translation initiation process *in vitro* (Kozak,
227 1994). In addition, in human, *in vitro* transcription/translation experiments demonstrated that the
228 substitution of -6G with a C decreased the efficiency of translation of the β-globin chain by about 30%
229 translation (De Angioletti et al. 2004).

230 Similarly, a polymorphism 5 bp upstream of the initiation codon in the Kozak sequence directly
231 influenced the *CSN1S2* translation in Norwegian Red cattle (Sodeland et al. 2011). Furthermore, in mouse
232 and human, a SNP at position -1 is associated with a significant reduction of CD40 gene product and with
233 a reduction in the translation efficiency (Jacobson et al. 2005; Pineda et a. 2008), analogous to what we
234 observed for donkey *CSN3*. Mechanistically, SNPs occurring at position -1 of the Kozak consensus
235 sequence would interfere with the ability of the ribosome to initiate translation, although not affecting the
236 ability of RNA polymerase to transcribe mRNA (Jacobson et al. 2005).

237 The ORF length is another element potentially affecting the translation efficiency. Valleriani et al.
238 (2011) demonstrated that the translational ratio decreases with increasing mRNA length. In this respect,
239 the calcium-sensitive casein genes in donkeys showed a higher translation efficiency of the *CSN1S1 vs*
240 *CSN2* and *CSN1S2* I genes, which is consistent with the length of their coding sequence: 212 codons
241 (GeneBank FN386610) *vs* 241 (GeneBank FN598778) and 236 (GeneBank FM946022), respectively.
242 Therefore, based on these data, it is reasonable to suppose that the reduced ORF length counteracts the
243 negative effect of the "weak consensus site" and the impact of the SNP in position -6 on the *CSN1S1*
244 translation efficiency.

245 The coding region length could also explain some of the differences in translation efficiency
246 observed among the species. Donkey *CSN1S2* I and *CSN2* transcripts, which show a lower translation
247 efficiency than the homologous genes in ruminants, are characterized by a higher coding sequence length.
248 In particular, 236 codons for the donkey *CSN1S2* I *vs* 223 of goat and sheep (GenBank NM_001285585,
249 NM_001009363, respectively) and *vs* 222 of cattle, buffalo and yak (GenBank NM_174528, FM865618
250 and XP_014335716, respectively). Similarly, 241 codons for the donkey *CSN2 vs* 222 for goat

(AJ011018) and sheep (NM_001009373), *vs* 224 for cattle (KC993858), buffalo (FM946182) and yak (ELR51814).

A common feature in all species examined is the relatively low efficiency of translation of *CSN1S2* compared to *CSN2*. The analysis of the mammary tissue collected from yak, goats, sheep and cows has revealed that *CSN2* and *CSN1S2* mRNA are expressed at similar levels, but the β-casein accumulation in milk is 4-5 times that of the α_{s2}-casein (Bevilacqua et al. 2006; Bai et al. 2013). In the mammary tissue of water buffalo, the *CSN2* and *CSN1S2* represent 23 and 56% of casein transcripts, respectively, while their corresponding protein concentrations in milk are 54 and 5%, respectively, of total caseins, indicating approximately 10-fold more efficient translation of *CSN2* (Cosenza et al. 2011). Analogously, in donkey lactating mammary gland the *CSN2* and *CSN1S2* I transcripts represent respectively 70.85 and 14.23% of the total casein mRNAs, while the corresponding protein concentration is 54.28 and 7.19 % respectively, with a greater *CSN2* translation efficiency of about 1.5 times. In the bovine species, Kim et al. (2015) show that the usage of the last 28 codons of *CSN1S2* is the main regulatory element attenuating its expression, and it is responsible for the differential translational expression of the *CSN1S2* and *CSN2*. In particular, the authors reported that the codon usage and order influenced the accuracy and the speed of translation.

Although the analysis of the sequences flanking the start codon, codon usage frequencies and the coding sequence length can help to formulate hypotheses concerning some of the observed differences in translation efficiency, other elements need to be analysed to fully understand the regulation mechanisms of their expression. Factors like gene ontology enrichment scores, biochemical and physicochemical features, minimum free energy, 5'UTR and 3'UTR length, number of transcription factors known to bind the promoter region, number of RNA binding proteins known to bind its mRNA product, protein abundance, mRNA and protein half-life, might affect gene expression (Huang et al. 2011). By simultaneously measuring translational efficiencies (thus indirectly levels of protein synthesis) and mRNA abundance, global analyses have shown evidence of significant mRNA destabilization and translational repression. Since only slightly more translational repression is observed than mRNA

277 destabilization, it is possible that most of the loss in protein synthesis could directly result from effects on
278 mRNA stability (Djuranovic et al. 2012).
279
280 **Conclusions**
281 DM was characterized by a lower protein content with respect to ruminants milk and the different
282 proportions of caseins were closer to the human casein-type composition. β-CN was predominant with
283 respect to the alpha (s1), which may reduce allergenicity. This compositional feature might be responsible
284 for the soft curd produced in the stomach, determining a better digestibility of DM than cow's milk.
285 Moreover, the casein composition of DM could also be decisive for using it as a substitute when breast-
286 feeding is not possible.
287 The results obtained showed also a significant difference in the expression of donkey casein genes,
288 which revealed dissimilar patterns in comparison to those of the main species of ruminants (cattle,
289 buffalo, sheep, goats and yak). These data represent an important first step in the understanding of the
290 mechanisms regulating the expression of these genes in donkeys aimed at improving the milk production,
291 which fulfill special consumer requirements..
292
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295
296 **Conflict of interest**
297 None of the authors have any conflict of interest to declare.
298
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Figure 1.

PAGE analysis at pH 8.6 of the donkey's casein samples, after CBB staining (A) and identification of the four casein fractions by immunoblotting with polyclonal antibodies against α_{s2} (B), α_{s1} (C), β (D) and κ-CN (E).

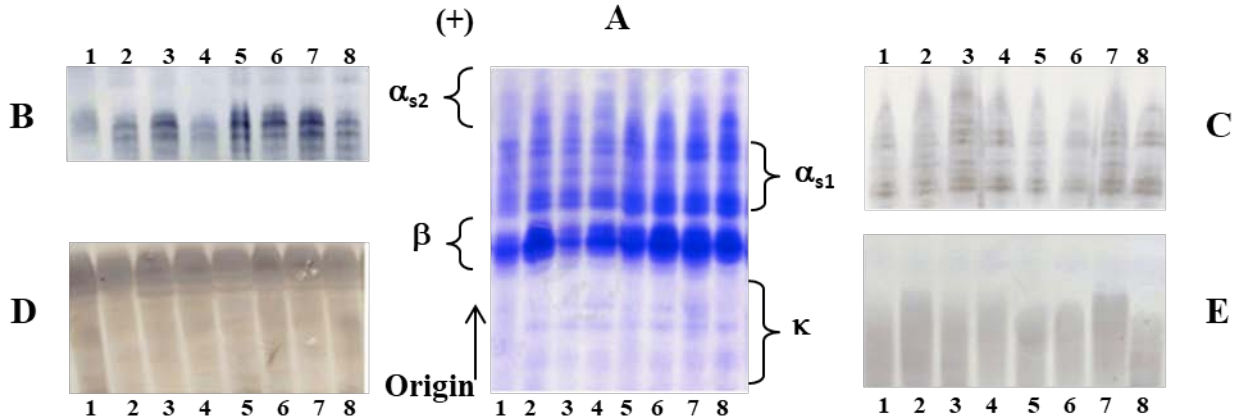

Table 1.
Total casein and caseins' fraction content in DM in comparison with pony horse, cattle, buffalo, goat, yak and camel milk.

Species	α_{s1}		β		α_{s2}		κ		Total casein
	%	mg mL^{-1}	%	mg mL^{-1}	%	mg mL^{-1}	%	mg mL^{-1}	mg mL^{-1}
Donkey[a]	35.59	1.82	54.28	2.77	7.19	$3.68 \cdot 10^{-1}$	2.79	$1.42 \cdot 10^{-1}$	5.12
Pony Horse[b]	17.92	2.50	78.85	11.00	1.43	0.20	1.80	0.25	13.95
Cattle[b]	36.77	10.00	36.77	10.00	13.69	3.70	12.86	3.50	27.20
Buffalo[c]	16.19	7.62	42.08	19.81	32.70	15.39	9.03	4.25	47.07
Goat[b]	26.12	7.00	41.05	11.00	15.67	4.20	17.16	4.60	26.80
Yak[d]	30.80	10.50	48.20	16.50	8.70	2.90	12.30	4.20	34.10
Camel[e]	22.00	5.20	65.00	15.60	9.60	2.30	3.30	0.80	24.00

[a] Present work
[b] Miranda et al. (2004)
[c] Cosenza et al. (2011)
[d] Bai et al. (2013)
[e] Kappeler et al. (1998)

Table 2.
Comparison of average quantitative transcript levels for α_{s1}- (*CSN1S1*), β- (*CSN2*), α_{s2}- (*CSN1S2*) and κ-casein (*CSN3*) in donkey and in the main ruminant species.

Species	***CSN1S1* (%)**	***CSN2* (%)**	***CSN1S2* (%)**	***CSN3* (%)**
Donkey [a]	6.28 ± 1.93	70.85 ± 8.96	14.23 ± 6.82	8.65 ± 1.21
Cattle, sheep, goat [b]	~ 25	~ 25	~ 25	~ 25
Buffalo [c]	16.48 ± 4.99	23.18 ± 5.41	55.87 ± 8.22	4.47 ± 0.96
Yak [d]	17.5 ± 1.80	31.9 ± 1.90	29.6 ± 2.50	20.9 ± 2.10

[a] Present work
[b] Bevilacqua et al. (2006)
[c] Cosenza et al. (2011)
[d] Bai et al. (2013)

Table 3.
Comparison of start codon flanking sequences of the 4 casein transcripts in donkey.

Position[1]										Sequence[2]
-6	-5	-4	-3	-2	-1	+1	+2	+3	+4	
G	C	C	R	C	C	A	U	G	G	Kozak consensus sequence
U	C	A	G	C	C	A	U	G	A	*CSN2*
A	U	A	A	C	C	A	U	G	A	*CSN1S2* I
A	C	A	A	G	C	A	U	G	A	*CSN1S1*
G	C	A	A	C	G	A	U	G	A	*CSN3*

[1]The start codon (AUG) in the four casein transcripts is underlined; gray colour identifies a conserved nucleotide in comparison with the Kozak consensus sequence.
[2]Kozak consensus sequence = the optimal context for initiation of translation in mammals. *CSN2*, *CSN1S2* I, *CSN1S1* and *CSN3* are the genes encoding β, α_{s2}, α_{s1} and κ-casein, respectively.