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

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Research Article

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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.

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 of casein to whey protein that is closer to human milk than that 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.*, 2009*a*, 2009*b*; Chianese *et al.*, 2010). In particular, the complete primary structure of α_{s1}-casein (202 amino acids, Cunsolo *et al.*, 2009*a*), β (226 amino acids, Cunsolo *et al.*, 2009*b*) 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

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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 characterizes 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 g (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 (×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 and 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 sequences 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 analyzed, 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 human and mare's milk (Malacarne *et al.*, 2002). The nutritional and biological significance of this milk fraction is still far from being completely understood, but it seems to be related to the development of the infant (Lonnerdal, 1994). It has been suggested that the high amount of whey protein (49.81%) in donkey's milk, similar to mare's milk, may make it more favorable for human nutrition than cow's milk, because of the relatively higher acute postprandial availability of essential amino acids.

Qualitative and quantitative characterization of donkey's caseins by Urea-PAGE at pH 8.6, immunoblotting and densitometry analysis

The individual casein samples analyzed by Urea-PAGE at pH 8.6 and shown in Fig. 1, were stained with either Coomassie Brilliant Blue (CBB) or specific polyclonal antibodies against α_{s2}, α_{s1}, β and κ-CN to identify each casein fraction in the electrophoretic pattern. In the Urea-PAGE profiles, at least three components exhibiting the highest mobility toward the anode and migrating head α_{s1}-CN were detected as α_{s2}-CN after immunoblotting; each component accounted for 10, 11 and 12 P/mole as previously reported (Chianese *et al.*, 2010). The α_{s1}-CN fraction showed a complex heterogeneity, after immunostaining with specific antibodies, since five main components were identified as α_{s1}-CN, exhibiting an intermediate anodic mobility between donkey β- and α_{s2}-CN. The compositional heterogeneity of donkey α_{s1}-CN could be due to different phosphorylation degree of its components as well as the presence of deleted forms (Cunsolo *et al.*, 2009a), as in mare counterparts (Miranda *et al.*, 2004; Mateos *et al.*, 2009) as well as in ruminants (Martin *et al.*, 2003). After immunodetection the β-CN was constituted of two/three main components, differing for the phosphorylation degree (5, 6 and 7 P/mole) (Chianese *et al.*, 2010), as found in mare's milk also (Girardet *et al.*, 2006).

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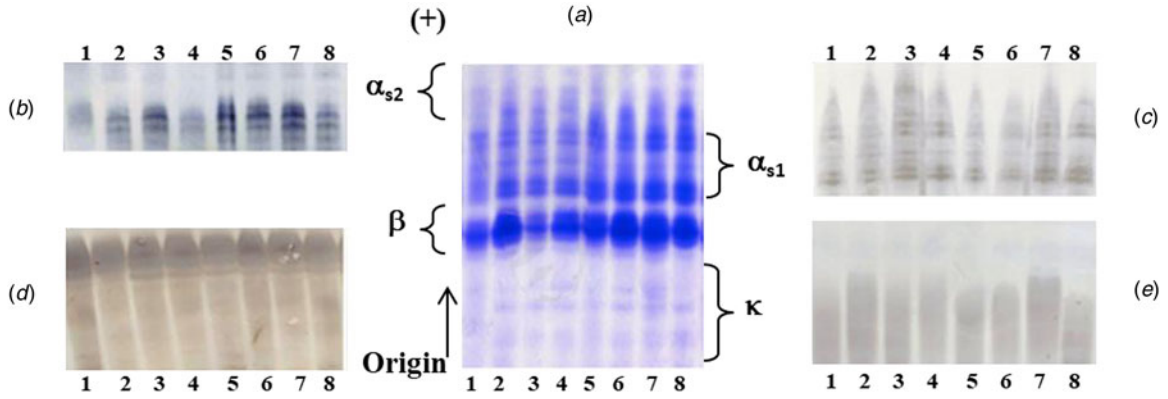

Fig. 1. PAGE analysis at pH 8.6 of the donkey's casein samples, after CBB staining (A) and identification of the four casein fractions by immunoblotting with polyclonal antibodies against α_{s2} (B), α_{s1} (C), β(D) and κ-CN (E).

The electrophoretic profiles stained with CBB were quantitatively evaluated by densitometric analysis. Taking into account the high intensity of electrophoretic bands, the donkey β-CN may be the most abundant casein fraction. Finally, the CBB stained bands, characterized by a lower negative charge 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 breast milk. 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. 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 ($\beta > \alpha_{s2} > \alpha_{s1} > \kappa$) (Cosenza *et al.*, 2011).

These data confirm that the casein-type composition (as well as the protein/fat ratio) varies between different 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

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

	α_{s1}		β		α_{s2}		κ		Total casein
Species	%	mg/ml	%	mg/ml	%	mg/ml	%	mg/ml	mg/ml
Donkey[a]	35.59	1.82	54.28	2.77	7.19	3.68×10^{-1}	2.79	1.42×10^{-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).

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 and 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 Angioletti *et al.*, 2004). An outstanding example exists in rabbit, where the substitution of the G at −6 with a T in the β-globin 5′UTR reduced the efficiency of the translation initiation process in vitro (Kozak, 1994). In addition, in human, in vitro transcription/translation experiments demonstrated that the substitution of −6G with a C decreased the efficiency of translation of the β-globin chain by about 30% translation (De Angioletti *et al.*, 2004).

Similarly, a polymorphism 5 bp upstream of the initiation codon in the Kozak sequence directly influenced the *CSN1S2* translation in Norwegian Red cattle (Sodeland *et al.*, 2011). Furthermore, in mouse and human, a SNP at position −1 is associated with a significant reduction of CD40 gene product and with a reduction in the translation efficiency (Jacobson *et al.*, 2005; Pineda *et al.*, 2008), analogous to what we observed for donkey

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

Position[a]										
−6	−5	−4	−3	−2	−1	+1	+2	+3	+4	Sequence[b]
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*

[a]The start codon (AUG) in the four casein transcripts is underlined.
[b]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.

CSN3. Mechanistically, SNPs occurring at position −1 of the Kozak consensus sequence would interfere with the ability of the ribosome to initiate translation, although not affecting the ability of RNA polymerase to transcribe mRNA (Jacobson *et al.*, 2005).

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

The coding region length could also explain some of the differences in translation efficiency observed among the species. Donkey *CSN1S2* I and *CSN2* transcripts, which show a lower translation efficiency than the homologous genes in ruminants, are characterized by a higher coding sequence length. In particular, 236 codons for the donkey *CSN1S2* I *vs.* 223 of goat and sheep (GenBank NM_001285585, NM_001009363, respectively) and *vs.* 222 of cattle, buffalo and yak (GenBank NM_174528, FM865618 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 analyzed 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 destabilization, it is possible that most of the loss in protein synthesis could directly result from effects on mRNA stability (Djuranovic *et al.*, 2012).

Conclusions

DM was characterized by a lower protein content with respect to ruminants milk and the different proportions of caseins were closer to the human casein-type composition. β-CN was predominant with respect to the alpha (s1), which may reduce allergenicity. This compositional feature might be responsible for the soft curd produced in the stomach, determining a better digestibility of DM than cow's milk. Moreover, the casein composition of DM could also be decisive for using it as a substitute when breast-feeding is not possible.

The results obtained showed also a significant difference in the expression of donkey casein genes, which revealed dissimilar patterns in comparison to those of the main species of ruminants (cattle, buffalo, sheep, goats and yak). These data represent an important first step in the understanding of the mechanisms regulating the expression of these genes in donkeys aimed at improving the milk production, which fulfill special consumer requirements.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029919000256

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References

Aschaffenburg R and Drewry J (1959) New procedure for the routine determination of the various non-casein proteins of milk. In London: XVth International Dairy Congress. vol. **3**, pp. 1631–1637.

Afshar-Kharghan V, Li CQ, Khoshnevis-Asl M and Lopez JA (1999) Kozak sequence polymorphism of the glycoprotein (GP) Iba gene is a major determinant of the plasma membrane levels of the platelet GP Ib-IX-V complex. *Blood* **94**, 186–191.

Bai WL, Yin RH, Jiang WQ, Ajayi OO, Zhao SJ, Lu GB, Zhao ZH and Imumorin IG (2013) Molecular analysis of alpha(s1)-, beta-, alpha(s2)- and kappa-casein transcripts reveals differential translational efficiency in yak lactating mammary gland. *Livestock Science* **152**, 74–78.

Bevilacqua C, Martin P, Candalh C, Fauquant J, Piot M, Roucayrol AM, Pilla F and Heyman M (2001) Goats' milk of defective alpha(s1)-casein genotype decreases intestinal and systemic sensitization to beta-lactoglobulin in Guinea pigs. *Journal of Dairy Research* **68**, 217–227.

Bevilacqua C, Helbling JC, Miranda G and Martin P (2006) Translational efficiency of casein transcripts in the mammary tissue of lactating ruminants. *Reproduction Nutrition Development* **5**, 567–578.

Businco L, Giampietro PG, Lucenti P, Lucaroni F, Pini C, Di Felice G, Lacovacci P, Curadi C and Orlandi M (2000) Allergenicity of mare's milk in children with cow's milk allergy. *Journal of Allergy and Clinical Immunology* **105**, 1031–1034.

Chianese L, Quarto M, Pizzolongo F, Calabrese MG, Caira S, Mauriello R, De Pascale S and Addeo F (2009) Occurrence of genetic polymorphism at the α_{s1}-casein locus in Mediterranean water buffalo milk. *International Dairy Journal* **19**, 181–189.

Chianese L, Calabrese MG, Ferranti P, Mauriello R, Garro G, De Simone C, Quarto M, Addeo F, Cosenza G and Ramunno L (2010) Proteomic characterization of donkey milk 'caseome'. *Journal of Chromatography* **A1217**, 4834–4840.

Cosenza G, Pauciullo A, Annunziata AL, Rando A, Chianese L, Marletta D, Iannolino G, Nicodemo D, Di Berardino D and Ramunno L (2010) Identification and characterization of the donkey *CSN1S2* I and II cDNAs and polymorphisms detection. *Italian Journal of Animal Science* **9**(e40), 206–211.

Cosenza G, Pauciullo A, Coletta A, Di Francia A, Feligini M, Gallo D, Di Berardino D and Ramunno L (2011) Translational efficiency of casein transcripts in Mediterranean river buffalo. *Journal of Dairy Science* **94**, 5691–5694.

Cosenza G, Pauciullo A, Gallo D, Colimoro L, D'avino A, Mancusi A and Ramunno L (2008) Genotyping at the CSN1S1 locus by PCR-RFLP and AS-PCR in a Neapolitan Goat Population. *Small Ruminant Research* **74**, 84–90.

Criscione A, Cunsolo V, Bordonaro S, Guastella AM, Saletti R, Zuccaro A, D'Urso G and Marletta D (2009) Donkeys' milk protein fraction investigated by electrophoretic methods and mass spectrometric analysis. *International Dairy Journal* **19**, 190–197.

Cunsolo V, Cairone E, Fontanini D, Criscione A, Muccilli V, Saletti R and Foti S (2009*a*) Sequence determination of αs1-casein isoforms from donkey by mass spectrometric methods. *Journal of Mass Spectrometry* **44**, 1742–1753.

Cunsolo V, Cairone E, Saletti R, Muccilli V and Foti S (2009*b*) Sequence and phosphorylation level determination of two donkey beta-caseins by mass spectrometry. *Rapid Communication in Mass Spectrometry* **23**, 1907–1916.

Cunsolo V, Saletti R, Muccilli V, Gallina S, Di Francesco A and Foti S (2017) Proteins and bioactive peptides from donkey milk: the molecular basis for its reduced allergenic properties. *Food Research International* **99**, 41–47.

D'Alessandro AG, Rosanna De Petro R, Claps S, Pizzillo M and Martemucci G (2009) Yield and quality of milk and udder health in Martina Franca ass: effects of daily interval and time of machine milking. *Italian Journal of Animal Science* **8**, 697–699.

De Angioletti M, Lacerra G, Sabato V and Carestia C (2004) β + 45 G —> C: a novel silent β-thalassaemia mutation, the first in the Kozak sequence. *British Journal of Haematology* **124**, 224–231.

Djuranovic S, Nahvi A and Green R (2012) miRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay. *Science* **336**, 237–240.

Giorgis V, Rolla G, Raie A, Geuna M, Boita M, Lamberti C, Nebbia S, Giribaldi M, Giuffrida M and Brussino L (2018) A case of work-related donkey milk allergy. *Journal of Investigational Allergology and Clinical Immunology* **28**, 197–199.

Girardet JM, Miclo L, Florent S, Mollè D and Gaillard JL (2006) Determination of the phosphorylation level and deamination susceptibility of equine β-casein. *Proteomics* **6**, 3707–3717.

Guo HY, Pang K, Zhang XY, Zhao L, Chen SW, Dong ML and Ren FZ (2007) Composition, physiochemical properties, nitrogen fraction distribution, and amino acid profile of donkey milk. *Journal of Dairy Science* **90**, 1635–1643.

Jacobson EM, Concepcion E, Oashi T and Tomer Y (2005) A Graves' disease-associated Kozak sequence single-nucleotide polymorphism enhances the efficiency of CD40 gene translation: a case for translational pathophysiology. *Endocrinology* **146**, 2684–2691.

Hobor S, Kunej T and Dovc P (2008) Polymorphisms in the kappa casein (CSN3) gene in horse and comparative analysis of its promoter and coding region. *Animal Genetics* **39**, 520–530.

Huang T, Wan S, Xu Z, Zheng Y, Feng KY, Li HP, Kong X and Cai YD (2011) Analysis and prediction of translation rate based on sequence and functional features of the mRNA. *PLoS One* **6**, e16036.

IDF (1993) *Milk. Determination of Nitrogen Content. Standard Method 20B*. Brussels, Belgium: International Dairy Federation.

Kappeler S, Farah Z and Puhan Z (1998) Sequence analysis of *Camelus dromedarius* milk caseins. *Journal of Dairy Research* **65**, 209–222.

Kim JJ, Yu J, Bag J, Bakovic M and Cant JP (2015) Translation attenuation *via* 3′ terminal codon usage in bovine CSN1S2 is responsible for the difference in αs2- and β-casein profile in milk. *RNA Biology* **12**, 354–367.

Kozak M (1984) Point mutations close to the AUG initiator codon affect the efficiency of translation of rat preproinsulin in vivo. *Nature* **308**, 241–246.

Kozak M (1987) Analysis of 50-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Research* **15**, 8125–8148.

Kozak M (1994) Features in the 5′ non-coding sequences of rabbit α and β-globin mRNAs that affect translational efficiency. *Journal of Molecular Biology* **235**, 95–110.

Lonnerdal B (1994) Nutritional importance of Non-protein nitrogen protein. Metabolism during infancy. In Niels CR Raiha (ed), *Nestle Nutrition Workshop Series*. New York: Ed. Nestec Ltd., Vevey/Raven Press, Ltd., p. 33.

Malacarne M, Martuzzi F, Summer A and Mariani P (2002) Protein and fat composition of mare's milk: some nutritional remarks with reference to human and cow's milk. *International Dairy Journal* **12**, 869–877.

Martin P, Ferranti P, Leroux C and Addeo F (2003) Non-bovine caseins: quantitative variability and molecular diversity. In Fox PF and McSweeney PLH (eds), *Advanced Dairy Chemistry Volume 1: Proteins*, 3rd edn. Kluwer Academic/Plenum Publishers.

Mateos A, Miclo L, Molle D, Dary A, Girardet JM and Gaillard JL (2009) Equine αs1- casein: characterization of alternative splicing isoforms and determination of phosphorylation levels. *Journal of Dairy Science* **92**, 3604–3615.

Miranda G, Mahé MF, Leroux C and Martin P (2004) Proteomic tools to characterize the protein fraction of Equidae milk. *Proteomics* **4**, 2496–2509.

Monti G, Viola S, Baro C, Cresi F, Tovo PA, Moro G and Bertino E (2012) Tolerability of donkey's milk in 92 highly-problematic cow's milk allergic children. *Journal of Biological Regulators and Homeostatic Agents* **26** (Suppl 3), 75–82.

Ochirkhuyag B, Chobert JM, Dalgalarrondo M and Haertlé T (2000) Characterization of mare caseins. *Identification of αs1- and αs2-caseins. Lait* **80**, 223–235.

Pineda B, Laporta P, Hermenegildo C, Cano A and García-Pérez MA (2008) A C > T polymorphism located at position −1 of the Kozak sequence of

https://doi.org/10.1017/S0022029919000256 Published online by Cambridge University Press

CD40 gene is associated with low bone mass in Spanish postmenopausal women. *Osteoporosis International* **19**, 1147–1152.

Roncada P, Piras C, Soggiu A, Turk R, Urbani A and Bonizzi L (2012) Farm animal milk proteomics. *Journal of Proteomics* **75**, 4259–4274.

Salimei E, Fantuz F, Coppola R, Chiofalo B, Polidori P and Varisco G (2004) Composition and characteristics of asses' milk. *Animal Research* **53**, 67–78.

Sodeland M, Grove H, Kent M, Taylor S, Svendsen M, Hayes BJ and Lien S (2011) Molecular characterization of a long range haplotype affecting protein yield and mastitis susceptibility in Norwegian Red cattle. *BMC Genetics* **12**, 70.

Usuki F and Maruyama K (2000) Ataxia caused by mutations in the alpha-tocopherol transfer protein gene. *Journal of Neurology, Neurosurgery and Psychiatry* **69**, 254–256.

Valleriani A, Zhang G, Nagar A, Ignatova Z and Lipowsky R (2011) Length-dependent translation of messenger RNA by ribosomes. *Physical Review. E, Statistical, Nonlinear, and Soft Matter Physics* **83**(4 Pt 1), 042903.

https://doi.org/10.1017/S0022029919000256 Published online by Cambridge University Press