



Review

Proteins and bioactive peptides from donkey milk: The molecular basis for its reduced allergenic properties

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ABSTRACT

The legendary therapeutics properties of donkey milk have recently been supported by many clinical trials who have clearly demonstrated that, even if with adequate lipid integration, it may represent a valid natural substitute of cow milk for feeding allergic children. During the last decade many investigations by MS-based methods have been performed in order to obtain a better knowledge of donkey milk proteins. The knowledge about the primary structure of donkey milk proteins now may provide the basis for a more accurate comprehension of its potential benefits for human nutrition. In this aspect, experimental data today available clearly demonstrate that donkey milk proteins (especially casein components) are more closely related with the human homologues rather than cow counterparts. Moreover, the low allergenic properties of donkey milk with respect to cow one seem to be related to the low total protein content, the low ratio of caseins to whey fraction, and finally to the presence in almost all bovine IgE-binding linear epitopes of multiple amino acid differences with respect to the corresponding regions of donkey milk counterparts.

1. Introduction

Since its domestication, about 5000 years ago, donkey (*Equus asinus*) has been used principally as pack and riding animal and for millennia its main activity was to carry weights and pull carts. At the end of XX century, donkey was reassessed and for the first time was used as a partner in pet-therapy projects and “onodidattica” (education through donkeys) activities for children with and without disabilities. So that, with the beginning of XXI century an ancient knowledge was rediscovered: the value of donkey milk (DM). Indeed, since ancient times DM has been used for infant nutrition and its therapeutic properties were widely known. Hippocrates as well as Pliny the Elder believed that it could act therapeutically in numerous cases, such as liver problems, infectious diseases, fevers, asthma, etc. (Hippocrates; translated by Adams, 1843; Pliny the Elder, translated by Bostock, & Riley, 1855). The use of DM as a moisturiser in cosmetics is even more fabled. Cleopatra and other privileged ladies of ancient times were taking their bath in DM in order to keep their skin fresh and shiny. Nowadays, the alleged therapeutic and cosmetic properties of DM seem to be validated by many trials (Brumini, Criscione, Bordonaro, Vegarud, & Marletta, 2016). DM is rich in vitamins and polyunsaturated fatty acids (Aspri, Economou, & Papademas, 2016) and contains anti-ageing, anti-oxidant and regenerating compounds, which are described as naturally active in

skin hydration and skin ageing prevention. The lipid fraction is characterized by high levels of essential fatty acids and low saturated fatty acids (Gastaldi et al., 2010). Compared with ruminants' milk, the considerable presence of unsaturated fatty acids found in DM make it very useful in the prevention of the cardiovascular, auto-immune and inflammatory diseases (Martemucci & D'Alessandro, 2012; Martini, Altomonte, & Salari, 2014). In this aspect, the high content of ω 3-polyunsaturated fatty acids, characteristic constituents of the fish oils, can counteract the above-mentioned pathologies through the synthesis of anti-inflammatory, anti-aggregant and non-immunosuppressant substances, like lipidic prostaglandins, leukotrienes, cytokines or interleukins (Salimei & Fantuz, 2012). Moreover, like human milk (HM), DM contains a substantial amount of lactose (about 7%), which determines its flavour and facilitates calcium absorption. The high lactose content together with the low protein amount, the low casein (CN)/whey protein (WP) ratio and the high level of the non-protein nitrogen (NPN) fraction reveal that DM differs considerably from that of the principal dairying species (i.e. cow, goat and sheep), whereas it is the most similar to breast HM and mare milk as well (Table 1). However, it should be noted that DM, with respect to breast HM, has a low caloric value and relatively low lipid content. So that, DM is inadequate as exclusive food in infants for the first year of life, and therefore adequate lipid integration is needed for toddlers' diet (D'Auria, Mandelli, Ballista,

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Table 1
Gross composition^a of the milk from different species.

	Donkey	Mare	Human	Cow	Goat	Sheep
Proteins ^b	16	23	13	34	35	56
Fat ^b	8	11	40	36	38	69
Lactose ^b	66	63	66	50	41	49
Caseins/Whey Ratio	1.3	1.1	0.4	4.6	3.5	3.2

^a Values are reported as average of different literature data (see text for bibliography).

^b Values are expressed as g/kg milk.

Di Dio, & Giovannini, 2011). Compared with mare and cow milk (CM), DM has a lower AA content, probably related to its lower protein content. On the other hand, the overall amount of essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine, and valine) in DM is higher than that in bovine milk. Moreover, DM shows noticeably higher levels of serine, glutamic acid, and arginine, and a lower value of cysteine with respect to CM. An exhaustive description of the gross composition of DM is beyond the scope of this review. However, an excellent survey about this topic and a comparison among different mammals' milk can be found in some review articles (Guo et al., 2007; Medhammar et al., 2012; Uniacke-Lowe, Huppertz, & Fox, 2010). DM is known also to possess natural protective antimicrobial factors and a specific epidermal growth factor (EGF) that suggest its beneficial impact on gastrointestinal mucosa health and integrity, a claim particularly valuable for children, the elderly, and convalescents, who have a reduced immune defense system (Brumini et al., 2016; Scafizzari et al., 2009). In fact, many clinical studies have clearly demonstrated that DM may be very useful in the prevention of atherosclerosis (Tafaro et al., 2007), has an effect on the osteogenesis process, as well for the rehabilitation of patients with coronary heart disease or premature senescence, and in hypocholesterolemic diets (Chiofalo, Salimei, & Chiofalo, 2001). In raw DM the antimicrobial activity is mainly attributed to the very high content of lysozyme (Uniacke-Lowe et al., 2010) that, working together with lactoferrin and immunoglobulins, inhibits microbial growth in the digestive tract and contributes to reduce the incidence of gastrointestinal infections, especially during infancy and in childhood (Baldi et al., 2005; Gubić et al., 2016). Finally, taking into account the very similar composition between DM and HM, an increasing number of literature data shows that DM may represent, for a large number (about 90%) of the investigated subjects, the best natural substitute of CM for children affected by Cow Milk Protein Allergy (CMPA) (Bertino et al., 2010; Carroccio et al., 2000; Monti et al., 2007; Monti et al., 2012). As a consequence, in the last decade the interest of researchers, above all paediatric allergologists, towards DM has increased. Many investigations have been performed in order to obtain a better knowledge of DM components, particularly proteins, to detect unintended contaminations, improve dairy productions, and to accomplish a more accurate comprehension of its potential benefits for human nutrition (Claeys et al., 2014; Cunsolo, Muccilli, Saletti, & Foti, 2013; Medhammar et al., 2012). In this aspect, the continuous and rapid evolution of mass spectrometry-based techniques, which arguably represent the core tools in proteomic approaches, has provided an efficient platform for the characterization of food-derived proteins (Cunsolo, Muccilli, Saletti, & Foti, 2014).

In this review the main results of research oriented to the elucidation of donkey milk proteins characterization will be summarized. Moreover, structural differences between proteins of donkey and other mammalian milks, their significance in human nutrition and potential cross-reactivity of milk proteins from different species in relation to the treatment of CMPA will be discussed. This overview will be preceded by a brief description of the most common MS-based proteomic techniques.

2. MS-based approaches in milk proteomics

No one can deny the fact that the bovine is certainly the primary dairy animal species dominating global milk production and consequently, among mammalian milks, this brand has been the longest and the broadest investigated (D'Alessandro, Zolla, & Scaloni, 2011). On the contrary, although extensive reviews about the composition, physico-chemical and nutritional properties of DM have been published, the characterization of the protein fraction until ten years ago was very scanty and limited to a handful of whey proteins, whereas the knowledge of the donkey caseins was totally absent (Cunsolo, Saletti, Muccilli, & Foti, 2007). In the last years, an extensive investigation of DM aimed to the characterization of its molecular composition, allowed to expand the information regarding the most abundant whey proteins and to identify and characterize the casein components previously completely unknown (primary structure, disulphide bridges and other post-translational modifications such as phosphorylation and glycosylation). An important step forward has occurred in 2011 when an in-depth proteomic investigation described the presence of about one hundred proteins in DM (Cunsolo et al., 2011). The recent advances in the proteomic analysis of DM have been possible because the continuous and rapid evolution of techniques usually employed for the investigation and characterization of complex protein mixtures. Particularly, the impressive increasing in performance and versatility of the MS-instrumentation has contributed to the development of new analytical strategies for proteins identification and structure determination, evidencing how mass spectrometry arguably represents an indispensable tool in proteomics. On the other hand, concurrent optimization of the separation techniques and the availability of efficient methods for gene sequencing, resulting in an extensive enlargement of the protein sequences database, have provided an efficient platform for large scale protein investigation. In the current proteomic approaches, the characterization of protein mixtures is usually performed combining highly efficient separation techniques, such as reversed-phase ultra high performance liquid chromatography (RP-UHPLC), mono-(SDS-PAGE) or two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) with biochemical methods (e.g. to immunospecific staining), enzymatic digestions and mass spectrometric analyses in order to obtain data suitable for searching, by specific software, protein databases. Taking into account that the general strategy applied depends on the experimental question and the scope of the research, two complementary MS-based approaches are currently used in proteomics: the so-called "bottom-up" and "top-down", each of them having advantages and drawbacks (Kelleher et al., 1999). Briefly, bottom-up strategy represents the most widespread proteomic workflow and can be carried out by different ways; as example, in "break-then-sort" approach, the protein mixture is directly digested into a collection of peptides, which are separated by multidimensional chromatography (e.g. strong cation exchange followed by RP-HPLC) on-line coupled to ESI tandem MS analysis. Instead, in the so-called "sort-then-break" approach, proteins are firstly separated by 2D electrophoresis; then, the isolated proteins are subjected to enzymatic digestion followed by LC-MS/MS analysis. If the "bottom-up" represents the traditional approach, the so-called "top-down" represents the emerging MS-based strategy in proteomic studies, providing information on both intact protein mass and its amino acid sequence. The top-down proteomics approach utilizes molecular and fragment ion mass data obtained by the ionization and fragmentation of a protein in the mass spectrometer, without prior proteolytic digestion. In particular, the proteins present in a complex mixture are firstly fractionated and separated into pure single proteins or less complex protein mixtures. This step is followed by off-line static infusion or by on-line LC/MS analysis of sample into the mass spectrometer for high resolution mass measurement of intact protein multi-charged molecular ions. MS/MS of mass-selected multi-charged ions then provides fragment mass values for its structural characterization (McLafferty et al., 2007). Top-down and bottom-up

Table 2
Properties of donkey caseins.

Protein	Calculated MW (Da) ^a	pI ^b	GRAVY score ^c	AA	Post-translational modifications ^d	UniProt Acc. No.	Amino acid differences ^e	Disulphide bonds	Free sulphhydryl groups
α_{s1} -CN					N-glycosylation Phosphorylation	P86272			
α_{s1} -CN A	24,406.4	5.96	-1.115	202	5P, 6P		-	0	0
α_{s1} -CN A ₁	24,278.3	5.96	-1.103	201	5P, 6P		Missing Gln ⁸⁸	0	0
α_{s1} -CN B	23,785.8	5.85	-1.075	197	5P, 6P		Missing His ³⁴ , Glu ³⁸	0	0
α_{s1} -CN B ₁	23,657.6	5.85	-1.063	196	5P, 6P		Missing His ³⁴ , Glu ³⁸ and Gln ⁸⁸	0	0
α_{s2} -CN					Phosphorylation	B7VGF9			
α_{s2} -CN A	26,030.2	5.68	-0.908	221	10P, 11P, 12P		-	1	0
α_{s2} -CN A ₁	25,432.5	5.50	-0.883	216	10P, 11P, 12P		Missing Asn ¹⁷⁶ , Gln ¹⁸⁰	1	0
α_{s2} -CN A ₁ Δ4,5,6	21,942.6	5.65	-0.929	185	8P, 9P, 10P		Missing Asp ¹² , Glu ⁴² and Asn ¹⁷⁶ , Gln ¹⁸⁰	0	0
α_{s2} -CN A ₂	25,203.2	5.68	-0.987	214	10P, 11P, 12P		Missing Tyr ²¹² , Leu ²¹⁸	1	0
α_{s2} -CN A ₂ Δ4,5,6	21,713.3	5.85	-1.051	183	8P, 9P, 10P		Missing Asp ¹² , Glu ⁴² and Tyr ²¹² , Leu ²¹⁸	0	0
β -CN					N-glycosylation Phosphorylation	P86273			
β -CN A	25,529.3	5.44	-0.406	226	5P, 6P, 7P		-	0	0
β -CN A ^{Δ5}	24,606.3	5.24	-0.390	218	5P, 6P, 7P		Missing Glu ²⁷ , Lys ³⁴	0	0
β -CN ^f	25,557.0	-	-	226	5P, 6P, 7P		n.a. ^g	0	0
κ -CN					O-glycosylation	F0V6V5			
κ -CN	18,485.2	9.36	-0.413	162			-	0	1

^a Molecular masses were calculated considering the cysteines in reduced form (-SH).

^b Isoelectric point calculated by GPMW considering the cysteines in reduced form (-SH), and based on the algorithm of [Skoog & Wichman, 1986](#).

^c Grand average hydropathy (GRAVY) score is calculated by GPMW using the scale of [Kyte & Doolittle, 1982](#).

^d The phosphorylation level for each isoform is reported. For details see the text.

^e Amino acid differences are reported with respect to the isoform A of each protein.

^f Molecular mass of this isoform was experimentally measured; characterization of the sequence is lacking; number of amino acid residues is hypothesized.

^g n.a.: not available.

represent complementary approaches, as evidenced by a number of proteomic studies performed by coupling these two strategies ([Aebersold & Mann, 2016](#); [Zhang, Wu, Stenoien, & Paša-Tolić, 2014](#)). In general, the bottom-up method is widely accepted for the routine identification of proteins in complex mixtures. As example, in biomedical research the identification of the gene that encodes for a protein is usually more important than the full characterization of the protein itself. In other cases, when a more extensive comprehension of the protein structure or specific data such as on polymorphisms or complete post-translational modifications (PTMs) are needed, top-down proteomics becomes an unavoidable tool. However, it is important to note that the proteins present in most biological mixture are expressed over a vast concentration range (up to 10 ÷ 12 orders of magnitude in human sera) and, independently from the adopted methodological approach, the most abundant proteins complicate the detection of low-abundant or trace components, including also allergen species, which could remain “hidden”. During the last years several strategies, such as pre-fractionation and immuno-depletion methods, have been employed to remove the most abundant proteins and for enhancing the sensitivity and efficiency in the identification of low-abundant proteins. Very recently, the characterization of minor components in complex protein systems has been revolutionized by the introduction of the combinatorial peptide ligand libraries technology (CPLL) ([Boschetti & Righetti, 2008](#); [Righetti & Boschetti, 2008](#)). This new methodology, commercially named ProteoMiner, is based on the use of hexa-peptide baits to capture and normalize the relative concentrations of the components of the proteome under investigation. The major advantage of this technique, which has recently been applied to the exploration of different mammals' whey proteomes ([Cunsolo et al., 2015](#); [Cunsolo et al., 2017](#); [D'Amato et al., 2009](#); [Ha et al., 2015](#); [Liao, Alvarado, Phinney, & Lönnerdal, 2011](#)) in comparison with other pre-fractionation methods, is that not only it reduces the concentration of the more abundant proteins, but also concentrates low-abundance and even trace components, thus providing access to the “invisible” proteome.

3. Characterization of donkey milk proteome

Protein content of mature milk and the relative protein composition may vary among species, breeds within the same species, and even among individual animals within the same breed. On the other hand, it is well known that exists a strong qualitative resemblance about the principal classes of proteins (i.e. caseins and most abundant whey proteins) in all types of milk. Milk proteins can be grouped into three main classes according to their different solubility: caseins (CNs), whey proteins and milk fat globule membrane (MFGM) proteins. They can be easily separated as following: MFGM proteins fraction can be obtained by centrifugation (typically at 5000 × g for 15 min at 4 °C) of whole milk which separates a floating layer, containing MFGM proteins, from the skimmed milk fraction. Acidification (in the range of pH 4.2 ÷ 4.6) of skimmed milk allows to separate caseins, as insoluble fraction, from whey proteins, which instead remain as soluble fraction.

3.1. Donkey caseins

CNs are a group of proteins coded by four tightly linked autosomal genes (*CSN1S1*, *CSN1S2*, *CSN2* and *CSN3*), and classically subdivided according to the homology of their primary structures into four families: α_{s1} -, α_{s2} -, β - and κ -CN ([Swaisgood, 1992](#)), of which the first three are calcium-sensitive. The amount of CNs shows remarkable species variation; indeed while in mature bovine milk they account for 80% (w/w) of all proteins, in humans ([Lönnerdal, 2003](#)) and *Equidae* ([Malacarne, Martuzzi, Summer, & Mariani, 2002](#)), caseins represent only 35% and 50% of total protein content, respectively. They show great heterogeneity due to post-translational processing, alternative splicing and genetic polymorphism ([Caroli, Chessa, & Erhardt, 2009](#); [Martin, Szymanowska, Zwierchowski, & Leroux, 2002](#); [Ng-Kwai Hang & Grosclaude, 1992](#)). DM primarily includes α_{s1} - and β -CNs whereas α_{s2} - and κ -CN constitute minor components. In particular, as in HM, where β -CN can account for up to 80% of the total caseins ([Kroening, Pradip Mukerji, & Hards, 1998](#)), this component also

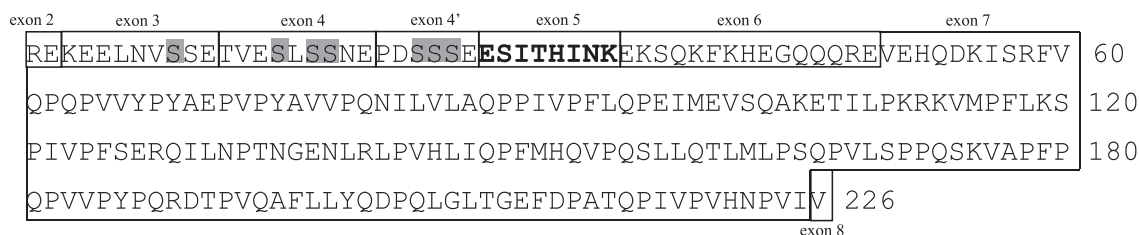


Fig. 1. Exon modular structure of donkey β -CN A (UniProt Acc. No. P86273). The exon nomenclature is that used by Miranda et al. (2004). The variant A Δ 5 is lacking of the domain E27SITHINK34, here reported in bold. The hypothesized phosphorylation sites are shaded.

represents the predominant protein in DM casein fraction. Notwithstanding the renewed growing interest for DM, until ten years ago the knowledge of the donkey's casein components was practically absent, and only in the last decade this fraction was characterized at molecular level. Nowadays, the characterization of donkey caseins is still at a relatively early stage of progress and, compared with bovine milk, only limited data are related to its genetic polymorphism.

3.1.1. β -casein

β -CN is the most abundant casein component and its occurrence has been reported by means of isoelectric focusing (IEF) and MS analysis through fitting of its tryptic hydrolysate with the known mare counterpart (Criscione et al., 2009). Subsequently the entire primary structure of two donkey β -CNs, with a molecular weight (MW) of 25,529 (226 amino acids) and 24,606 (218 amino acids) Da, respectively, has been characterized (Cunsolo, Cairone, Saletti, Muccilli, & Foti, 2009) (Table 2). They differ for the presence in the full-length component of the peptide E²⁷SITHINK³⁴ encoded by exon 5 (Fig. 1), as occurring in the mare β -CNs (nrNCBI Acc. N. Q9GKK3) used as references (Girardet, Miclo, Florent, Molle, & Gaillard, 2006). In view of these analogies the same nomenclature of mare β -CNs was adopted to name the two donkey β -CN variants. Therefore, the full-length (226 amino acids) β -CN was termed variant A, whereas the shorter one (218 amino acids), originated by an alternative splicing process at level of exon 5, was termed variant A Δ 5. Based on their amino acid sequences donkey β -CN A and A Δ 5 show an isoelectric point (pI) of 5.44 and 5.24, respectively. Moreover, based on the hydropathy scale proposed by Kyte and Doolittle (1982), donkey β -CNs show a grand average hydropathy (GRAVY) score of -0.406 (variant A) and -0.390 (variant A Δ 5). GRAVY score is related to the relative ratio of hydrophobic and hydrophilic amino acid residues in a protein, with a positive value reflecting an overall hydrophobic and a negative value an overall hydrophilic nature of the protein. In this aspect, a comparison with bovine, having a GRAVY score of -0.355 , and human β -CN (GRAVY score -0.289) reveals that donkey β -CNs are slightly more hydrophilic than their bovine and human counterparts. The two donkey β -CNs are both equally phosphorylated with 5, 6 and 7 P/mol. As such, donkey β -CNs are more phosphorylated than the homologous mare β -CNs, in which the predominant phosphorylated isoform carries five phosphate groups. The primary structure of donkey β -CN A contains six Ser (Ser⁹, Ser¹⁵, Ser¹⁸, Ser²³, Ser²⁴ and Ser²⁵) located in Ser-Xxx-Glu/SerP motif (with SerP = phosphoserine residue) and one Ser (Ser¹⁷) in a Ser-Xxx-

Xxx-Glu motif, together with two other potential phosphorylation sites located at Thr¹² (Thr¹²-Val-Glu¹⁴) and Thr²⁰⁷ (Thr²⁰⁷-Gly-Glu²⁰⁹) (Fig. 1), analogously to the homologous mare β -CN. Taking into account that several literature data (Bingham & Groves, 1979; Sørensen & Petersen, 1994) evidence that the sequence motif Thr-Xxx-Glu is a poor substrate for the mammary gland enzyme and is usually not phosphorylated, likely in the homologous mare's β -CN, the seven serine residues above mentioned probably constitute the phosphorylation sites of donkey β -CN. In addition, recent data, which however need to be directly confirmed, revealed that donkey β -CNs might also exist as glycosylated isoforms, a feature that appears unique of DM (Bertino et al., 2010). More recently, a new genetic variant of donkey β -CN was identified. The primary structure was not reported, but available data showed that the new variant has a MW 28 mass units higher than the most common variant A and the same phosphorylation pattern (5P, 6P and 7P) (Chianese et al., 2010).

Caseins show a very flexible tertiary structure, and therefore are stable to denaturing agents (e.g. heat or urea) but they are very susceptible to proteolysis. In this respect, the β -CN appears to be the most susceptible to the action of the endogenous milk proteases (Bastian & Brown, 1996; Eigel et al., 1979) and the existence of β -CN fragments, originated by hydrolysis of the two variants A and A Δ 5 has been reported (Chianese et al., 2010). In addition, internally deleted isoforms of donkey β -CN A with an apparent MW around 17 kDa have been detected (Bertino et al., 2010). These components, apparently unique to *Equidae* species and absent in milk from ruminants, could be the donkey homologues of a low-MW β -CN from mare which displays, with respect to the full-length equine β -CN, a large deletion (residues 50–181), due to a cryptic splice site usage occurring within exon 7 during the course of primary transcripts processing (Miclo et al., 2007).

3.1.2. α _{s1}-casein

The primary structure of donkey α _{s1}-CN, classified as variant A, is shown in Fig. 2. The protein contains 202 amino acids and has a MW of 24,406 Da (Cunsolo, Cairone, Fontanini, et al., 2009), a pI of 5.96, and a GRAVY score of -1.115 (Table 2). As already reported for mare (Mateos et al., 2009), sheep (Ferranti et al., 1995; Ferranti et al., 1997) and goat milk (GM), donkey α _{s1}-CN also shows a great micro-heterogeneity depending on a variable degree of phosphorylation and on the presence of non-allelic forms generated by RNA incorrect splicing events (Cunsolo, Cairone, Fontanini, et al., 2009). Particularly, three isoforms of α _{s1}-CN named A₁ (201 amino acids; MW 24278 Da, pI 5.96,

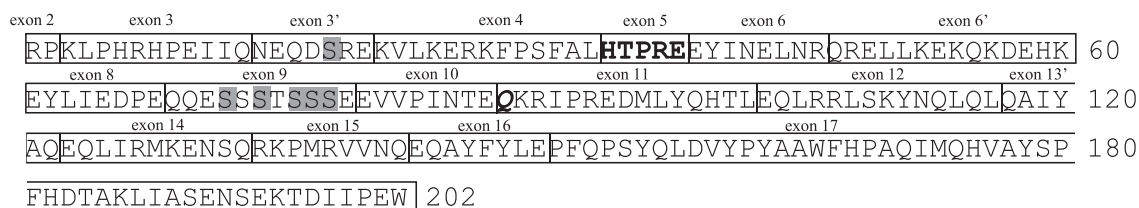


Fig. 2. Exon modular structure of the primary structure of donkey α _{s1}-CN (UniProt Acc. No. P86272). The exon nomenclature is that used by Miranda et al. (2004). The pentapeptide HTPRE only presents in the variants A and A1 and encoded by exon 5, is reported in bold. The glutamine residue (position 88) absent in the variants A1 and B1 is reported in bold italics. The hypothesized phosphorylation sites are shaded.

GRAVY score -1.103), B (197 amino acids; MW 23786 Da, pI 5.85, GRAVY score -1.075) and B₁ (196 amino acids; MW 23658 Da, pI 5.85, GRAVY score -1.063) have been detected and characterized. The isoform A₁ differs from the full-length component for the absence of a glutamine residue at position 88. The other two components, namely the isoforms B and B₁, are lacking of the pentapeptide HTPRE between Leu³³ and the Glu³⁴ (isoform B) with respect to the variant A, and additionally also of the glutamine residue in position 88 (isoform B₁). Taking into account the exon modular structure of the α_{s1} -CN in other species and in mare, it is reasonable to assume that the pentapeptide HTPRE between Leu³³ and the Glu³⁴ is encoded by the exon 5, which is instead constitutively spliced in mare α_{s1} -CNs (Lenasi, Rogelj, & Dovc, 2003; Miranda, Mahe, Leroux, & Martin, 2004). On the contrary, the exon 7, encoding the peptide DTSNESTE and alternatively spliced in mare α_{s1} -CNs (Mateos et al., 2009), in donkey counterparts is constitutively skipped. Moreover, the existence of the two isoforms lacking of a glutamine residue is probably related to a cryptic splice site occurring at the first codon (CAG) of exon 11 (encoding this glutamine residue) as occurring in mare α_{s1} -CNs (Lenasi et al., 2003; Mateos et al., 2009). Even if detected as minor components and characterized with few small fragments, the existence of two other α_{s1} -CN isoforms, with experimentally measured MW 25142 and 25,272 Da, respectively, has been also reported (Cunsolo, Cairone, Fontanini, et al., 2009). All the α_{s1} -CNs show two levels of phosphorylation with 5 and 6 phosphate groups. The primary structure of donkey α_{s1} -CN A contains six Ser residues (Ser¹⁸, Ser⁷², Ser⁷⁴, Ser⁷⁶, Ser⁷⁷, and Ser⁷⁸) located in Ser-Xxx-Glu/SerP motif together with another potential phosphorylation site located at Thr¹⁰¹ (Thr¹⁰¹-Leu-Glu¹⁰³) (Fig. 2). As above discussed and by similarity to the homologous mare α_{s1} -CN, it is reasonable to hypothesize that the phosphate groups are linked to the six serines at positions 18, 72, 74, 76, 77, and 78. In comparison with mare α_{s1} -CNs, which present several phosphate groups ranging from 2 to 6 or 8, donkey counterparts are less phosphorylated. In this aspect, it should be noted that, while in donkey α_{s1} -CNs the exon 7 is constitutively spliced, the mare counterparts could carry a maximum of 6P or 8P depending on the loss or conservation of exon 7, respectively, because the amino acid sequence coded by this exon in mare milk, namely DTSNESTE, contains two potential sites of phosphorylation. A recent paper (Bertino et al., 2010), even if only by indirect data, reported that donkey α_{s1} -CN may also exist as glycosylated forms, in spite of the fact that neither human or bovine α_{s1} -CNs have been reported to be glycosylated. As above reported for the β -CN, also donkey α_{s1} -CNs show a lower GRAVY score with respect to bovine (GRAVY score -0.704) and human (GRAVY score -1.013) homologues, reflecting their more hydrophilic nature.

3.1.3. α_{s2} -casein

Donkey α_{s2} -CN is a very minor component, whereas in CM represents approximately 10% of the total casein fraction. On the contrary, this protein is absent in HM as, the human gene, due to a premature stop-codon (Rijnkels, Elnitski, Miller, & Rosen, 2003), encodes only a peptide containing 27 amino acids encompassing the signal peptide. The complete sequence of donkey α_{s2} -CN was firstly derived from the cDNA (GenBank Acc. No. CAV00691) as reported by Ramunno et al. (Ramunno, 2008). Subsequently, direct structural characterization allowed confirming the correctness of the cDNA derived sequence (Chianese et al., 2010). This protein contains 221 amino acids, a disulphide bond linking Cys³⁷ and Cys⁴¹ and shows a MW of 26,028 Da (Fig. 3; Table 2). Analogously to the α_{s1} -type caseins, α_{s2} -CN also shows a great micro-heterogeneity depending on a variable degree of phosphorylation. Indeed, the existence of multiple-phosphorylated components of this casein, containing ten to twelve phosphate groups, was reported (Chianese et al., 2010). Sequence of α_{s2} -CN shows fourteen Ser (positions: 8, 9, 17, 32, 38, 40, 62, 63, 64, 65, 66, 67, 142, and 154) located in a Ser-Xxx-Glu/SerP motif. Even if a complete characterization of all phosphorylation sites has not been reported, available data

have evidenced that ten of twelve phosphorylated sites in α_{s2} -CN A may be identified with serine residues at position 9, 17, 40, 62, 63, 64, 65, 66, 67, and 142. The great micro-heterogeneity of α_{s2} -CN also depends on the presence of internally-deleted isoforms generated by RNA incorrect splicing events. In this aspect, four α_{s2} -CN isoforms, with experimentally determined MW of 25,429, 21,939, 25,203 and 21,713 Da, were recently isolated and characterized by means of RP-HPLC, 2D-PAGE, enzymatic digestion and mass spectrometry (Saletti et al., 2012). In detail, the protein with MW 25429 Da differs from the full-length α_{s2} -CN for the deletion of the Asn¹⁷⁶-Gln¹⁸⁰ pentapeptide, whereas in the protein with MW 21939 Da, in addition to the same peptide, the sequence Asp¹²-Glu⁴² is also absent (Fig. 3). In the third isoform (MW 25203 Da), the Tyr²¹²-Leu²¹⁸ heptapeptide is absent in comparison with the full-length α_{s2} -CN, whereas in the primary structure of the component with MW 21713 Da, both the heptapeptide Tyr²¹²-Leu²¹⁸ and the sequence Asp¹²-Glu⁴² are missing. On the basis of the exon modular structure of the donkey α_{s2} -CN (Cosenza et al., 2010) (Fig. 3) it can be observed that: i) the sequence Asn¹⁷⁶-Gln¹⁸⁰, absent in the isoforms with MW 25429 and 21,939 Da, represents the first five amino acids encoded by the exon 17; ii) the sequence Asp¹²-Glu⁴², absent in the primary structure of the isoforms with MW 21939 and 21,713 Da, is encoded by exons 4, 5 and 6; iii) the heptapeptide Tyr²¹²-Leu²¹⁸, absent in the primary structure of the isoforms with MW 25203 and 21,713 Da, is the terminal part of the sequence encoded by the exon 17. On the light of these considerations, it can be hypothesized that exons 4, 5 and 6 are completely skipped in some events. On the other hand, cryptic 5' and 3' splicing sites inside exon 17 may determine the absence of the first 5 amino acids or the final 7 amino acids encoded by this exon in the final products. Taking into account these events, the full-length donkey α_{s2} -CN and the four internally-deleted isoforms have been named as following: isoform A (MW 26028 Da; pI 5.68, GRAVY score -0.908), A₁ (MW 25429 Da; pI 5.50, GRAVY score -0.883), A₁^{A4,5,6} (MW 21939 Da; pI 5.65, GRAVY score -0.929), A₂ (MW 25203 Da; pI 5.68, GRAVY score -0.987) and A₂^{A4,5,6} (MW 21713 Da; pI 5.85, GRAVY score -1.051) (Table 2). This polymorphism, as occurring for the α_{s1} -CN, seems to be correlated with the complex intron/exon modular structure of the genes which encode these proteins. Particularly, these genes consist of a large number of short exons, that may undergo differential splicing events during primary transcript processing, thus originating isoforms with different amino acid length (Lenasi et al., 2003). It is interesting to note that the skipping event involving the exons 4, 5 and 6, which encode for the long amino acid sequence Asp¹²-Glu⁴², led to the two internally-deleted isoforms A₁^{A4,5,6} and A₂^{A4,5,6} which are missing of two phosphorylation sites (e.g. Ser¹⁷ and Ser⁴⁰) and of the unique disulphide bond linking the two cysteines present in the full-length isoform A. Moreover, the above described internal deletions determine different GRAVY scores for each isoform. In this aspect, the A₂^{A4,5,6} isoform, having the lower GRAVY score (e.g. -1.051), represents the most hydrophilic protein. The complex polymorphism of donkey α_{s2} -CN is also characterized by the existence of two different CSNIS2 cDNAs, called CSNIS2 I and CSNIS2 II (Cosenza et al., 2010). The first one, constituted by 19 exons, encodes for the full-length α_{s2} -CN A and the related deleted isoforms above described. The second cDNA, analogously to the human CSNIS2 A gene, is characterized by the presence of 16 exons and encodes for a predicted protein of 160 amino acids (calculated MW of mature protein, 16,721 Da), named α_{s2} -CN B [GenBank Acc. No. CAX65660.2] (Ramunno, 2009). However, conversely to what happens in humans, for which the same locus is inactive, in donkey the CSNIS2 II gene seems to be functional, leading to an effective translation of α_{s2} -like proteins. Indeed, partial characterization of two proteins with experimentally measured MW of 16,316 and 17,238 Da, and present as minor casein components in an individual milk sample, allowed their identification as gene products of this duplicate gene CSNIS2 II (data not published). Particularly, characterization of their primary structures reveals differences with respect to the sequence predicted by cDNA translation,

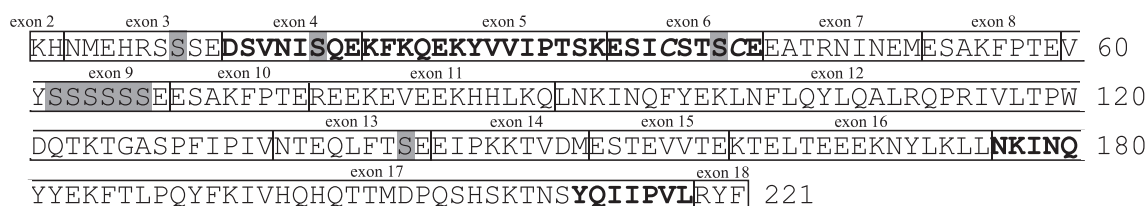


Fig. 3. Exon modular structure of the primary structure of donkey $\alpha 2$ -CN (UniProt Acc. No. B7VGF9). The exon nomenclature is that used by Miranda et al. (2004). The amino acid traits lacking in the internally-deleted $\alpha 2$ -CN isoforms are reported in bold. The experimentally characterized phosphorylation sites are shaded. The cysteine residues involved in the unique disulphide bridge are reported in bold italics.

probably related with post-transcriptional modifications (alternative splicing).

3.1.4. κ -casein

κ -casein is the only “calcium-insensitive” protein (i.e. it is not precipitated by Ca^{2+} ions) of the casein fraction. The presence of this protein in the casein fraction of *Equidae* milk has been debated for many years (Ochirkhuyag, Chobert, Dalgalarondo, & Haertle, 2000; Visser, Jenness, & Mullin, 1982). Nowadays, it is well known that κ -CN in DM represents a very minor component, with a concentration that appears lower than in mare milk and far lower than in CM or HM, and therefore its detection may appear very difficult without specific immunostaining with polyclonal antibodies (Chianese et al., 2010). A draft version of cDNA derived amino acid sequence of donkey κ -CN was reported by Hobor et al. [UniProt Acc. No. B1PLB8] (Hobor, Kunej, & Dove, 2008). Subsequently, this sequence was revised by Di Gregorio et al. [UniProt Acc. No. F0V6V5] (Di Gregorio, Laviano, & Rando, 2011) which inserted both the N- and C-terminal amino parts. It shows 162 amino acids, a MW of 18,485 Da, a pI of 9.36 and a GRAVY score of -0.413 (Fig. 4; Table 2). Unlike its bovine counterpart, carrying two cysteine residues, donkey κ -CN shows only one cysteine as well as the human homologue. Moreover, both donkey (pI 9.36) and human κ -CN (pI 8.68) show a considerably higher pH than bovine counterpart (pI 5.93), whereas the higher GRAVY score of donkey κ -CN, with respect to that of bovine (GRAVY score -0.557) and human (GRAVY score -0.528) homologue, indicates its less hydrophilic nature. Literature data about donkey κ -CN report the occurrence of genetic polymorphism at this locus as well as the presence of a micro-heterogeneity related to post-translational modifications (Chianese et al., 2010). In this aspect, contrarily to what happens for mare milk in which κ -CN has only been detected in highly glycosylated forms (Egito et al., 2001), donkey κ -CN may exist as both glycosylated and non-glycosylated forms.

3.2. Donkey whey proteins

The donkey whey proteins represent 35–50% of the nitrogen fraction, while in cow milk they are only 20%. The main components of whey proteins, as reported by Salimei et al. (2004) are represented by β -lactoglobulins (β -LGs; 29.9% of the total whey proteins), α -lactalbumin (α -LA; 22.6%), lysozyme (LYS; 21%), immunoglobulins (Igs; 11.5%), serum albumin (SA; 6.2%), and lactoferrin (LF; 4.5%), but numerous minor proteins (i.e. the low-abundance proteins), including enzymes, enzyme inhibitors, and metal-binding proteins are also present.

3.2.1. β -Lactoglobulins

β -Lactoglobulin, accounting for $> 50\%$ of the whey CM fraction, is the major bovine whey protein and also represents one of the most allergenic cow milk proteins (allergen Bos d 5; <http://www.allergen.org/index.php>) (Swaigood, 1995). On the contrary, this protein is not expressed in HM and, even if still represents the major whey protein component, it constitutes $< 30\%$ of the total whey proteins in DM. Particularly, donkey β -LG, as in mare milk, consists of two structurally diverse forms, the major β -LG I and the minor β -LG II, where the minor component is expressed by a second gene which seems to be originated by gene duplication (Conti, Godovac-Zimmermann, Liberatori, & Braunitzer, 1984; Godovac-Zimmermann, Conti, Liberatori, & Braunitzer, 1985; Godovac-Zimmermann, Conti, Sheil, & Napolitano, 1990). The expression of two different forms of β -LG represents a peculiar characteristic of equine milk with respect to the ruminants' milks. Indeed, duplicated copies of β -LG encoding gene have been also characterized in cattle, goat and sheep (Folch, Coll, Hayes, & Sanchez, 1996; Mercier & Vilotte, 1993; Passey & Mackinlay, 1995), but analogously to what has been already discussed for the human *CSNIS2* A gene, they do not lead to an effective translation of a protein (pseudogene). Donkey β -LG I and the minor β -LG II show differences in their electrophoretic mobility, chain length and primary structure. In particular, the sequence of β -LG I isoform A (Godovac-Zimmermann, Conti, & Napolitano, 1988) consists of 162 amino acids, as in ruminant milks, which results in a molecular mass of 18,524 Da, a pI of 4.53, and a GRAVY score of -0.404 , whereas β -LG II A (Godovac-Zimmermann et al., 1990) is 163 amino acid residues long, as in mare milk and, despite having one more amino acid, shows a lower molecular mass (18,258 Da; pI 4.45, GRAVY score -0.265 ; see Fig. 5 and Table 3). To date, only another genetic variant of β -LG I has been identified. This variant named as β -LG I B differs, with respect to the variant A, for three amino acid exchanges which led to a molecular mass of 18,510 Da (pI 4.59, GRAVY score -0.385) (Herrouin et al., 2000). On the contrary, donkey β -LG II locus shows a remarkable genetic polymorphism and, apart from β -LG II A, other four genetic variants (named variants B, C, D and E) have been identified (Chianese et al., 2013; Cunsolo, Costa, Saletti, Muccilli, & Foti, 2007; Herrouin et al., 2000). A micro-heterogeneity seems to be also related to the apparent N-glycosylation (Bertino et al., 2010), which represents a peculiar property of donkey's β -LGs. Moreover, unlike ruminant β -LGs (having five cysteines), and with the exception of the β -LG II A (carrying five Cys residues per molecule of protein), all the identified donkey β -LGs show four Cys residues, arranged in two disulphide bridges at the same chain position (in β -LG I A, Cys¹⁰⁶–Cys¹¹⁹ and Cys⁶⁶–Cys¹⁶⁰). As a consequence, another interesting structural characteristic differentiating equine β -LGs with respect to ruminants, in

EVQNQEQTPTCRKNDERFFDLKTVKYIPVYYVLNSSPRNEPIYYQHRLAVLINNHMPYQY 60
 YARPAAVRPHVQIPQWQVLPNIYPSTVVRHPRPHPSFIAIPPKKLQEKTVIPKINTIATV 120
 EPTPIPTPEPTVNNVAPDASSEFIIASTPETTTVPVTSPPV 162

Fig. 4. The cDNA derived amino acid sequence of donkey κ -CN (UniProt Acc. No. F0V6V5). The unique cysteine residue is underlined.

Beta-LG IA	TNIPQTMQDLDLQEVAG K WHSVMA A ASDISLLDSE E APLRVY I EKL RPTPEDNLEILRE 60
Beta-LG IIA	TDIPQTMQDLDLQEVAG R WHSVMA V ASDISLLDSE S APLRVY V EEL RPTPEGNLEILRE 60
Beta-LG IA	GENKGC A EKK I FAEKTES P AE F KINYLDEDT V FALD S DY K NYL F L M KNA A -TPGQSLVC 119
Beta-LG IIA	G A NHV C VERN I VAQ K TEDPAV F TVNY Q GERKISVLD T DY A H M F F C V GP C LPSAEHGMVC 120
Beta-LG IA	QYLARTQ M VDEE I MEK F RRALQPLPG R VQ I VPDLTRMAERCRI 162
Beta-LG IIA	QYLARTQ K VDEE V MEK F SRALQPLPG H VQ I IQ D PSGG Q ERC G F 163

Fig. 5. Alignment of the primary structure of donkey β -LG I A (UniProt Acc. No. P13613) and β -LG II A (UniProt Acc. No. P19647). The amino acid differences between the β -LG type I and II are reported in bold. The cysteine residues involved in disulphide bridges are reported in italics. The additional fifth cysteine (position 110) only present in β -LG I A is underlined.

which this protein exists predominantly as a dimer, is the maintenance of a monomeric structure at the pH of milk (Chianese et al., 2013; Polidori & Vincenzetti, 2012). Finally, with respect to the most common variants of bovine β -LG, namely A and B, having a GRAVY score of -0.167 and -0.162 , respectively, donkey β -LGs always show a considerably lower GRAVY score (see Table 3), which is related to their more hydrophilic nature.

3.2.2. α -Lactalbumin

α -Lactalbumin is a calcium-protein, in which Ca^{2+} plays a fundamental role in folding process, including the re-formation of the correct disulphide bridges after its reduction (Rao & Brew, 1989). This protein is involved in lactose synthesis in the mammary gland and represents the major source of essential amino acids in HM. α -LA in cow, camel and mare milk is present in multiple genetic forms (Conti, Godovac-Zimmermann, Napolitano, & Liberatori, 1985; Shewale, Sinha, & Brew, 1984; Uniacke-Lowe et al., 2010). On the contrary, even if the existence of two donkey α -LA isoforms was previously hypothesized, a unique genetic variant of this protein has been identified and characterized in DM (Giuffrida, Cantisani, Napolitano, Conti, & Godovac-Zimmermann, 1992). Similarly to the α -LAs of cow, goat, sheep, camel, human, and mare milk, the sequence of donkey α -LA consists of 123 amino acid residues and includes eight cysteines (Fig. 6, Table 3). α -LAs from ruminants, Equidae, and human share a high level of similarity (see below) and show similar properties. In this aspect, donkey (pI 4.88; GRAVY score -0.416) and bovine (pI 4.80; GRAVY score -0.503) α -LA have very similar pI and hydropathy, whereas the human

counterpart appears the most hydrophobic (GRAVY score -0.255) In human and bovine α -LAs, the eight cysteine residues are linked to form four intra-molecular disulphide bonds as follows: Cys⁶ to Cys¹²⁰, Cys²⁸ to Cys¹¹¹, Cys⁶¹ to Cys⁷⁷ and Cys⁷³ to Cys⁹¹. Taking into account the high similarity among donkey, human and cow α -LAs it is reasonable to hypothesize that also donkey α -LA presents the same four intra-molecular disulphide bonds occurring as in both human and bovine counterparts. Moreover, similarly to several α -LAs of both ruminant and non-ruminant species, recent results evidenced that donkey α -LA may occur as two slightly N-glycosylated isoforms (Bertino et al., 2010). However, it is important to highlight that in both ruminant and non-ruminant α -LAs, the glycosylation site is the Asn⁴⁵ in the usual consensus triplet sequence Asn-X-Ser. Instead, primary structure of donkey α -LA lacks this typical consensus triplet due to the Ser⁴⁷ $\rightarrow$ Lys substitution. So that it is possible to suppose that in donkey α -LA the glycosylation might occur at the unusual triplet Asn⁷¹-Ile-Cys, as has already been reported for the human counterpart (Giuffrida et al., 1997).

3.2.3. Lysozyme

Lysozyme is an enzyme closely related to α -LA showing similar amino acid sequence and three-dimensional structure. This protein belongs to the glycoside hydrolases, and particularly, since it catalyses the hydrolysis of glycosidic bonds of mucopolysaccharides in bacterial cell walls, it is known to be a natural antimicrobial agent. In comparison with other milks, but similarly to that from mare, DM shows a high LYS content (1.0 mg/mL) (Vincenzetti et al., 2008) that may be

Table 3
Properties of the most abundant donkey whey proteins.

Protein	Calculated MW (Da) ^a	pI ^b	GRAVY score ^c	AA	Post-translational modifications	UniProt Acc. No.	Amino acid differences ^d	Disulphide bonds	Free sulphhydryl groups
β -LG I					N-glycosylation	P13613			
β -LG I A	18,528.3	4.53	-0.404	162			–	2	0
β -LG I B	18,514.3	4.59	-0.385	162			Ser ³⁶ ; Thr ⁹⁷ ; Ile ¹⁵⁰	2	0
β -LG II					N-glycosylation	P19647			
β -LG II A	18,262.7	4.45	-0.265	163			–	2	1
β -LG II B	18,226.6	4.45	-0.306	163			Pro ¹¹⁰ ; Thr ¹¹⁸	2	0
β -LG II C	18,240.6	4.47	-0.306	163			Glu ⁹⁶ ; Pro ¹¹⁰ ; Thr ¹¹⁸	2	0
β -LG II D	18,314.7	4.39	-0.309	163			Pro ¹¹⁰ ; Asp ¹⁶²	2	0
β -LG II E	18,257.7	4.45	-0.320	163			Asn ² ; Lys ¹⁸ ; Ala ²⁵ ; Pro ¹¹⁰	2	0
α -LA	14,214	4.88	-0.416	123	N-glycosylation	P28546		4	0
LYS						P11375			
LYS A	14,687.7	8.01	-0.624	129			–	4	0
LYS B	14,639.6	7.77	-0.641	129			Asp ⁴⁹ ; Ser ⁵² ; Asn ⁶¹	4	0
SA	65,618.2	5.60	-0.427	583		Q5XLE4	–	17	1
LF	75,356.6	7.69	-0.363	689	N-glycosylation	O77811^e	–	17	0

^a Molecular masses were calculated considering the cysteines in reduced form ($-\text{SH}$).

^b Isoelectric point calculated by GPMW considering the cysteines in reduced form ($-\text{SH}$), and based on the algorithm of Skoog & Wichman, 1986.

^c Grand average hydropathy (GRAVY) score is calculated by GPMW using the scale of Kyte & Doolittle, 1982.

^d Amino acid differences are reported with respect to the isoform A of each protein.

^e The Accession Number is referred to the mare lactoferrin.

KQFTKCELSQVLKSMGDKGVTLPEWICTIFHSSGYDTQTIVKNNNGKTEYGLFQINNKMW 60
 CRDNQILPSRNICGISCNKFLLDDDLTDDVMCAKKILDSEGIDYWLAKHPLCSEKLEQWLC 120
 EEL 123

Fig. 6. Primary structure of donkey α -LA (UniProt Acc. No. P28546). The eight cysteine residues involved in disulphide bridges are reported in *italics*. The N-glycosylation site (asparagine at position 45) is shaded.

KVFSKCELAHKLKAQEMDGFGGYSLANWVCMAYESNFNTRAFNGKNANGS**Y**DYGLFQLN 60
 SKWWCKDNKRSSSNACNIMCSKLLDDNIDDDISCAKRVVRDPKGMSAWKAWVKHCKDKDL 120
 SEYLASCNL 129

Fig. 7. Primary structure of donkey LYS isoform A (UniProt Acc. No. P11375). The eight cysteine residues involved in disulphide bridges are reported in *italics*. The three amino acid differing with respect to the variant B are reported in **bold**.

responsible for the low microbial load found in this milk. Moreover, the high concentration of LYS together with the presence of other proteins including immunoglobulins, lactoferrin and lactoperoxidase, may be useful in the infant digestive tract to reduce the incidence of gastrointestinal infections (Tidona et al., 2011). In DM two variants of LYS, differing for three amino acid substitutions (e.g. N⁴⁹ → D, Y⁵² → S, S⁶¹ → N) have been identified (Godovac-Zimmermann et al., 1988; Herrouin et al., 2000). The two isoforms, namely A and B, show 129 amino acid residues and a molecular mass, in the oxidized form, of 14,680 (pI 8.01; GRAVY score −0.624) and 14,631 Da (pI 7.77; GRAVY score −0.641), respectively (Fig. 7, Table 3). Both the variants have eight cysteine residues which are linked to form four intra-molecular disulphide bonds as follows: Cys⁶ to Cys¹²⁷, Cys³⁰ to Cys¹¹⁵, Cys⁶⁵ to Cys⁸⁰ and Cys⁷⁶ to Cys⁹⁴. Donkey LYS, with respect to its bovine (GRAVY score −0.424) and human (GRAVY score −0.485) counterparts, has a lower GRAVY score which reflects its more hydrophilic nature.

3.2.4. Serum albumin

Serum albumin is the major protein found in blood serum, but occurs in many body tissues and secretions. This protein, also present in milk, is not synthesized in the mammary gland but it is presumed to enter the milk by leaky junctions between the mammary epithelial cells or by uptake with other components such as immunoglobulins. In DM, the primary structure of SA (UniProt Acc. No. Q5XLE4) was firstly derived from the corresponding cDNA sequence (Li, Tang, & Pingfan, 2004). Subsequently, direct characterization by MS analysis (Cunsolo, Saletti, et al., 2007) confirmed about 98% of the cDNA deduced sequence but also evidenced, similarly to the almost all mammalian's serum albumins, the presence of a valine residue at position 497 with respect to an isoleucine reported in the cDNA deduced sequence (Fig. 8). Mature chain of donkey SA shows 583 amino acid residues, a calculated molecular mass of 65,584 Da (Table 3) and a comparable hydropathy (GRAVY score −0.427) with respect to the bovine (GRAVY score −0.475) and human (GRAVY score −0.395) counterpart. Analogously to the SA of other species, it has thirty-five cysteines residues. One of them is present as cysteinyl residue, while the other thirty-four are linked to form seventeen disulphide bridges per molecule.

3.2.5. Lactoferrin

LF is an iron-binding protein which shows different levels of glycosylation and displays many biological functions such as regulation of iron homeostasis, cellular growth, anti-microbial and anti-viral functions, and protection against cancer development and metastasis (Ward, Paz, & Conneely, 2005). This protein (also called lactotransferrin) is present in all mammalian milk and constitutes, with the serotransferrins present in serum of all vertebrates and with the ootransferrins in the egg white of birds, a homogenous family of proteins

called transferrins. Due to its high content in HM (6–8 mg/mL in colostrum and 2–4 mg/mL in mature milk) (Garcia-Montoya, Cendon, Arevalo-Gallegos, & Rascon-Cruz, 2012), and considering its important multifunctional roles, LF has attracted huge scientific interest since 1960. LF concentration in ruminant milks is 10–100 times lower (in the range of 0.02–0.2 mg/mL) (Farrell et al., 2004; Hiss, Meyer, & Sauerwein, 2008) than in HM, whereas in *Equidae* milk (Uniacke-Lowe et al., 2010) it shows an intermediate concentration between the higher content of HM and the lower of ruminants. Only recently, the amino acid sequence of donkey LF has almost been completely characterized (99% sequence coverage) by Gallina et al. coupling cationic exchange purification, enzymatic digestions, 4-sulfo-phenyl isothiocyanate (SPITC) derivatization of tryptic digest, and different mass spectrometric platforms (Gallina et al., 2016a). Primary structure of donkey LF, at least for the covered sequence (Fig. 9), is closely related with the genomic deduced sequence from mare (UniProt Acc. No. O77811), differing only by five point substitutions, located at positions 91 (Arg → His), 328 (Thr → Ile/Leu), 466 (Ala → Gly), 642 (Asn → Ser), and 668 (Ser → Ala). It shows thirty-four cysteines linked to form seventeen disulphide bonds. Analysis of the glycosylated donkey LF showed that 26 different N-glycans structures are linked to the protein via an amide bond to asparagine residues located at the positions 137, 281, and 476 (Gallina et al., 2016b). Finally, donkey LF has a GRAVY score of −0.363, a value that is comparable to that of bovine (GRAVY score −0.350) and human homologue (GRAVY score −0.415), probably due to their similar hydropathy.

3.2.6. Immunoglobulins and low-abundance proteins

Three classes of Igs are commonly found in milk: IgG, IgA, and IgM, varying in the structure, molecular weight and saccharide content. Together with enzymes like lactoferrin, lactoperoxidase and lysozyme, Igs protect the immature neonate's immune system. Particularly, secretory Igs are resistant to proteolytic activity of gastrointestinal tract and prevent the adherence of bacteria to mucosal surfaces neutralizing toxins from microorganisms. Due to their antimicrobial functions, the amount of Igs is very high in colostrum while decreases during lactation stages. Particularly, IgG represents the principal immunoglobulin (65%–95% of total Igs) in mammalian colostrum. With respect to cow milk, equine whey milk protein fraction presents a higher level of total Igs (Uniacke-Lowe et al., 2010), that account for about 20% of mature milk whey proteins and about 60% of colostrum whey proteins. As lactation proceeds, Igs content changes, with differences related to species. In mature milk from bovine, IgG is the predominant immunoglobulin; on the contrary, in human and equine mature milk Immunoglobulins A are the most representative antibodies (Tizard, 1987).

The immunoglobulins, together with caseins, α -LA, β -LGs, LF, LYS, and SA constitute > 90% of the mass of total milk proteins in donkey

DTHKSEIAHRFNDLGEKHFGLVLVAFSQYLQQCPFEDHVKLNVNEVTEFAKKCAADESAE 60
 NCDKSLHTLFGDKLCTVATLRTYGEADCCCKQEPERNECFLTHKDDHPNLPKLKPEPD 120
 AQCAAFQEDPDKFLGKLYEVARRHPYFYGPPELLFHAEYKADFTECCPADDKAGCLIPK 180
 LDALKERILLSSAKERLKCSSFQKFGERAFAKWSVARLSQKFPKADFAEVSKIIVTDLTKV 240
 HKECCHGDLLECADDRADLTKYICEHQDSISGKLKACCDKPLLQKSHCIAEVKEDDLPSD 300
 LPALAADFAEDKEICKHYKDAKDVLGTFLYEYSRRHPDYSVSLLLRIAKTYEATLEKCC 360
 AEADPPACYATVFDQFTPLVEEPKSLVKKNCDLFEEVGEYDFQNALIVRYTKKAPQVSTP 420
 TLVEIGRTLGVGSRCKLPESERLPCSENHLALALNRLCVLHEKTPVSEKITKCCTDSL 480
 AERRPCFSALELDEGY**V**PKEFKAETFTFHADICTLPEDEKQIKKQSALAEVLVHKPKATK 540
 EQLKTVLGNFSAFVAKCCGAEDKEACFAEEGPKLVASSQLALA 583

Fig. 8. Primary structure of donkey serum albumin (UniProt Acc. No. Q5XLE4). The thirty-five cysteine residues are reported in italics. The presence of a valine residue at position 497 with respect to an isoleucine, as reported in the cDNA deduced sequence, is reported in bold.

milk. The remaining 10%, comprises hundreds of very low-abundance proteins (including the milk fat globule membrane proteins described in next paragraph) that are very difficult to detect without specific sample preparation, but represent an important population of biological interest. So that, methods to reduce the complexity of protein mixtures by removing highly abundant species are rapidly becoming an essential first step in proteomic analyses. As above reported, the combinatorial peptide ligand library technology represents one of the most powerful technique allowing access to very-low-abundance proteins. In particular, every time the CPLL was applied to protein extracts, the number of species detected was significantly increased respect to the control, ranging from a factor of two to a factor of five. Recently, the CPLL technology allowed discovering and identifying a total of 106 unique gene products in DM (Cunsolo et al., 2011), 88 of which (corresponding to 83% of the total identification) were not described in previous proteomic studies, and therefore represent the most extensive profiling of this milk at present. Gene ontology (GO) analysis performed about these low-abundant species revealed that proteins associated with nutrient transport and the immune system response are significantly enriched in the donkey whey fraction.

3.3. Milk fat globule membrane proteins

Milk protein fraction also contains a group of proteins associated with the milk fat globule membranes (MFGM). Proteomic analysis of these membranes may highlight not only some of the signalling and secretory pathways used by the mammary gland, but also provides an additional insight into this membrane's cellular origin (Reinhardt, & Lippolis, 2006). MFGM proteins in mature milk account only for 1–4% of total protein content, but in colostrum represent the principal components together with immunoglobulins. Over the last decade growing attention has been paid at MFGM proteins for their involvement in various defense mechanisms in the newborn (Cavaletto, Giuffrida, & Conti, 2008). It should be highlighted that different milk treatment technologies lead to different types of MFGM-enriched fractions, the so-called whey protein concentrates and buttermilk protein concentrates, showing different characteristics in terms of protein content and composition (Affolter, Grass, Vanrobaeys, Casado, & Kussmann, 2010). To date, the proteomic investigation of MFGM proteins is limited to human (Fortunato et al., 2003; Spertino et al., 2012), cow (Affolter et al., 2010), water buffalo (D'Ambrosio

APRKSVRWCTISPAAEAAKCAKQFQRNMKKVRGSPVSCIRKTSSFEICQAI AANKADAVTLD 60
 GGLVYEAGLHPYKLRPVAAEVYQTRGKPQTHYYAVAVVKKGSGFQLNQLQGVKSCHTGLG 120
 RSAGWNIPIGTLPYL**N**WTGPPEPLQKAVANFFSASCVP CADGKQYPNL CRLCAGTEADK 180
 CACSSQEPYFGYSGAFKCLENGAGDVAFVKDSTVFENLPDEADRDYELLCPDNTRKPV 240
 AFKECHLARVP SHAVVARSDGREDLIWRL LHRAQE EFGR**N**KSSAFQLFKSTPENKDLLF 300
 KDSALGFVRIPSQIDSGLYLGANYLTAL**L**QNLRETA AEVAARRERVVWCAVGPEEERKCKQ 360
 WSDVSNRKVACASASTTEECIALVLKGEADALNLDGGFIYVAGKGLVPVLAENQKSQNS 420
 NAPDCVHRPPEGYLAVAVVRKSDADLTWNSLSGKKSCHTGVGRTAGWNIPMGLLF**N**QTGS 480
 CKFDKFFSQSCAPGADPQSSLCALCVGNNEENENKMPNSEERYGYTGAFRCLA EKAGDV 540
 AFVKDVTVLQNTDGKNSEPWAKDLKQEDFELLCLDGRKPVAEAESCHLARAPNHAVVSQ 600
 SDRAQHLLKKVLFLQQDQFGNGPDCPGKFLFKSETKNLLF**A**DNTECLAELQGKTTYEQY 660
 LGSEYVT**A**ITNLRRCSSSPLEACAFLRA 689

Fig. 9. Primary structure of donkey lactoferrin (UniProt Acc. No. O77811 for mare LF). The five amino acid differences with respect to the mare lactoferrin taken as reference are reported in bold. The thirty-four cysteine residues involved in disulphide bridges are reported in italics. The three N-glycosylation sites (asparagines 137, 281 and 476) are shaded.

et al., 2008), goat (Cebo, Caillat, Bouvier, & Martin, 2010), sheep (Pisanu et al., 2011), and mare (Barello et al., 2008). On the contrary, a systematic proteomic investigation of donkey MFGM is still lacking. However, the presence of some major (e.g. butyrophilin, adipophilin) and minor (e.g. apolipoprotein A, clusterin, polymeric immunoglobulin receptor) proteins associated to MFGM, already found in other milks, was reported in donkey whey protein concentrates (Cunsolo et al., 2011).

4. Bioactive peptides from donkey milk

In addition to the properties reported in the introduction section, DM shows other interesting biological activities, such as antioxidant activity, immunostimulating ability, anti-inflammatory effects, antimicrobial and antiviral action (Brumini et al., 2013; Bucevic-Popovic, Delas, Medugorac, Pavela-Vrancic, & Kulisic-Bilusic, 2014; Jirillo, Jirillo, & Magrone, 2010). So that, recently many investigations have been focused on the bioactivity of milk peptide fraction and their potential use in food formulations. In this aspect, it is should be noted that, with the exception of α -lactalbumin, lysozyme and lactoferrin, the most abundant milk proteins show little or no bioactivity in their native state, but represent the main source of food-derived biologically active peptides. These peptides remain inactive within the sequence of native protein but can become active upon hydrolysis by proteolytic enzymes during gastrointestinal transit or during food processing (ripening, fermentation and cooking) (Carrasco-Castilla, Hernandez-Alvarez, Jimenez-Martinez, Gutierrez-Lopez, & Davila-Ortiz, 2012; Schanbacher, Talhouk, & Murray, 1997). Otherwise, a lot of peptides with biological activity can be part of the endogenous milk peptidome. Recently, an extensive peptidomic study in commercial donkey milk samples was carried out by two different peptide purification strategies, providing a total of 1330 peptide identifications (Piovesana et al., 2015). As already assessed for milk from different mammals also for DM, the majority of peptides, mainly arising from the action of plasmin, belongs to the most abundant milk proteins (α _{s1}- and β -caseins) (Baum, Fedorova, Ebner, Hoffmann, & Pischetsrieder, 2013). Bioactivity assays of the pool of these peptides revealed an Angiotensin Converting-Enzyme (ACE) inhibitory activity of 67% and an antioxidant activity of 35%. On the other hand, the characterization of the peptidic map derived from the in vitro simulated gastrointestinal digestion of donkey milk (Bidasolo, Ramos, & Gomez-Ruiz, 2012; Tidona et al., 2011) allowed to confirm that most of the peptides are produced by the rapid digestion of α _{s1}- and β -caseins. The β -lactoglobulin I and lactoferrin are two other sources of peptides, while α -lactalbumin and lysozyme, as already observed in other mammalian milks (Inglingstad et al., 2010), represent the most resistant proteins to gastrointestinal enzymes. Among the identified peptides, many fragments exhibit antimicrobial activity, numerous sequences possess the typical characteristic of ACE-inhibitory peptides (Zenezini Chiozzi et al., 2016), while others show potential antioxidant activity. Even if further studies are needed to evaluate other bioactivities and to confirm the potential of donkey milk as functional food, these results offer a preliminary insight into the possible protein degradation mechanism of donkey milk and may provide an appropriate model to investigate the release of gastrointestinal resistant peptides and the effects on gut health.

5. Effects of thermal treatments on donkey milk

The high content of lysozyme and other non-specific antimicrobial compounds gives to raw fresh DM a long natural shelf life and makes a prolonged heat treatment, normally required for preserving hygienic quality and nutritional properties, unnecessary. However, because thermal treatments are needed in order to guarantee milk healthiness and quality over time, the effects that processing has on the valuable DM components have been assessed (Cosentino et al., 2016; Giacometti et al., 2016). The most common method of sanitization is the

pasteurization at 63 °C, which generally does not affect the main nutritional and health properties of milk. Other preservation methods, such as drying (spray-drying and freeze-drying), frozen at – 20 °C and high temperature sterilization, allow a longer storage but can significantly affect milk protein composition and their functional and nutritional properties. It has been shown that both whey proteins and fat soluble nutrients of DM decrease with increasing temperature and heat processing time (Sorrentino et al., 2005). Major modification of the nitrogenous profile occurs after heating milk at 90 °C for 1 min. The thermal stability of equine lactoferrin and serum albumin is comparable with that of their bovine homologues; on the contrary, equine α -LA and β -LG show a higher heat stability with respect to the corresponding bovine proteins (Bonomi, Iametti, Pagliarini, & Solaroli, 1994), a feature that may be related to the rare cases of weak DM allergenicity (Gall, Kalveram, Sick, & Sterry, 1996). Frozen at – 20 °C and the spray-drying powdering process (90 °C for 25 s, then dried at 170 °C) don't significantly affect the total whey proteins content, which however decreases with respect to the raw DM. On the contrary, the content in total caseins notably decreases in frozen and, particularly, in powdered milk, which shows the lowest amount if compared to the fresh and frozen DM (Polidori & Vincenzetti, 2013). The lysozyme content and its activity remain the same in fresh as well as in frozen milk, indicating that the freezing and thawing out process in DM does not affect the activity of this important enzyme. In contrast, although lysozyme shows a high thermal stability, its denaturation starts from temperatures above 70 °C. In spray-dried powdered milk, even if the amount of lysozyme doesn't change with respect to that of fresh DM, it shows a 30% residual activity with respect to fresh and frozen milk, indicating that the spray-dry process, utilized in the powderization of milk, strongly affects the activity of this enzyme. In view of this, the lyophilization, or freeze-drying, a powdering process carried out at low temperature and vacuum, seems to be more protective to the sensitive components of the milk. In fact, fresh and freeze-dried (lyophilized) DM show a similar protein composition in terms of caseins and total proteins, except for α -LA. Moreover, lysozyme maintains its high level and enzymatic activity (Polidori & Vincenzetti, 2013). More recently, a comparison of the effects on the physicochemical quality and shelf-life of treated and raw (untreated) DM by ultra-high pressure homogenization (UHPH) treatments and different pasteurization processes have been reported (Addo & Ferragut, 2015). In this aspect, the UHPH treatment appears to be a processing technology comparable or better than pasteurization in terms of microbiological quality with a shelf-life of 28 days at least. It proves also to have a positive effect on the lysozyme activity similar to pasteurization, maintaining the activity over the storage period. Available data about the effects that thermal treatments have on DM components seems to indicate very similar quality parameters for fresh, UHPH and lyophilized DM compared with frozen or powdered DM. Even if further investigations are needed, these results may be important in the choice of valid methods that should be adopted for the storage or milk processing by the commercial donkey milk processing industries.

6. Donkey milk as alternative choice in the treatment of cow milk protein allergy: the clinical trials

Among allergenic foods, it is well known that milk represents the main source of allergens in early childhood: in developed countries, approximately 2–3% of infant younger than one year of age are allergic to cow milk proteins (CMP) (Sicherer & Sampson, 2010). The proteins most frequently and most intensively recognized by IgE are the α _{s1}-CN and β -LG, even if the other caseins, α -LA and components such as lactoferrin, IgG and bovine serum albumin are capable of inducing immune responses (Wal, 2004). On the other hand, even the balance between caseins and whey proteins seems to play an important role in the sensitization capacity of CM and its modification (e.g. with a casein/whey protein ratio of 40:60) may reduce its allergenicity (Lara-

Table 4

List of the major (recognition > 60%) and minor frequently recognized IgE-binding epitopes of the major cow's milk allergens in transient and persistent CMPA. In bold are reported the IgE-binding epitopes recognized only by the patient with persistent CMPA.

Allergen (Bibliog. ref.)	Recognized AA regions by patients with < 3 years age		Recognized AA regions by patients (> 3 years age) with persistent CMPA	
	Major epitopes	Minor epitopes	Major epitopes	Minor epitopes
α s ₁ -CN (Chatchatee, Jarvinen, Bardina, Beyer, et al., 2001; Jarvinen et al., 2002)	–	17–36 39–48 (very poorly recognized) 93–102 (very poorly recognized) 109–120 (very poorly recognized) 123–132 (very poorly recognized) 139–154 159–174	17–36 69–78 93–102 109–120 173–194	39–48 123–132 139–154 159–174
α s ₂ -CN (Busse, Jarvinen, Vila, Beyer, & Sampson, 2002; Jarvinen et al., 2002)	145–154 159–168	87–96	83–100 143–158 157–172 165–188 171–180 105–114 117–128 191–200	31–44 33–42 43–56 93–106 105–114 117–128 191–200
β -CN (Chatchatee, Jarvinen, Bardina, Vila, et al., 2001)	1–16 45–54 83–92 107–120 135–144	57–66	1–16 45–54 55–70 83–92 107–120 135–144	149–164 167–184
κ -CN (Busse et al., 2002; Jarvinen et al., 2002)	17–26 21–44 53–64	–	9–26 17–26 21–44 47–68 67–78	95–116 111–126 137–148 149–166
α -LA (Jarvinen et al., 2002; Jarvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001; Maynard, Jost, & Wal, 1997)	–	13–22	1–16	13–26 47–58 (very poorly recognized) 93–102 (very poorly recognized)
β -LG (Jarvinen et al., 2002; Maynard et al., 1997)	49–60 75–84 119–128 143–152	67–76 127–136	47–60 67–78 75–86 127–144 141–152	1–16 31–48
SA (Tanabe et al., 2002)	107–123 364–382 451–459	–	107–123 336–345 364–382 451–459	–

Villoslada, Olivares, & Xaus, 2005). Although most children outgrow CMPA by 3 years of age, in a minority of allergic subjects it can become persistent. So that, taking into account that the basic treatment of CMPA is avoidance of CMP, and considering that breast feeding is not always possible, indicated or sufficient, alternative supplies are indispensable. Many clinical trials demonstrated that sheep and GM (Ah-Leung et al., 2006), and partially hydrolysed formulas (Carroccio et al., 2000; Walker-Smith, 2003) should not be used because adverse reactions (i.e. cross-reactivity) have been experienced with these products. Soy infant formula represents an additional alternative choice, even if it is not recommended for young children with CMPA because of the great risk of developing allergy to soy (Candeva et al., 2015; Zeiger et al., 1999). Cow milk based extensive hydrolysate formulas (EHFs) remains the first option for the majority of patients, while amino acid formulas

must be used for the most severe cases. However, the use of EHFs is hampered by a poor, bitter taste and because of the possibility of residual antigenicity/allergenicity even to EHFs for highly sensitised individuals. Moreover, also the amino acid formulas, considered the only non-allergenic breast-milk substitute, have unpleasant bitter taste that often precludes their use in infant diet (Host & Halken, 2004). As mentioned above, many clinical trials showed that DM adequately supplemented, even if did not achieve the 90% tolerability value required to define a hypoallergenic formula, appears a good natural substitute of CM in most children with severe IgE-mediated CMA (Mansueto et al., 2013). In this aspect, it is important to note that the use of a natural milk as feeding resources rather than formulas should be encouraged because it contains many functional components which are involved in the protection of the newborn against infections and stimulate the functional recovery and development of the neonate intestine. In the first clinical evidence (Iacono et al., 1992), 9 unweaned infants, with severe symptoms of CMA and multiple food hypersensitivity, were fed using supplemented DM. Authors reported that DM was well tolerated by all patients, with satisfactory growth rate and without negative clinical reactions. The follow-up of the patients showed that DM was tolerated without any problems up to an age ranging from 15 to 20 months, when CM was reintroduced in some patients. Subsequent clinical studies confirmed DM tolerability (Carroccio et al., 2000; Monti et al., 2007; Monti et al., 2012). Particularly, a crossover randomized-controlled trial aimed to compare the tolerability of DM and GM in twenty-six children (age 6 months-3 years) with atopic dermatitis evidenced that DM may represent a better substitute of CM than the currently used GM (Vita et al., 2007). In fact, the results of this study indicated that DM was tolerated by 88% of the children with CMPA and produced a significant improvement in atopic dermatitis. On the contrary, in all the children receiving GM, symptoms remained unchanged or even worsened. On the other hand, the results today available, even if suggest that DM can be regarded as a valid dietary alternative for children with CMA IgE- and non IgE-mediated, cannot be considered conclusive. So that its tolerability must firstly be tested by a supervised titrated oral challenge test (Businco et al., 2000; Tesse, Paglialunga, Braccio, & Armenio, 2009).

7. Donkey milk as valid alternative in the treatment of cow milk protein allergy: the molecular basis

As known, CMPA represents the most common food allergy in children. Therefore, as milk represents the main source of nutrition for infants, one of the major objectives of paediatrics is the finding of appropriate alternative supplies miming the breast milk composition, with a pleasant taste, good nutritional values, and not evoking allergic reactions. Regarding the possibility of cross-reactivity, it should be considered that few biochemical characteristics (e.g. abundance in the food, presence of multiple linear epitopes, resistance to digestion and processing etc.) can be associated with food allergens, and consequently we cannot as yet predict the allergenicity of a given food protein (Bannon, 2004; Breiteneder & Mills, 2005). However, it is known that cross-reactive allergenic proteins, regardless of their source, usually show very similar three-dimensional conformations (conformational epitopes) or even similar linear epitopes (Restani, Beretta, Fiocchi, Ballabio, & Galli, 2002). Many linear IgE-binding epitopes of CM allergens have been mapped for both patients with persistent (i.e. long-life) or transient (i.e. who subsequently outgrew their allergy) CMPA, and are reported in literature (Monaci, Treggoat, van Hengel, & Anklam, 2006) (see Table 4). Most of them range in length from 8 to 18 amino acids, but no obvious sequence motif shared by all peptides has been identified. Clinical trials evidence that people with persistent CMPA show significantly elevated levels of casein-specific IgE antibodies compared with younger children and may recognise IgE-binding epitopes unrecognised by any of the patients with transient CMPA (Chatchatee, Jarvinen, Bardina, Beyer, & Sampson, 2001; Jarvinen

et al., 2002). With the aim to identify “critical” amino acids within allergenic epitopes, mutational analyses of the major sequential IgE-binding epitopes of CM allergens have been carried out. It was found that the substitution of one or two amino acids (especially hydrophobic residues) in the central portion of the epitope in the most of cases decreases or induces a loss of antibody when pooled sera from children with CMA are used. However, analyses carried out using individual patient sera have shown a more heterogeneous pattern of critical amino acids necessary to eliminate IgE antibody in some of these epitopes (Cocco, Jarvinen, Han, Beyer, & Sampson, 2007; Cocco, Jarvinen, Sampson, & Beyer, 2003; Han et al., 2008). However, it is important to note that also post-translation modifications such as glycosylation and phosphorylation may also influence the IgE immunoreactivity. Indeed, N-glycosylation can have a significant stabilizing or rigidifying effect on protein structure, conferring resistance to denaturation (Pedrosa, De Felice, Trisciuzzi, & Ferreira, 2000), whereas modification or elimination of the major phosphorylation sites of caseins may decrease the IgE response (Bernard, Meisel, Creminon, & Wal, 2000). So that, up to day it is difficult to predict new IgE-binding epitopes, and only those sharing very high sequence identity with already determined epitopes may potentially cause adverse reactions.

As above reported, many clinical trials have clearly demonstrated that, even if with adequate lipid integration, DM may represent a valid natural substitute of cow milk for feeding allergic children. However, to date, the molecular basis of DM tolerance for a large number of cow milk allergic subjects has not yet been fully clarified. It is reasonable to assume that the reduced allergenic properties of DM are related to the protein fraction composition, which is much closer to HM than to CM, and in particular to the structural differences of its protein components with respect to the bovine homologues. On the other hand, it should be highlighted that, even if the cross-reactive proteins of CM and DM are still unknown, a minor number of highly problematic cow milk allergic children may show weak and aspecific reactivity vs. donkey milk proteins (Monti et al., 2007). Taking into account these considerations, the information obtained during the last decade about the primary structure of the main components of DM provide the basis for the comparison not only with the bovine and human counterparts, but also with the homologues from goat and sheep milk which are often suggested as alternative to CM, even if their use remains strongly debated. To this aim, sequence relationships between the most abundant milk proteins of these species were performed by the Clustal Omega Tool (<http://www.clustal.org/omega/>). This comparison, reported in Table 5, shows that the primary structure of donkey caseins presents significant differences with respect to that of the other species, but is always more closely related with human counterparts. As above reported, even if whole bovine casein fraction may act as a potent allergen, α_{s1} - and α_{s2} -CNs represent the most intensively recognized components of this fraction (Monaci et al., 2006). These two proteins have a reduced tertiary structure and therefore their major allergenic epitopes are sequential (linear epitopes) rather than conformational. In this aspect, it is interesting to note that most of the amino acid sequences of the major allergenic linear epitopes (see Fig. S1, Supplementary material) of these two bovine caseins have remarkable differences with respect to the corresponding domains present in donkey counterparts (Cunsolo, Cairone, Fontanini, et al., 2009; Saletti et al., 2012). Moreover, as above described, donkey milk shows two internally-deleted α_{s2} -CN isoforms (e.g. A₁^{A4,5,6} and A₂^{A4,5,6}) which don't present two phosphorylation sites and the unique disulphide bond present in the full-length isoform A. Taking into account that elimination of phosphorylation sites may reduce IgE response to caseins (Bernard et al., 2000), and the absence of disulphide bonds may decrease the resistance to denaturation, the post-transcriptional events generating these two internally-deleted isoforms may contribute to the low allergenic properties of DM. On the other hand, donkey α_{s2} -CNs present the short amino acid sequence YEKFTLPQY, which differs only for two “conservative” (i.e. substitutions involving amino acids sharing similar characteristics)

(French, & Robson, 1983) amino acid substitutions (E → Q; T → A) with respect to the bovine trait Y¹⁷¹QKFALPQY¹⁸⁰, a linear epitope exclusively recognized by patients with persistent CMA. These considerations, together with the lower amount of α_s -caseins in DM with respect to cow's one, could be related to the documented low allergenic properties of DM, as well as to the very weak cross-reactivity in a minority of highly problematic cow milk allergic children. In this aspect, it should be also highlighted that HM does not contain the α_{s2} -CN, whereas the primary structure of human α_{s1} -CN, a minor component of the human casein fraction, shows huge differences (only 29% of residues are conserved) with respect to that of homologue from CM and it is more closely related with the donkey counterpart (Table 5). Similarly, both β - and κ -caseins from human and donkey are more closely related to each other than the bovine counterparts, which again contain linear epitopes having remarkable differences with respect to the corresponding domains present in donkey homologues (see Fig. S2, Supplementary material). In this aspect, it is well known that evolutionary distance from human homologues reflects allergenicity of animal food proteins. Indeed, investigation about the structural relationships of animal food allergens and their evolutionary relatedness to human homologues showed that, in general, proteins with a sequence identity ≤ 54% to human homologues might be in principle allergenic, whereas those with a sequence identity > 63% are rarely recognized by the human immune system (Jenkins, Breiteneder, & Mills, 2007).

Table 5 also evidences that the caseins from cow, goat and sheep are much more closely related each other than the human and donkey counterparts. In addition, the alignment of α_{s1} -, α_{s2} -, β - and κ -CNs from cow, goat and sheep (Fig. S3, S4, S5 and S6) shows that most of bovine linear epitopes have a strict resemblance (one or two amino acid substitutions, many of which are “conservative”) or share the same primary structure with respect to the corresponding domains present in caprine and ovine counterparts. Taking into account that these three ruminants belong to the *Bovidae* family, and therefore present strict phylogenetic relationships, these data are not surprising and may also explain why most of the patients allergic to CM cannot also tolerate goat or sheep milk. Similarly, the most abundant whey proteins from these ruminants are strictly related each other (see Table 5) and again evidencing a strong resemblance or the same sequence between the bovine epitopes and the corresponding domains present in caprine and ovine counterparts (Fig. S7). On the other hand, it is interesting to note that, unlike casein components, three whey proteins (e.g. α -LA, SA and LF) from donkey and human milk share high level of identity (> 70%) with their bovine homologues. However, a comparison of the linear epitopes of bovine α -LA and SA (Tanabe et al., 2002) (see Fig. S8 and S9, Supplementary material) with the corresponding domains present in the donkey's homologues reveals that almost all show strong differences, with the exception of two epitopes: S⁴⁷TEYGLFQINNK⁵⁸ of α -LA and H³³⁶PEYAVSVLL³⁴⁵ of SA. In this aspect, the epitope Ser⁴⁷-Lys⁵⁸ of bovine α -LA, which represents a poorly recognized antigenic region, differs for a single (e.g. Ser⁴⁷ → Lys) “semi-conservative” substitution (i.e. substitution involving amino acids sharing weak similar characteristics) with respect to the corresponding trait of donkey α -LA. Moreover, as above reported, a single substitution within IgE-binding epitopes involving residues with different chemical and physical properties usually decreases or may induce a complete abrogation of IgE binding. Similarly, the epitope His³³⁶-Leu³⁴⁵ of bovine SA show three “conservative” substitutions (e.g. Glu³³⁸ → Asp, Ala³⁴⁰ → Ser and Val³⁴³ → Leu) with respect to the corresponding trait of donkey counterpart. Altogether, these data may explain the observed low allergenic properties of DM or may be related to the weak cross-reactivity in a minority of highly problematic cow's milk allergic children.

Finally, the most abundant and most recognized allergen of bovine whey fraction, namely the β -LG, shares low sequence identity (< 56% of identity) with respect to its equine counterparts, whereas it is absent in HM (Table 5). This sequence discrepancy also involves the allergenic epitopes, which show remarkable amino acid differences with respect

Table 5

Sequence comparison by Pairwise Sequence Alignment EMBOSS Needle tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) of major milk proteins from different species and their human homologues.

Proteins (No. amino acids)	Swiss-Prot Acc. No.	Sequence comparison (% identity)			
		Bovine homologue	Caprine homologue	Ovine homologue	Human homologue
α_{s1} -casein					
Donkey (202 AA)	P86272	40	42	40	41
Cow (199 AA)	P02662	100	87	87	29
Goat (199 AA)	P18626	87	100	97	30
Sheep (199 AA)	P04653	87	97	100	29
Human (170 AA)	P47710	29	30	29	100
α_{s2} -casein					
Donkey (221 AA)	B7VGF9	56	57	57	–
Cow (207 AA)	P02663	100	87	88	–
Goat (208 AA)	P33049	87	100	98	–
Sheep (208 AA)	P04654	88	98	100	–
Human	–	–	–	–	–
β -casein					
Donkey (226 AA)	P86273	53	55	55	56
Cow (209 AA)	P02666	100	91	91	50
Goat (207 AA)	P33048	91	100	99	51
Sheep (207 AA)	P11839	91	99	100	51
Human (211 AA)	P05814	50	51	51	100
κ -casein					
Donkey (162 AA)	F0V6V5	52	55	55	64
Cow (169 AA)	P02668	100	83	83	49
Goat (171 AA)	P02670	83	100	95	50
Sheep (171 AA)	P02669	83	95	100	51
Human (162 AA)	P07498	49	50	51	100
α -Lactalbumin					
Donkey (123 AA)	P28546	71	72	72	76
Cow (123 AA)	P00711	100	94	97	76
Goat (123 AA)	P00712	94	100	98	76
Sheep (123 AA)	P09462	97	98	100	76
Human (123 AA)	P00709	76	76	76	100
β -Lactoglobulin					
Donkey β -LG I (162 AA)	P13613	56	56	56	–
Donkey β -LG II (163 AA)	P19647	50	50	51	–
Cow (162 AA)	P02754	100	96	96	–
Goat (162 AA)	P02756	96	100	99	–
Sheep (162 AA)	P67976	96	99	100	–
Human	–	–	–	–	–
Serum albumin					
Donkey (583 AA)	Q5XLE4	74	75	75	76
Cow (583 AA)	P02769	100	92	92	76
Goat (583)	B3VHM9	92	100	98	74
Sheep (583 AA)	P14639	92	98	100	75
Human (585 AA)	P02768	76	74	75	100
Lactoferrin					
Donkey (689 AA)	O77811 ^a	73	73	74	75
Cow (689 AA)	P24627	100	92	92	70
Goat (689 AA)	Q29477	92	100	98	71
Sheep (689 AA)	D3G9G3	92	98	100	71
Human (691 AA)	P02788	70	71	71	100

^a The UniProt Accession Number of horse's lactoferrin is reported.

to the corresponding domains present in donkey's counterparts (see Fig. S10, Supplementary material).

In conclusion, although the development of an allergic reaction is a complex process involving a receptive immune system (i.e. a genetic predisposition to propagation of an IgE response) and further biochemical studies and clinical experiences are needed, the experimental data today available suggest that the low allergenic properties of DM with respect to cow one seem to be related to some of its peculiar characteristics: i) the low total protein content (very similar to that of HM); ii) the low ratio of caseins to whey fraction; iii) the remarkable differences in the primary structure of its protein components with respect to the main cow milk allergens, and finally iv) the presence in

almost all bovine IgE-binding linear epitopes of multiple amino acid differences with respect to the corresponding regions of DM counterparts.

8. Conclusions and perspectives

Taking into account the increasing number of subjects allergic to cow milk proteins and considering that many clinical trials have confirmed the legendary therapeutics properties of donkey milk, in the last decade the interest towards this milk has increased. In particular, many investigations have been performed with the aim to characterize its protein components, and therefore to obtain a more accurate

comprehension of its potential benefits for human nutrition. The improved performance and versatility of the MS-instrumentation together with the concurrent optimization of the separation techniques and the availability of efficient methods for gene sequencing, have led to an extensive characterization of the primary structure, post-transcriptional and post-translational modifications of donkey milk proteins. The knowledge about the primary structure of the main components of donkey milk now provides the basis for a more precise elucidation of the relationship between protein composition and its nutritional properties. Moreover, a comprehensive sequence comparison of the main components of donkey milk with the corresponding homologue proteins from both human and the most widespread ruminant species (e.g. cow, goat and sheep) may contribute to explain the reduced allergenic properties of DM as well as the rare cases of allergy to this milk (Peeters, Herman, & Baeck, 2017; Verhulst, Kerre, & Goossens, 2016). The information today available clearly demonstrates that donkey milk proteins (especially casein components) are more closely related with the human homologues rather than cow counterparts. β -LG, the most abundant and the most recognized bovine allergen, in DM is present at a lower concentration, whereas it is absent in HM. In this light, the identification of animals producing milk lacking in β -LG II protein (Criscione et al., 2009) appears promising to solve potential residual cases of reactivity (Wal, 2002). Moreover, bovine β -LG shares a low sequence identity with respect to its equine counterparts and shows a different structure organization. In fact, this protein in ruminant milk occurs naturally as dimer, whereas it has a monomeric structure in DM. Consequently bovine β -LG is very resistant to peptic digestion and can cause allergenic reaction on consumption. On the contrary, donkey β -LG shows a better digestibility in newborns due to higher digestibility and absorption of soluble monomer proteins (Lönnerdal, 2003; Barłowska, Sz wajkowska, Litwińczuk, & Król, 2011). Interestingly, donkey β -LG is highly degraded (70%) in vitro by human gastric (HGJ) and duodenal juice (HDJ) (Tidona et al., 2011; Tidona et al., 2014) that is twice as much in comparison to cow counterpart (Inglingstad et al., 2010). On the other hand, it should be noted that equine β -LG is more thermally stable than the bovine counterpart; a feature that may be related to its weak allergenicity (Gall et al., 1996). Finally, sequence comparison of the known epitopes of bovine milk allergens reveals that almost all show remarkable amino acid differences with respect to the corresponding traits of donkey milk homologues. On the other hand, this comparison also shows that three bovine epitopes, coming from α_2 -CN, α -LA and SA, share a very similar primary structure with the corresponding domains present in donkey counterparts. All these observations, may be related to the documented low allergenic properties of DM, as well as to the very weak cross-reactivity in a minority of highly problematic cow milk allergic children, and might constitute the rationale for the production of new formulas based on donkey milk even if new both in vitro and in vivo clinical studies must be designed in order to confirm or update the reported results.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.foodres.2017.07.002>.

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A)

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SP | P86272 | CASA1_EQUAS  RPKLPHRHPEIIQNEQDSREKVLKERKFPSFALHTPREEYINELNRQRELLKEKQKDEHK 60
SP | P02662 | CASA1_BOVIN  RPKHPIKHQGLPQ-----EVL17NENLLRFFVAPFPEV-F--GKEKVN36EL39SKDIGSE48TE 50
                        *** * : * : * : *** : * : * : : : ** * : . : :
SP | P86272 | CASA1_EQUAS  EYLIEDPEQQESSSTSSEEVVPINTEQKRIREDMLY---QHTLEQLRRLSKYNQLQLQ 117
SP | P02662 | CASA1_BOVIN  DQAMEDIKQMEAESISS69EEIVPNSVEQ78KHIQKEDVPSE93RYL102GYLEQLRLK109KYKVPQLE 110
                        : : ** : * * : * * : ** : . : ** : * : ** :      * * * * * * : ** : ** :
SP | P86272 | CASA1_EQUAS  AI--YAQEQLIRMKE--NSQRKPMRVVNQEQA120YFYLEPFQPSYQLDVYPYA123AWFH132PAQI 172
SP | P02662 | CASA1_BOVIN  IVPNSAEERLHSMKEGIHAQQ139KEPMIGV154NQELAYFYPELFRQFYQLDAYPSGAWYYVPLG159 170
                        : * : * : * * * . * : ** * * * * * * * : * * * . * * . * : :
SP | P86272 | CASA1_EQUAS  MQHVAYSPFHDTAKLIASENSEKTDI174IPEW 202
SP | P02662 | CASA1_BOVIN  TQYTDAPSFSDIPNPIGSENSEKT194-TMPLW 199
                        * : . * * : * . * * * * * : * *

B)
SP | B7VGF9 | CASA2_EQUAS  KHNMEHRSSSE31DSVNISQEKFKQEKYVVIPTSKESICSTSC33EEATRNINEMESAKFPTEV 60
SP | P02663 | CASA2_BOVIN  KNTMEHVSSSEE-SIISQETYKQEK42NMAINP44SKENLCSTFCKEVVRNANEE-----E 51
                        * : . *** * * * : * * * . : * * * : * * * * : * * . * * *
SP | B7VGF9 | CASA2_EQUAS  YSSSSSSEESAKFPTEREEKEVEEKHHLKQLNKINQFYEKLNFLQYLQALRQPRIVLTPW 120
SP | P02663 | CASA2_BOVIN  YSIG56SSSEESA83EVATEEVKITVDDKH93YQKAL100NEINQFYQK106-FPQYLQYLYQGPIVLNPW 109
                        ** . * * * * * : . * * : * : * * : * * : * * * * * * * * * * *
SP | B7VGF9 | CASA2_EQUAS  DQTKTGASPFIPVNT114EQLFTSEEIPKKTVDMESTE117VTEKTELTEEEKNYLKL128NKINQ 180
SP | P02663 | CASA2_BOVIN  DQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTE143VFTK145TKLT154TEEEK158NRLN165FLKKISQ 169
                        * * . * * : * : * * * * * * * * * * * * * * * * * : * : * : * *
SP | B7VGF9 | CASA2_EQUAS  YYEKFTLPQYFKIVHQHQTMDPQSHSKTNSYQIIPVLR171YF 221
SP | P02663 | CASA2_BOVIN  RYQKFALPQYLKTVYQH180QKAMKPWIQPKTK188-191-VIPYVRYL200 207
                        * : * * : * * * : * : * * : * : * : * : * : * :

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Figure S1. Alignment of donkey and cow $\alpha 1$ - (A) and $\alpha 2$ -CNs (B) obtained by the Clustal Omega Tool (<http://www.clustal.org/omega/>). Major and minor IgE-binding epitopes in transient and persistent CMPA are in italics and underlined, respectively (see Table 5). Epitopes exclusively recognized by persistent allergic patients are shown in bold. Alignment displays the consensus symbols, which denote the degree of conservation observed in each column. The trait Asp¹²-Glu⁴², lacking in two donkey $\alpha 2$ -CNs is shaded. The symbol "*" (asterisk) indicates positions which have a single, fully conserved residue. The symbol ":" (colon) indicates conservation between groups of strongly similar properties (conservative substitutions). The symbol "." (period) indicates conservation between groups of weakly similar properties.

A)

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SP|P86273|CASB_EQUAS REKEELNVSETVESLSSNEPDSSSEESITHINKEKSQKFKHEGQQQREVEHQDKISRFBV 60
SP|P02666|CASB_BOVIN RELEELNVPGEIVES16SS-----SEESITRINK-KIEKFQSEEQQQTEDE45LQDKIHPFA 53
                      ** ***** . * ***** *****:*** * :*: * * * * * * * * * * .

SP|P86273|CASB_EQUAS QPQPVVYPYAEPVPYAVVPQNILVLAQPP--IVPFLQPEIMEVSQAKETILPKRKVMPFL 118
SP|P02666|CASB_BOVIN QTQSLVYPFPGPI5457PN6670SL-PQNIPPLTQTPV83VPPFLQPE92VMGVSKVKEAMAPKH107KEMPFP 112
                      * * :***: *:* :: ***** *: * * : *****:* * *:.**: :*: * * *

SP|P86273|CASB_EQUAS KSPIVPFSEERQILNPTNGENLRPLVHLIQPFMHQVPQSLLQTLMLPSQPVLSPPQSKVAP 178
SP|P02666|CASB_BOVIN KYPVEP129FTESQSLTLTDVENLH135LP144PL149QSWMQPH164QLPPTVMFPPQSVLS167QSKVLP 172
                      * *:***:* * * *: *****:*** *: * * *:*** * * *:*** * * * * * *

SP|P86273|CASB_EQUAS FPQPVVYPYQRDTPVQAFLLYQDPQLGLTGEFDPATQPIVPVHNPVIV 226
SP|P02666|CASB_BOVIN VPQKAV184PYPQRDMP IQAFLLYQEPVLGPVRGPFPIIV----- 209
                      .** .***** *:*****:* ** . *

B)
TR|F0V6V5|F0V6V5_EQUAS EVQNQEPTCRKNDERFFDLKTVKYIPVYYVLNSSPRNEPIYYQHRLAVLINNQHMPYQY 60
SP|P02668|CASK_BOVIN QEQNQEPI9IRCEKDER17FFSD21KIAKYI26PIQYVLSRYP44SYGLNYYQ47QKPVALIN53QFLPYP 60
                      : ***** :***** . * .*****: *** . * *****:..*****.:** *

TR|F0V6V5|F0V6V5_EQUAS YARPAAVRPHVQIPQWQVLPNIYP-----STVVRHPRPHPSFIAIPPKKLQEKTVIP 112
SP|P02668|CASK_BOVIN YAKPA64AVR68SPAQILQ76WQVLS95NTVPAKSCQAQPTT111MARHPH116PHLSFMAIPPKKNQDKTEIP 120
                      **:***** .** ***** * * :*:***:* * *:***** *:*** **

TR|F0V6V5|F0V6V5_EQUAS KINTIATVEPTPIPTPEPTVNNAVIPDASSEFIIASTPETTTVPVTSPVV 162
SP|P02668|CASK_BOVIN TINTI126ASGEPT137STPTTE148AVESTVATLE-DSPEVIESPPEINTVQVT166STAV 169
                      .*****: *** ** * . . . . : . * :* * * * .** *** .*
```

Figure S2. Alignment of donkey and cow κ -CNs (A) and κ -CNs (B) obtained by the Clustal Omega Tool (<http://www.clustal.org/omega/>). Major and minor IgE-binding epitopes in transient and persistent CMPA are in italics and underlined, respectively (see Table 5). Epitopes exclusively recognized by persistent allergic patients are shown in bold. Alignment displays the consensus symbols, which denote the degree of conservation observed in each column. The symbol "*" (asterisk) indicates positions which have a single, fully conserved residue. The symbol ":" (colon) indicates conservation between groups of strongly similar properties (conservative substitutions). The symbol "." (period) indicates conservation between groups of weakly similar properties.

```

SP|P02662|CASA1_BOVIN RPKHPIKHQGLPQEVLENLLRFFVAPFPEVFGKEKVN39ELSKDIGSE48STEDQAMEDIKQM 60
SP|P18626|CASA1_CAPHI RPKHPINHRGLSPEVPNENLLRFVAPFPEVFRKENINELSKDIGSESTEDQAMEDAKQM 60
SP|P04653|CASA1_SHEEP RPKHPIKHQGLSSEVLNENLLRFVAPFPEVFRKENINELSKDIGSESIEDQAMEDAKQM 60
*****.:*:* ** *****.***** **.:***** ***** **
SP|P02662|CASA1_BOVIN EAESISSEEIVPNSVEQKHIQKEDVPSE83RYLGYLEQLLRL102KKYKVPQLEIVPNSAEERL 120
SP|P18626|CASA1_CAPHI KAGSSSSSEEIVPNSAEQKYIQKEDVPSE83RYLGYLEQLLRL102KKYNVPQLEIVPKSAEEQL 120
SP|P04653|CASA1_SHEEP KAGSSSSSEEIVPNSAEQKYIQKEDVPSE83RYLGYLEQLLRL102KKYNVPQLEIVPKSAEEQL 120
.: * * *****.***.:*****.:*****.:*****.:*
SP|P02662|CASA1_BOVIN HSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFS 180
SP|P18626|CASA1_CAPHI HSMKEGNPAHQKQPMIAVNQELAYFY83QQLFRQFYQLDAYPSGAWYYLPLGTQYTDAPSFS 180
SP|P04653|CASA1_SHEEP HSMKEGNPAHQKQPMIAVNQELAYFY83QQLFRQFYQLDAYPSGAWYYLPLGTQYTDAPSFS 180
***** :*:*:* *****.:*****.:*****.:*****
SP|P02662|CASA1_BOVIN DIPNPIGSENSEKTTMPLW 199
SP|P18626|CASA1_CAPHI DIPNPIGSENSGKTTMPLW 199
SP|P04653|CASA1_SHEEP DIPNPIGSENSGKITMPLW 199
***** * *****

```

Figure S3. Alignment of cow, goat and sheep ~s1-CNs obtained by the Clustal Omega Tool (<http://www.clustal.org/omega/>). Bovine epitopes and the corresponding domains present in caprine and ovine counterparts sharing the same primary structure are reported in bold. Alignment displays the consensus symbols, which denote the degree of conservation observed in each column. The symbol "*" (asterisk) indicates positions which have a single, fully conserved residue. The symbol ":" (colon) indicates conservation between groups of strongly similar properties (conservative substitutions). The symbol "." (period) indicates conservation between groups of weakly similar properties.


```

SP|P02666|CASB_BOVIN  RELEELNVPGEIVESLSSEESITRINKKIEKFQSEEQQQTEDEL45QDKIHPFAQ54TQSLVY 60
SP|P33048|CASB_CAPHI  REQEELNVVGETVESLSSEESITHINKKIEKFQSEEQQQTEDELQDKIHPFAQAQSLVY 60
SP|P11839|CASB_SHEEP  REQEELNVVGETVESLSSEESITHINKKIEKFQSEEQQQTEDELQDKIHPFAQAQSLVY 60
      ** ***** ** *****:*****:*****
SP|P02666|CASB_BOVIN  PFPGPIPN107SLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFT120 120
SP|P33048|CASB_CAPHI  PFTGPIPN107SLPQNILPLTQTPVVVPPFLQPEIMGVPKVKETMVPKHKEMPFPKYPVEPFT120 120
SP|P11839|CASB_SHEEP  PFTGPIPN107SLPQNILPLTQTPVVVPPFLQPEIMGVPKVKETMVPKHKEMPFPKYPVEPFT120 120
      ** ***** *****:*** *:*****
SP|P02666|CASB_BOVIN  ESQSLTLTDVENLHLPLPLLQSWMHQPH149QLPPTVMFPPQSVLS164SLSQSKVLPVPQKAVPY 180
SP|P33048|CASB_CAPHI  ESQSLTLTDVEKLHLPLPLVQSWMHQPPQPLSPTVMFPPQSVLSLSQPKVLPVPQKAV - - 178
SP|P11839|CASB_SHEEP  ESQSLTLTDVEKLHLPLPLVQSWMHQPPQLPPTVMFPPQSVLSSLSQPKVLPVPQKAV - - 178
      *****:*****:***** ** *****
SP|P02666|CASB_BOVIN  PQRDMPIQAFLLYQEPVLGPVRGPFPIIV 209
SP|P33048|CASB_CAPHI  PQRDMPIQAFLLYQEPVLGPVRGPFPIIV 207
SP|P11839|CASB_SHEEP  PQRDMPIQAFLLYQEPVLGPVRGPFPIIV 207
      *****:*****

```

Figure S5. Alignment of cow, goat and sheep -CNs obtained by the Clustal Omega Tool (<http://www.clustal.org/omega/>). Bovine epitopes and the corresponding domains present in caprine and ovine counterparts sharing the same primary structure are reported in bold. Alignment displays the consensus symbols, which denote the degree of conservation observed in each column. The symbol "*" (asterisk) indicates positions which have a single, fully conserved residue. The symbol ":" (colon) indicates conservation between groups of strongly similar properties (conservative substitutions). The symbol "." (period) indicates conservation between groups of weakly similar properties.

```

SP|P02668|CASK_BOVIN  QEQNQEPIRCEKDERFFSD21KIAKYIPIQYVLSRYPSYGLNYY44QKPVALINN53QFLPYPY 60
SP|P02670|CASK_CAPHI  QEQNQEPICCCKDERFFDDKIAKYIPIQYVLSRYPSYGLNYYQQRVALINNQFLPYPY 60
SP|P02669|CASK_SHEEP  QEQNQEQRICCEKDERFFDDKIAKYIPIQYVLSRYPSYGLNYYQQRVALINNQFLPYPY 60
                        ***** * ***** . ***** . *****
SP|P02668|CASK_BOVIN  YAKP64AAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPLSFMAIPPKKNQDKTEIP 120
SP|P02670|CASK_CAPHI  YAKPVAVRSPAQTLQWQVLPNTVPAKSCQDQPTTLARHPHPLSFMAIPPKKDQDKTEVP 120
SP|P02669|CASK_SHEEP  YAKPVAVRSPAQTLQWQVLPNAVPAKSCQDQPTAMARHPHPLSFMAIPPKKDQDKTEIP 120
                        ***** . ***** * : ***** * * : : ***** : ***** : *
SP|P02668|CASK_BOVIN  TINTIASGEPTS- -TPTTEAVESTVATLEDSPVIESPPEINTVQVTSTAV 169
SP|P02670|CASK_CAPHI  AINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV 171
SP|P02669|CASK_SHEEP  AINTIASAEPTVHSTPTTEAVVNAVDNPEASSESIASAPETNTAQVTSTEV 171
                        : ***** . ***      ***** : . : * . * * * * * * * * * * *

```

Figure S6. Alignment of cow, goat and sheep ©-CNs obtained by the Clustal Omega Tool (<http://www.clustal.org/omega/>). Bovine epitopes and the corresponding domains present in caprine and ovine counterparts sharing the same primary structure are reported in bold. Alignment displays the consensus symbols, which denote the degree of conservation observed in each column. The symbol "*" (asterisk) indicates positions which have a single, fully conserved residue. The symbol ":" (colon) indicates conservation between groups of strongly similar properties (conservative substitutions). The symbol "." (period) indicates conservation between groups of weakly similar properties.

SP P00711 LALBA_BOVIN	EQLTKCEVFRELKDLKGYGGVSLPEWVCTTFHTSGYDTQAIVQNND ⁴⁷ STEYGLFQINN ⁵⁸ KIW	60
SP P00712 LALBA_CAPHI	EQLTKCEVFQKLKDLKDYGGVSLPEWVCTAFHTSGYDTQAIVQNND STEYGLFQINN KIW	60
SP P09462 LALBA_SHEEP	EQLTKCEVFQELKDLKDYGGVSLPEWVCTAFHTSGYDTQAIVQNND STEYGLFQINN KIW	60
*****.:*****.*****.:*****.*****.*****		
SP P00711 LALBA_BOVIN	CKDDQNPHSSNICNISCDKFLDDDLTDDIMCV ⁹³ KKILD ¹⁰² KVGIN YWLAHKALCSEKLDQWLC	120
SP P00712 LALBA_CAPHI	CKDDQNPHSRNICNISCDKFLDDDLTDDIVCA KKILD KVGIN YWLAHKALCSEKLDQWLC	120
SP P09462 LALBA_SHEEP	CKDDQNPHSRNICNISCDKFLDDDLTDDIMCV KKILD KVGIN YWLAHKALCSEKLDQWLC	120
*****.*****.:*.*.*****.*****.*****		
SP P00711 LALBA_BOVIN	EKL	123
SP P00712 LALBA_CAPHI	EKL	123
SP P09462 LALBA_SHEEP	EKL	123

[illegible]

Figure S7. Alignment of cow, goat and sheep α -Las (A) and γ -LGs (B) obtained by the Clustal Omega Tool (<http://www.clustal.org/omega/>). Bovine epitopes and the corresponding domains present in caprine and ovine counterparts sharing the same primary structure are reported in bold. Alignment displays the consensus symbols, which denote the degree of conservation observed in each column. The symbol "*" (asterisk) indicates positions which have a single, fully conserved residue. The symbol ":" (colon) indicates conservation between groups of strongly similar properties (conservative substitutions). The symbol "." (period) indicates conservation between groups of weakly similar properties.

```

SP|P28546|LALBA_EQUAS KQFTKCELSQVLKSM13MDGYKGVTLPEWICTIFHSSGYDTQTIVKNNGKTEYGLFQINNKMW 60
SP|P00711|LALBA_BOVIN 1EQLTKCEVFREL13KDL16KG22YGGV26SLPEWVCTTFHTSGYDTQAIVQNN47DSTEYGLFQINN58KIW 60
      *:*****: : **.:** **:*:*:*:*:* **:*:*:*:*:*:*:*:*:*:*:*
SP|P28546|LALBA_EQUAS CRDNQILPSRNICGISCNKF93LDDDLTDDVMCAKKILDSE102GIDYWLAHKPLCSEKLEQWLC 120
SP|P00711|LALBA_BOVIN CKDDQNPHSSNICNISC93DKFLDDDLTDDIMCV102KKILDKVGIN102YWLAHKALCSEKLDQWLC 120
      *:.* * ***.***:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*
SP|P28546|LALBA_EQUAS EEL 123
SP|P00711|LALBA_BOVIN EKL 123
      *:.*

```

Figure S8. Alignment of donkey and cow ~-LA obtained by the Clustal Omega Tool (<http://www.clustal.org/omega/>). Major and minor IgE-binding epitopes in transient and persistent CMPA are in italics and underlined, respectively (see Table 5). Alignment displays the consensus symbols, which denote the degree of conservation observed in each column. The symbol "*" (asterisk) indicates positions which have a single, fully conserved residue. The symbol ":" (colon) indicates conservation between groups of strongly similar properties (conservative substitutions). The symbol "." (period) indicates conservation between groups of weakly similar properties.


```

SP|P13613|LACB1_EQUAS TNIPQTMQDLDLQEVAGKWHSVAMAASDISLLDSEEAPLRVYIEKLRPTPEDNLEIILRE 60
SP|P02754|LACB_BOVIN LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQK 60
      :  ***: . ** : * : * * * . * : * : * * * * * * * * * : . * * * * * : * : * : * * * . : * * * : * : :
SP|P13613|LACB1_EQUAS GENKGCAEKKIFAECTESPAEFKINYLDDETVFALDSYKNYLFLCMKNAATPGQSLVCQ 120
SP|P02754|LACB_BOVIN WENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQ 120
      * *  * * : * * * : * * * : * *  * * : * : * : . * : * * * : * * : * * : * : * * * * * . * * * * *
SP|P13613|LACB1_EQUAS YLARTQMVDEEIMEKFRRALQPLPGRVQIVPDLTRMAERCRI 162
SP|P02754|LACB_BOVIN CLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI 162
      * . * *  * * : * : * * : * * : * *  : : : :  : * : : * : * : *

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SP|P19647|LACB2_EQUAS TDIPQTMQDLDLQEVAGRWHSVAMVASDISLLDSESAPLRVYVEELRPTPEGNLEIILRE 60
SP|P02754|LACB_BOVIN LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQK 60
      :  ***: . ** : * : * * * * * * * * * : . * * * * * * * * * : * * * * * : * * * : * : :
SP|P19647|LACB2_EQUAS GANHVCVERNIVAQKTEDPAVFTVNYQGERKISVLDTDYAHYMFFCVGPCLPSAEHGMVC 120
SP|P02754|LACB_BOVIN WENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSA-EPEQSLAC 119
      *  *  : : : : * : * : * * * : : . * . * : * * * * * : * : : * * : . .  * : : . *
SP|P19647|LACB2_EQUAS QYLARTQKVDEEVMEKFSSRALQPLPGHVQIIQDPSSGGQERCGF 163
SP|P02754|LACB_BOVIN QCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI 162
      *  * . * *  : * * : * . : * * * . : * * : * *  * *  * : : :  : * :  : * : * :

```

Figure S10. Alignment of donkey -LG I (A) and -LG II (B) with respect to the cow -LG obtained by the Clustal Omega Tool (<http://www.clustal.org/omega/>). Major and minor IgE-binding epitopes in transient and persistent CMPA are in italics and underlined, respectively (see Table 5). Alignment displays the consensus symbols, which denote the degree of conservation observed in each column. The symbol "*" (asterisk) indicates positions which have a single, fully conserved residue. The symbol ":" (colon) indicates conservation between groups of strongly similar properties (conservative substitutions). The symbol "." (period) indicates conservation between groups of weakly similar properties.