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Characterization of donkey milk proteins by a proteomic approach

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Abstract

The increased incidence of allergies to cow's milk proteins (CMP) in newborns enhanced the production of infant *formula* based on either soy proteins and/or hydrolysates of CMP. These *formula*, although having a good nutritional value, can be affected by the presence of peptides with a size stimulating the immunological response in predisposed subjects and/or an unpleasant taste for the infant consumers. Recent clinical studies indicated that patients with CMP allergy and also intolerant to these *formula*, showed a high tolerance towards the donkey milk, due to its greater quantitative similarities to human milk than cow's milk. Specifically, although the mechanism of this tolerance has not yet been clarified, it is reasonable to hypothesize that the reduced allergenic properties of donkey milk can be related to structural differences of its protein components with respect to cow's milk.

At present, compared with cow's milk, the characterization of donkey milk proteins (whey proteins and mainly caseins) is at a relatively early stage of progress, and only limited data are related to their heterogeneity. In this PhD thesis the complexity and specifically the qualitative and quantitative heterogeneity of donkey milk proteins of 77 animals reared in Italy was investigated using a proteomic approach. This analytical procedure for structural analysis of protein fractions is based on the combination of highly efficient separation techniques, such as one-dimensional electrophoretic techniques (PAGE pH 8.6, UTLIEF, PAGE-SDS) or, more efficiently, the two dimensional (2-DE) electrophoresis, coupled either to Coomassie Brilliant Blue staining or immunospecific staining with polyclonal antibodies, to chromatography analysis (RP-HPLC), interfaced to structural Mass Spectrometry analysis (LC/ESI/MS, MALDI/TOF/MS, ESI q-TOF/MS).

For donkey caseins, these combined methodologies allowed the contemporary identification of the four casein fractions (α_{s1}-, α_{s2}-, β- and κ-CN) in donkey milk together with their related heterogeneity due to post-translational phenomena such as the different phosphorylation degree of the caseins (α_{s1}-, α_{s2}-, β-CN) and the high glycosylation level of κ-CN, incorrect splicing of primary transcript in mRNA (non allelic deleted forms of α_{s1}-, α_{s2}-, β-CN) and genetic polymorphism of α_{s1}- and β-CN (one or more variants in individual samples). At this regard, for the first time the primary structure of a new α_{s1}-CN variant was determined, as well as and the amino acid sequences of three new genetic variants of β-CN (B, C, D) were characterized, using the known common α_{s1}- and β-CN phenotype from donkey as reference, respectively.

From a quantitative analysis, β-CN was always present in all analyzed donkey milk samples, as in human milk, representing the most abundant casein fraction followed by α_s-CN complex. The α_{s1}-CN showed an high variability in its expression level (0.2-2 g/L), confirming a quantitative polymorphism at this *locus* as in goat milk. The minor expression of α_{s1}-CN together with the absence of α_{s2}-CN in some donkey milk samples, as in the human milk, confirms the compositional similarity of two milks and represent a scientific basis for donkey milk's use in nutrition of infants with CMP allergy.

The screening carried out on the individual whey protein samples by the proteomic approach revealed a monomorphism either for α-La (A) and for β-Lg I (B). β-Lg II was instead polymorphic, for the occurrence of four variants (A, B, C, D) already known in literature and for the identification of a new variant E. The primary structure of β-Lg II E was determined and differs from β-Lg II D for D Asp^2/E Asn^2, D Arg^{18}/E Lys^{18}, D Val^{25}/E Ala^{25} amino acid substitutions with a M_r18256 Da. Moreover a quantitative polymorphism seemed to affect the donkey β-Lg II *locus* since different phenotypes, with a relative quantitative percentage respect to total whey protein ranging from 0% to 3.34%, were detected by RP-HPLC. This quali-quantitative polymorphism at donkey β-Lg II *locus* seems to affect donkey milk composition and therefore, its allergenic properties. Moreover, the achieved results also highlighted that all examined samples have a high lysozyme content (~ 2 g/L) which together with lactoferrin is important from an immunological point of view and for the shelf life of the milk.

Finally, the donkey milk samples were shown to be poor in the total proteins (1.48%) as human milk, and specifically they were characterized by a lower casein content (35-40%) with allergenic properties and a higher whey protein content (50-60%) with biological activities, compared to bovine milk, with a CN/WP ratio <1. However, in analyzed donkey milk samples, an individual variability either of each casein or whey protein amounts suggests further studies in this direction aimed at a selection of animals producing milk with a composition "qualitatively and quantitatively" more similar to human milk, and "functionalized" for infant feeding.

Abstract

L'incremento di allergie nei confronti delle proteine del latte vaccino (PLV) nei neonati ha incrementato la produzione di formulati per l'infanzia basati essenzialmente sulle proteine della soia e idrolizzati estensivi in PLV. Queste formule, pur avendo un buon valore nutrizionale, possono contenere peptidi in grado di indurre una risposta immunitaria nei soggetti predisposti e/o avere gusto sgradevole per il piccolo consumatore. Recenti studi clinici hanno mostrato che pazienti allergici alle PLV e refrattari anche a questi formulati, sono in grado di tollerare il latte di asina per la sua composizione quantitativa più simile al latte umano piuttosto che al latte vaccino. Specificamente, sebbene questo meccanismo di tolleranza non è chiaro, è ragionevole ipotizzare che le ridotte proprietà allergeniche del latte di asina possono essere correlate alle differenze strutturali dei suoi componenti proteici rispetto a quelli del latte di vacca.

Ad oggi, rispetto al latte di vacca, la caratterizzazione delle proteine asinine (sieroproteine e soprattutto caseine) è ancora agli stadi iniziali, con pochi dati disponibili a causa soprattutto della loro eterogeneità. In questa tesi di dottorato, la complessità e specificamente l'eterogeneità quali-quantitativa delle proteine del latte di asina prodotto da 77 animali allevati in Italia è stata investigata mediante un approccio proteomico. Questa metodologia analitica per l'analisi strutturale delle frazioni proteiche è basata sulla combinazione di tecniche di separazione altamente efficienti, come le tecniche elettroforetiche mono-dimensionali (PAGE pH 8.6, UTLIEF, PAGE-SDS), o più efficientemente, l'elettroforesi bidimensionale (2-DE), con la doppia colorazione al Blue Coomassie e con anticorpi policlonali specifici, all'analisi cromatografica (RP-HPLC), interfacciata all'analisi di Spettrometria di Massa (LC/ESI/MS, MALDI/TOF/MS, ESI q-TOF/MS).

Per le caseine asinine, la combinazione di queste tecniche ha permesso la contemporanea identificazione delle quattro frazioni caseiniche (α_{s1}-, α_{s2}-, β- and κ-CN) nel latte di asina assieme alla loro eterogeneità compositiva dovuta ai processi post-traduzionali come il differente grado di fosforilazione delle caseine (α_{s1}-, α_{s2}-, β-CN) e l'alto livello di glicosilazione della κ-CN, incorreto splicing del trascritto primario in mRNA (forme delete non alleliche dell'α_{s1}-, α_{s2}-, β-CN) e polimorfismo genetico dell'α_{s1}- e β-CN (una o più varianti nei latti individuali). A tal riguardo, per la prima volta è stata determinata la struttura primaria di una nuova variante dell'α_{s1}-CN, cosi come sono state caratterizzate le sequenze aminoacidiche di tre nuove varianti di β-CN (B, C, D) utilizzando i comuni noti fenotipi di α_{s1}- e β-CN di asina, come riferimento.

Da un punto di vista quantitativo, la β-CN è sempre stata presente in tutti i campioni di latte di asina analizzati, come nel latte umano, rappresentando anche la frazione caseinica più abbondante, seguita dal complesso α_s-CN. L'α_{s1}-CN ha mostrato un'elevata variabilità nel livello di espressione (0.2-2 g/L), confermando il polimorfismo quantitativo a tale *locus*, come nel latte di capra. Il minor livello di espressione dell'α_{s1}-CN, insieme all'assenza dell'α_{s2}-CN in alcuni campioni di latte di asina, come nel latte umano, conferma la similarità compositiva dei due latte e rappresenta una base scientifica per l'utilizzo del latte di asina nell'alimentazione di bambini con allergia alle PLV.

Lo screening effettuato sui campioni individuali di sieroproteine mediante l'approccio proteomico, ha mostrato un monomorfismo sia per α-La (A) che per la β-Lg I (B). La β-Lg II è risultata invece polimorfa, per l'identificazione delle quattro varianti (A, B, C, D) già note in letteratura e di una nuova variante E. La struttura primaria della β-Lg II E (M_r18256 D) è stata determinata e differisce dalla β-Lg II D per le seguenti sostituzioni aminoacidiche: D Asp^2/E Asn^2, D Arg^{18}/E Lys^{18}, D Val^{25}/E Ala^{25}. Inoltre un polimorfismo quantitativo sembra condizionare il *locus* della β-Lg II in quanto differenti fenotipi, con una percentuale quantitativa relativa rispetto alle sieroproteine totali compresa tra 0 e 3.34%, sono stati identificati mediante RP-HPLC. Questo polimorfismo quali-quantitativo al *locus* della β-Lg II sembra condizionare la composizione del latte di asina e perciò, le sue proprietà allergeniche. Inoltre i risultati ottenuti hanno anche evidenziato che tutti i campioni analizzati mostrano un alto contenuto di lisozima (~ 2 g/L) che assieme alla lattoferrina è importante da un punto di vista immunologico e per la shelf life del latte.

Infine, i campioni di latte di asina hanno mostrato, come il latte umano, un basso contenuto di proteine totali (1.48%). Specificamente, essi sono stati caratterizzati da un più basso contenuto (35-40%) di caseine allergeniche e un più alto contenuto di sieroproteine (50-60%) con attività biologiche, mostrando un rapporto CN/SP <1. Comunque, nei campioni analizzati, un'elevata variabilità nelle quantità di ciascuna caseina o sieroproteina, suggerisce futuri studi in questa direzione, finalizzati a una selezione di animali produttori di un latte con una composizione " qualitativa e quantitativa" più simile al latte umano, e "funzionalizzato" per l'alimentazione pediatrica.

0 Preface

The production and wider use of donkey milk suggests the potential economic value of donkey breeding, providing an economic justification for breeding donkey and preserving their natural environment, mostly represented by marginal and hilly areas, helping to protect certain donkey breeds from extinction in industriazed countries.
Donkey milk, having an organoleptic and compositional characteristics very similar to human milk, may become an important food in the feeding of infants with cow's milk protein allergy, frequently refractory to other treatments, also allowing the development of a normal and complete immune system in the newborn. In addition to its use for infants, some researchers (Carroccio et al., 2000a; Monti et al., 2007) have reported that donkey milk has an effect on the osteogenesis process, as well as in arteriosclerosis therapy, for rehabilitation of patients with coronary heart disease or premature senescence, and in hypocholesterolemic diets.
Today, the production of donkey milk has also an economic interest, not only for human consumption, but also in the pharmaceutical and cosmetic industries and in rehabilitation of patients with psycho-physical deficits (Vita et al., 2007; Tesse et al., 2009).
So, the biological and nutritional value of donkey milk urges a deep knowledge of this milk which could be used as a breast milk substitute and as a new dietetic-biofood.
In this context, since the knowledge about the genetic profiles of donkey "proteome" (caseins and whey proteins) of animals reared in Italy is very scarce, as well as there are few experimental evidences of the expression level of each individual milk proteins, the present research was aimed to filling this gap and to relating it to the milk nutritional quality by a proteomic approach.
A classic proteomic approach provides, after the extraction of proteins from a food, the separation of the protein mixture (e.g. by electrophoresis or chromatography) and identification of proteins combining techniques of classical biochemistry (immunospecific staining, enzymatic hydrolysis) to the mass spectrometry methods, followed by bioinformatic analysis of the data.
However, the proteome is the set of proteins expressed in a cell, in a tissue and therefore in an organism. The science which allows the depth study of the proteome is called the "proteomics". This science allows the study of the proteins both in the forms just transcribed by the genes and in the isoforms (due to alternative splicing) or post-translational modifications which may occur in the cell after transcription (after translation). The study of the isoforms or any post-translational modifications allows the comprehension of the interaction's mechanisms between the proteins; these mechanisms enable the activity and function of proteins. So, the proteomics is a science which seeks to investigate and determine the identity, quantity, structure and bio-chemical and cellular functions of all proteins of a tissue, cellular or sub-cellular compartment, describing how these properties are variable in time, in space and in certain physiological states.
All the information obtained from the proteomic studies, correlated with those obtained in the genome's study, could help to establish the actual role of the proteins, as well as the relationship between genotype and phenotype.

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1 STATE OF THE ART

1.1 The history of donkey's milk through the centuries

The domestic equid species (horse and donkey) belong to the taxonomic order Perissodactyla, family Equidae, genus Equus, specie asinus. The domestic donkey could derive from African widcat donkey which still lives along northern and eastern coasts of Africa, Syria, Persia, Tibet and Mongolia. The donkey was domesticated for the first time, at the end of Neolithic period in ancient Egypt, in the Nile valley, and later in Europe, in southern Italy (Bonadonna, 1976). The donkey has always been a cult object in East countries and in Africa, and it has always been used for work, for the production of mule, meat (fresh and sausages) and milk (also used for the production of fermented alcoholic beverages as koumiss). Today the donkey is still used for trekking but also for rehabilitation of disabled people (onotherapy). At this regard, the donkey still occupies a large place in folklore, art and literature of European and extra-European countries.
Since Erodoto (V century BC), the donkey was known for nutritional and beneficial properties of its milk; in fact, the curative recipes based on donkey's milk and spread by Plinio were very famous, as well as Cleopatra, Egypt queen, kept her youth and beauty of the skin immersing herself in donkey's milk; these healthy benefits required a herd of about 700 donkey every day. The Romans also used donkey's milk because of the great healing powers; Poppea, Nerone's wife, used this precious liquid in order to preserve the splendour and the elasticity of the skin; even Messalina, Rome Empress, cured her face with masks made of bread soaked in donkey's milk (Salimei et al., 1996a; Andena, 2007).
It is in Renaissance, that was the first scientific consideration of the donkey's milk, by the king of France, Francis I, which exhausted by wars and excesses, used this milk against stress and physical fatigue. In the nineteenth century, in the European countries, it was easy to find merchants who carried the donkeys. The elegant society of that period regularly consumed this precious drink, while poor families reserved the rare elixir only to stick children and to the weak and tired elderly. During this same period, donkey's milk began to be used for breastfeeding; the donkey lend itself to nurse infants that are submitted directly to the breast. In fact, also in France in the nineteenth century, Dr .Parrot of Hospital des Enfants Assistès spread the practice of bringing babies motherless directly to the donkey's nipple (Bullettin de l'Acadèmie de mèdicine, 1882).
The donkey's milk was then sold until the twentieth century to feed orphaned infants and to cure delicate children, the sick and the elderly. For this reason, in Italy, Belgium, Germany, Switzerland many donkey farm born. Today there is a renewed interest towards the donkey's milk because of its peculiar characteristics of composition (The story of donkey's milk through the centuries in www.eurolactis.com).
However, asinine breeds-populations are few and have a variability not very accentuated, except for the morpho-functional characteristics, such as height that in some breeds, is equal to 160 cm and in other breeds reduced less than one meter (Balasini, 1990). Breeding is mostly present in the countries of southern Europe (Spain, France, Italy and Balkan Peninsula) and in Africa, from which this species is originated. In Europe, there are asinine breeds in France (Poitou donkey), Spain (Catalan donkey), but especially in Italy where there are many breeds: Martina Franca donkey, Sardinian donkey, Romagnolo donkey, Viterbese donkey, Asinara donkey, Armata donkey, Pantelleria donkey and Ragusan donkey (Baroncini, 1987).
In 1950, donkeys were almost one million in Italy, but their consistency has dwindled over the years. Only, since 2000, a rapid ascent is started and many local breeds were saved from the extinction (Paolicelli, 2010) (Fondiz). In fact, today, there are about 55000 donkeys in Italy (www.anagrafeequidi.it).
The Italian region, which has preserved the good tradition of donkey is Sicily, which has the highest number of asinine farms (donkeys breeding) together with Abruzzo, Lombardia, Campania and Lazio. Generally, a donkey farm (breeding), aimed at milk's production, is composed of 20/25 dams with 1 or 2 stallions. The donkey breeds, reared for milk production, are heavier ones, such as Amiata (Toscana), Martina Franca (Puglia) and Ragusana (Sicilia) breeds. They are shown more adequate in terms of efficacy, because the produced milk is related to the animal's mole (Paolicelli, 2010).
In Italy, the protected asinine breeds are: Armiata (1614 animals), Martina Franca (964 animals), Ragusan (2017 animals), Sardinian (1349 animals), Romagnolo (461 animals), Asinara (70 animals), Pantesco (76 animals) and Viterbese (131 animals), for a total number of about 6682 donkeys officially reported in the populations' register (www.anagrafeequidi.it). In the last years, the loss of asinine genetic patrimony and the demand of a hypoallergenic and nutritionally adequate milk, have stimulated the interest of many farms, mainly in the inner areas of the Italy, which have rediscovered the donkey breeding, as important for environment and biodiversity. The preservation of asinine breeds is due to donkey's reintegration in programs of social and economic interest, such as, onotherapy, onodidactics, onotourism and onomilk (milk production). In fact, donkey's milk, is a natural product attributable to category of pharmafood/nutraceuticals useful for children and adults (Gatti, 2007).

1.2 Donkey milk production

Donkey milk production differs greatly from that of conventional dairy species, especially in terms of milk supply. The equid mammary gland has a low capacity (max 2,5 L) so that milking may be carried out two or three hours after separation from the foal (Doreau, 1991; Drogoul et al., 1992). Kinetics of milk ejection shows two peaks: the first represents the emission of the cisternal milk, while the second represents the emission of alveolar milk (75-85%) as natural response to oxytocin release during milking, which is often insufficient for complete milk removal from the udder of donkey. As a consequence, low fat contents are reported for donkey milk, whereas residual milk, accounting on average for 30% of total milk extracted, is the richest fraction in fat (Salimei et al., 2004b). However, when foals are not physically present, the milking routine is more manageable in terms of both human and animal safety, and for an optimal milk extraction (Simoni et al., 2004). Due to the small amount of milk per milking and depending on the wavering consumer demand, mostly limited by local availability and lack of communication, donkeys, as mares, may need to be milked many times a day (Doreau, 1991; Drogoul et al., 1992). In donkeys, although manual milking could be efficient in terms of quantity of milk extracted per milking, production varies less when donkeys are machine milked (Salimei, 2011) and the risk of contamination, a crucial issue, can be significantly reduced compared with manual milking (Sorrentino et al., 2010).

Milking routine is crucial in donkeys with regard to the specific management of the dams that live with their suckling foals until natural weaning (> 7 months of age). Donkeys should be milked from 20 to 90 d after foaling, three times a day, following a strict milking preparation and routine consisting of 3 h physical separation from foal, udder massage and teat cleaning (Bayle-Labouré, 2007; Doreau & Martin-Rosset, 2011). In general, the presence of foal during milking does not affect milk ejection in donkeys which always showed a quick adaption to the milking procedure (Salimei et al., 2000), which is a significant advantage compared with horses and allows the modification of the milking parlour, routine and facility.

Donkeys adapt quickly to the routine (including udder massage, teat cleaning, approaching and leaving the parlour) that requires about 3 min per animal (Simoni et al., 2004).

However, the variability of donkey milk production is due to many factors, such as individual milk ability (individual features of breast apparatus) also related to genetics (donkey breed), nutrition (type of forage) (donkey body condition), but also management of reproduction and milking management (Salimei & Fantuz, 2012). In donkey, the changes during lactation include both qualitative and quantitative aspects, and the definition of these trends is important for the nutritional characterization of milk, as well as aspects concerning the production process (D'Alessandro, 2007). Studies on donkey milk showed an highly variable productions between manual (466 ± 260 mL) (Guo et al., 2007; Alabisio et al., 2009) and mechanical milking (772 ± 148 mL) (Salimei et al., 2004a; Simoni et al., 2004; Fantuz et al., 2007; D'Alessandro et al., 2009; Giosuè et al., 2008; Fantuz et al., 2010). In fact, Salimei et al. (2004a) have demonstrated that average milk yield per mechanical milking was about 740 mL, even if it was significantly higher during the second year of lactation (606,5 mL vs 854,3 mL), attributable to the adaption of donkeys and (operator) to mechanical milking. However, the milk yield per machine milking is less rich in fat, being the residual milk after oxytocin injection about 37% of the total milk produced, and milk fat is the main retained milk component (Salimei et al., 2004b), consistently with the data on mares (Doreu et al., 1986). Considering the different milk yield obtained when donkeys were milked twice a day, in the morning and in the afternoon (Salimei et al., 2004a), the average milk yield of the morning milking was found to be statistically lower than that observed for the afternoon milking (549,2 mL vs 949,3 mL). The observed higher milk production during the middle part of the day, as also reported in dairy mares (Dell'Orto et al., 1994), supports the hypothesis of a coadaption of the dam to the suckling rhythm and activity pattern of the foal, although a circadian rhythm for suckling was not noted in horses.

D'Alessandro & Martemucci (2007) have investigated on the effects of daily milking number and frequency on donkey milk production: the results obtained in that study demonstrated that the highest milk yield corresponded to three milking per day every three hours, while daily milking regimen of 5-6 milking per day did not increase milk production and had a negative influence on the health of the mammary gland. Also Alabisio et al. (2006) have demonstrated that the highest milk yield can be obtained with three milking per day compared to two milking per day, with an increase fat content, too. Outside the milking hours, the sucking of breast milk to foal should be instead allowed, which is essential for milk secretion (Salimei, 2001).

Variability in milk yield could be related could be related also to genetic diversities and therefore to the breeds, even though Blasi et al. (2005) defined genetically similar Romagnola, Ragusana and Martina Franca populations; the Authors also showed reciprocal influences confirming the hypothesis formulated by Gandini & Rognoni (1997). However, the lactation, for dairy donkeys, shows high individual variability, explained by the total absence of any form of genetic selection of the animals; starting at 30 d post partum milk yield constantly decreases up to 4^{th} – 5^{th} month of lactation (Salimei et al., 2005). This trend is substantially confirmed by data on Ragusana and Martina Franca populations (Alabisio et al., 2005), also showing seasonal variation of milk yield, presumably due to foaling period and the related different availability of pasture at the onset of lactation (Giosuè et al., 2008).

In fact, the asinine species is considered a seasonal polyestrous one, but the latitude in which the farm is located can greatly influence the reproduction cycle; considering a recent investigation runned in the south of Italy, specifically in Sicily, the local latitude determined small photoperiod oscillations between the different seasons, and under these conditions the donkeys have a continuous reproductive cycle, the same was observed in sheep. In Sicily it is in fact possible to obtain donkey milk throughout the year by adequately planning the reproductive seasons; results showed that donkey foaling in winter and in summer produced more milk than those foaling in the other seasons (Giosuè et al., 2008).
However, individual variation is high in dairy donkeys, suggesting that the adoption of adequate selection programmes and reproductive management would lead to a substantial improvement in milk production, as already experienced with dairy horses (Doreau & Martuzzi, 2006).

1.3 Gross composition of donkey milk

The composition of donkey's milk differs considerably from that the milk of the principal dairying species (cow, buffalo, goat and sheep). In comparison with bovine milk, donkey's milk contains less fat, protein and inorganic salts but more lactose, with a concentration close to that human milk (Table1.1).
Both genetic and environmental factors affect the gross composition of milk, including the breed of mammal, individuality of animals, stage of lactation, frequency and completeness of milking, maternal age, health and type of feed.
The pH value, ranging from 7.14 to 7.22 (Salimei et al., 2004a; Guo et al., 2007), does not vary significantly throughout the lactation period as in mare's milk (Mariani et al., 2001), and this suggests that the pH value is not influenced by breed or stage of lactation. The average pH value (7.18) of donkey's milk is higher than that of cow's milk (6.6 - 6.7) and it is associated with a low average titratable acidity. These data may be explained by the lower casein and phosphate contents in donkey's milk than cow's milk (Salimei et al., 2004a). During the lactation, the density of donkey's milk also remains constant at 1.032. This value, because of the lack of fats, is higher than human and cow milk.

Table 1.1. Average percentage composition (g/100g) found in donkey's milk, compared with other domestic species (Polidori, 1994; Salimei et al., 2000).

	Fat	Proteins	Lactose	Dry matter	Ashes
Donkey	0.38	1.72	6.88	8.84	0.39
Human	3.83	1.25	6.81	12.20	0.21
Mare	1.36	2.10	6.16	10.04	0.42
Cow	3.70	3.20	5.00	12.70	0.80
Goat	4.00	3.10	4.25	12.05	0.80

The fat content, according to Salimei et al. (2004a) and Guo et al. (2007) shows marked variability (1.82-0.20%) indicating that could be affected by breed, breeding area and forage, milking technique, interval between milkings and mainly stage and year of lactation as also reported by Fox (2003), with a negative trend throughout lactation. Due to the high variability observed in milk fat content, the variation in energy value appeared to show much similarity to that of the fat content (Salimei et al., 2004a; Guo et al., 2007).
The lactose content of donkey's milk is similar to that human and mare milk (table 1.1) and is much higher than that of cow milk. According to Guo et al. (2007) the lactose content is constant throughout the lactation, because lactose is responsible for about 50% of the osmotic pressure of milk, which is equal to that of blood. The lactose content in donkey's milk is unaffected by breed, milking time, year and stage of lactation, as observed by Salimei et al. (2004a). In donkey's milk, the high lactose content not only makes this milk pleasant in taste, but also stimulates the intestinal absorption of calcium, that is essential for bone mineralization and for nervous system development in infants (Schaafsma, 2003). Moreover, the high lactose content suggests using donkey's milk also for probiotic purpose (Coppola et al., 2002) because it is an ideal substrate for a correct development of intestinal lactobacilli and, from a technological point of view, makes donkey's milk an ideal matrix for the preparation of probiotic drinks following the incubation with *Lactobacillus rhamnosus* strains (Coppola et al., 2001).
Despite the high lactose content of donkey's milk, the average energy content is lower (1,708Kj/Kg) compared with human milk (2,60 Kj/Kg). According to Oftedal & Jennes (1988) the low energy content of donkey's milk is related to its low fat content and to the large amounts of milk secreted to meet the nutritional requirements of the foal for its rapid growth. The hypolipid content of donkey's milk (table 1.1) must be taken into account from a nutritive point of view: for this purpose, when used in infant nutrition, donkey's milk is usually supplemented with vegetal oil (4mL/100mL) to conform to human milk energy (Iacono et al., 1992). In donkey's milk significant positive correlations between milk yield and total solids or fat content were observed. These results confirm that higher milk fat is associated with a more complete

udder evacuation in dairy donkey. Moreover, total solids were positively correlated to protein, fat and ash content, whereas the protein content was directly related with fat and ash contents but negatively correlated to lactose (Salimei et al., 2009). According to circadian studies, milk lipid and lactose contents have been observed to peak in donkey during the night, whereas the protein content peaked during the day (Piccione et al., 2008). However, internal rhythms are known to be affected by environmental factors, such as management of feeding and the light-dark cycles.

1.4 Donkey milk proteins

The seasonal trend in the protein's percentage showed a richer milk during spring season, and because the breeding system of donkeys provides a wide grazing period especially during the spring and summer months, when the forage's availability is maximum, it is hypothesized that in the period from March to June the high proportion of protein in diet positively influences milk proteins. The abundant forage supply provides to donkeys an amount of proteins, often not fully utilized by nitrogen metabolism, which are then excreted also as urea in the milk (Conte, 2007).
The protein content of donkey milk is similar to that human milk and was much lower than that cow's milk (table 1.1) (Salimei et al., 2004a). Although it is not significantly influenced by milking times and breed, varies during lactation, with a negative trend, as already described for milk yield and its fat percentage.
However, during lactation both in donkey and in human a decreasing trend of the protein level (1.72g/100g) was showed (Salimei et al., 2004a). Similar trends were observed in mare's milk (Mariani et al., 2001) and cow's milk (Fox, 2003). This shows that the total protein percentage in donkey's milk is affected by the stage of lactation, consistent with the finding of Fox (2003) in cow's milk. This may be related to the differential expression of milk proteins genes during lactation (Demmer et al., 1998).
The study of protein fraction has an essential role, because it is presumably responsible for hypoallergenicity of donkey's milk, entering in tolerance's mechanism of this milk. Casein, whey protein and NPN contents do not vary significantly throughout the lactation period and appear not be influenced by breed or milking time (Salimei et al., 2004a; Guo et al., 2007).
The non-protein nitrogen (NPN) accounts for an average of 10-16% of total nitrogen in mare and donkey milk, which is lower than values reported for human milk but higher than those of domestic ruminants (Uniacke-Lowe et al., 2010; Salimei, 2011). The average content of NPN is lower in winter, tending to increase during the summer, because in the summer the protein content of feed intake becomes excessive compared to the physiological requirements of the animal (Tidona et al., 2011). The nutritional and biological significance of this milk fraction (0,29g/100g milk) which includes urea, uric acid, creatinine, amino acids, nucleic acids and nucleotides is still far from being completely understood, but seems to be related to the development of the infant (Emmet & Rogers, 1997).
The average content in casein and whey proteins are very similar (Table 1.2), but while casein content has a decreasing trend, the whey protein content remains constant during the lactation (Alabisio et al., 2005). There is a similarity between donkey milk and mare milk in nitrogen distribution. The whey protein content of donkey milk (0,68g/ 100g milk) is close to that of human and mare milk (Doreau et al., 2002; Malacarne et al., 2002), instead the average casein content (0,87g/100g) is resulted higher than human milk (0,53g/100 g milk) but it is lower than equine (1,11g/100g milk) and ruminant milk (2,42 g/100g cow milk; 2,40g/100g goat milk) (Travia, 1996). Moreover, the average ratio of casein to whey protein in donkey's milk (0,94) is intermediate between the lower value of human milk and the higher value of cow's milk, consistent with the finding reported by Travia (1986). In fact, in the milk of ruminants this ratio is four times higher than donkey's milk, and seven times higher than human milk (Polidori and Vincenzetti, 2007).

Table 1.2. Average percentage composition of the nitrogen component of donkey milk, compared with other domestic species (Polidori, 1994; Salimei et al., 2000).

	Proteins (g/100m)	NPN (x6.38) (g/100 mL)	WP (g/100mL)	%WP	Casein (g/100m)	CN/WP
Donkey	1.72	0.29	0.68	38.4	0.87	0.94
Human	1.25	0.16	0.71	56.8	0.38	0.53
Mare	2.10	0.20	0.79	37.50	1.11	1.40
Cow	3.20	0.18	0.60	18.50	2.42	4.03
Goat	3.12	0.14	0.58	18.59	2.40	4.14

The high whey protein content of donkey's milk make it more favorable for human consumption (Curadi et al., 2001; Iacono et al., 1992). With reference to the protein content, it is important not to ignore the fact that patients who experience an intolerance to cow, goat, or sheep milk are able to tolerate donkey and mare milk (Businco et al., 2000; Carroccio et al., 2000a; Curadi et al., 2001). Although the mechanism for tolerance to donkey's milk is still unclear, it may be related to the specific levels of major allergenic components in the

milk. Among the potentially allergenic milk components, β-Lg is, in fact, probably the major milk allergen among infants and children, whereas casein is considered the predominant milk allergen among adults (Carroccio et al., 1999). Lara-Villoslada et al. (2005) found that the balance between casein and whey protein played an important role in the sensitization capacity of cow milk and showed that cow milk with a CN:WP ratio of 40:60 was less allergenic than native cow milk. As a result, it may be of interest to use donkey milk for infant feeding. The donkey milk may be a valid substitute for human milk and also an alternative for feeding children who experience an allergy to cow milk.

1.5 Donkey caseins

Casein micelles are primarily a source of amino acids, calcium, phosphate and bioactive peptides for neonates (Shekar et al., 2006). Although the mechanism of tolerance of donkey's milk has not yet been fully clarified, it is reasonable to hypothesize that the reduced allergenic properties of this milk can be related to structural differences of its protein component with respect to cow's milk. The lower casein concentration in donkey's milk (about 50% of total protein) compared with bovine milk (80% of total protein) (Zicker & Lonnerdal, 1994) and the relevant percentage of essential aminoacids make this milk a new dietetic food and a promising breast milk substitute (Guo et al., 2007).
The traditional method for separating caseins from whey proteins is isoelectric precipitation of the caseins at pH 4.6. The casein fraction of most milk types consists of four gene products synthesized in the mammary gland in response to lactogenesis hormones and other stimuli: αs1-, αs2-, β-, and κ-caseins, of which the first three are calcium-sensitive. They showed an high heterogeneity due to post-translation phenomena (phosphorilation, glycosylation), genetic polymorphism (one or more variants in individual samples, much more in bulk milk), non allelic deleted forms and proteolysis by action of endogenous proteases. All caseins display a distinct lack of secondary structure, which led to classify them as rheomorphic proteins (Holt & Sawyer, 1993). The lack of secondary structure may be attributed, at least partially, to the relatively high level of proline residues in casein. As a result, caseins do not denature or associate on heating (Paulson & Dejmek, 1990). The biological function of the caseins lies in their ability to form macromolecular structures, casein micelles, which transfer large amount of calcium to the neonate with a minimum risk of pathological calcification of the mammary gland.
However, compared with cow's milk, the characterization at molecular level of donkey milk caseins is at a relatively early stage and limited data are available for its genetic polymorphism.

1.5.1 alpha-s1 casein (α_{s1}-CN)

Donkey α_{s1}-CN contains 202 amino acids (aa) and has a molecular mass (M_r) 24406 Da prior to post-translational modifications; as such, it is considerably larger than its cow (199 aa) and human (170 aa) counterpart (Table 1.3) (Cunsolo et al., 2009a). According to Miranda et al. (2004), equidae α_{s1}-CN gene, as cow and other mammals, is constituted by 19 exons (Fig. 1.1 and 1.2) mapped on chromosome 6, even if the first and the last two exons represent 5' and 3' UTR (untranslated region, respectively). Moreover, the alignment with the other species revealed the absence of exon 5 in the equine α_{s1}-CN cDNA, which resulted in the total of 18 exons in the longer form of the equine α_{s1}-CN transcript (using the number of the bovine gene). This assumption is consistent with cDNA sequences published by Milenkovic et al. (2002) and Lenasi et al. (2003), who reported alternative spicing and cryptic splice site usage.

Table 1.3: Properties of Equine, Bovine, Human and donkey α_{s1}-CN. Values were calculated from the amino acid sequences of the mature protein provided on SWISS-PROT database (http://au.expasy.org/tools).

Protein	Species	Primary Accession Number	Amino acid residues	Molecular Mass (Da)	pI	GRAVY	Cys residues
α_{s1}-CN	Equine	-	205	24614.4	5.47	–1.127	0
	Bovine	P02662	199	22974.8	4.99	–0.704	0
	Human	P47710	170	20089.4	5.17	–1.013	3
	Donkey	P86272	202	24406.4	5.95	–1.115	0

The α_{s1}-CN primary structure displays polymorphic patterns, due to alternative splicing processes leading to casual exon skipping events involving exons of the gene coding for the protein. In fact Cunsolo et al. (2009a) identified and completely characterized four donkey α_{s1}-CN components, using mare's α_{s1}-CN as reference. The two donkey's α_{s1}-CNs with a M_r 23786 (197aa) and 23658 Da (196aa) differ in the absence of a

glutamine residue at position 83 in the shorter form (196aa), which explains the difference of 128 Da in their molecular mass. In addition, they show two amino acid substitutions ($Q^{8}\rightarrow$ H and $H^{115}\rightarrow$Y) with respect to the homologous mare's α_{s1}-CN. The other two α_{s1}-CN components with M_r 24406 (202 aa) and 24278 Da (201), which also differ in the presence of a glutamine residue at position 88 in the full-length component, show the insertion of the pentapeptide HTPRE between Leu^{33} and Glu^{34}, which accounts for the increase of 620 Da in their observed molecular masses. Taking into account the exon modular structure of the α_{s1}-CN in the other species and in mare (Lenasi et al., 2003; Miranda et al., 2004), it is reasonable to assume that the pentapeptide HTPRE between Leu^{33} and Glu^{34} is encoded by the exon 5, which is constitutively spliced as in mare's α_{s1}-CNs, where it should express the sequence SIPREVRK (Lenasi et al., 2003). On the other hand, the existence of the two non-allelic deleted forms lacking a glutamine residue, which is the first amino acid encoded by exon 11, is probably related to an alternative splicing event occurring during primary transcript processing. On the other hand, the existence of a cryptic splice site occurring at the first codon (CAG) of exon 11, encoding this glutamine residue, has already reported for ewe (Ferranti et al., 1995), goat (Ferranti et al., 1997), cow and water buffalo (Ferranti et al., 1999) α_{s1}-CNs. The two α_{s1}-CNs bearing the pentapeptide insertion are named variant A (202 aa; Mr 24406) and A1 (201 amino acids; Mr 24278), whereas the two α_{s1}-CNs without the pentapeptide are named variants B (197 aa; Mr 23786) and B1 (196 aa; Mr 23658) (Table 1.4).

Table 1.4: Proposed nomenclature for the full-length and deleted donkey's α_{s1}-CNs (Cunsolo et al., 2009a).

Nomenclature of the isoforms	Calculated M_r	Amino acid differences
A	24406	-
A^1	24278	$-Q^{88}$
B	23786	$-H^{34}TPRE^{38}$
B^1	23658	$-H^{34}TPRE^{38}$; $-Q^{83}$

The absence of pentapeptide HTPRE confers to α_{s1}-CNs components, B and B1, a more acidic nature (theoretical pIs of 6.01) with respect to the components A and A1 (theoretical pIs of 6.12). The existence of a full-length component and deleted forms in α_{s1}-CN protein show genetic polymorphism in donkey milk and reflect the complex exon/intron structure of α_{s1}-CN, which consists of many short exons and allows alternative splicing of shorter exons from pre-mRNA (Lenasi et al., 2003).

α_{s1}-CN's primary structure from donkey milk has been characterized by Cunsolo et al.(2009a) who did not mention the possible phosphorilation degree of the protein, but looking its primary structure it can contains six potential phosphorylation sites (http://au.expasy.org/tools) (Ser^{18}, Ser^{73}, Ser^{74}, Ser^{76},Ser^{77} and Ser^{78}), as mare α_{s1}-CN (Ser^{18}, Ser^{75}, Ser^{77}, Ser^{79},Ser^{80} and Ser^{81}) (Lenasi et al., 2003; Mateos et al., 2009b), which are very close approximately and can thus form a phosphorylation centre, which is important in structure of casein micelles. Regarding mare's α_{s1}-CN, Mateos et al. (2009b) revealed the existence of a full-length protein and deleted forms resulting from posttranscriptional modifications; that is exon skipping involving exon 7, exon 14 or both and use of cryptic splice site encoding a glutamine residue. According to Mateos et al. (2009b) the full length α_{s1}-CN existed in equine milk, even if, additionally, the major components of α_{s1}-CN were Δ7 and Δ7,14 splicing variants, suggesting that the mechanism of alternative splicing mainly involved exon 7. Exon skipping is frequently observed in α_{s1}-CN of other mammalian species as human (Johnsen et al., 1995), caprine (Ferranti et al., 1997) and ovine milk (Passey et al., 1996), and it is not only restricted to ruminants (Martin et al., 2002). The formation of several splicing variants could be explained by the α_{s1}-CN gene structure that is divided into many short exons (19) (Martin et al., 2002). Equine α_{s1}-CN and human α_{s1}-CN (Johnsen et al., 1995) are the only α_{s1}-CNs for which exon 7 can be alternatively spliced and for the latter, the removal of exon 7 leads to the formation of a protein with 162 AA (M_r 19158 Da) for the expulsion of sequence D^{61}-Q^{68}. For each component of α_{s1}-CN, including the full-length component, a shorter component lacking a glutamine residue was characterized. This supported the occurrence of an alternative splice mechanism using a cryptic splice site located at the beginning of exon 11 and corresponding to Gln-91 (Lenasi et al., 2003). The deletion of the first codon of exon 11 (in mare α_{s1}-CN) is a rather frequent phenomenon occurring in other species (Martin et al., 2002) including ewe (Ferranti et al., 1995), goat (Ferranti et al., 1997), cow and water buffalo (Ferranti et al., 1999). In the human, the first codon of exon 6' (a supplementary exon in the gene of human α_{s1}-CN between exon 6 and exon 7) encoding a Gln-37 residue is spliced (Johnsen et al., 1995), with the formation of a deleted form with 169 AA and a molecular weight lower of 128 Da than the corresponding full-length protein. According to Martin et al. (2002), the loss of a Gln residue could be explained by a splicing error by the spliceosome. The codon CAG (encoding a Gln residue) at the beginning of an exon could be confused with the AG splice acceptor site of the adjacent intron and could be used alternatively (Smith et al., 1993).

Mateos et al. (2009b) also determined the different phosphorilation level of mare α_{s1}-CN, which varies from two to six or eight phosphate groups depending on the loss or conservation of exon 7 which encodes for an amino acid sequence with two potential phosphorilation sites (Ser^{58} and Ser^{61}).
In comparison, bovine α_{s1}-CN contains eight or nine phosphorilation sites (Swaisgood, 2003), while goat and ewe α_{s1}-CNs lead from 7 to 10 phosphate and from 5 to 11 respectively, where the highest phosphorilation level depends on the type of genetic variant considered. In contrast, the human α_{s1}-CN is resulted less phosphorylated than the other mammals, because in the sequence Ser^{70}-Glu^{78}, which is a phosphorylation cluster common to all ruminant α_{s1}-CNs, no trace of phosphate group has been found (Sorensen et al., 2003). In human α_{s1}-CN, Ser^{18} and Ser^{26} encoded by 3 and 5 exons of α_{s1}-CN gene and not always present in ruminants, are essentially phosphorylated. However, the human protein contains only four identified phosphorilation sites (Ser^{18}, Ser^{26}, Ser^{73} and Ser^{75}), despite the presence of 9 potential phosphorilation sites (Kjeldesen et al., 2007). Moreover human α_{s1}-CN (170 aa) is in mature milk as an heteropolymers with κ-CN, because it has three cysteines at position 75, 99 and 104. Two of these amino acids form an intramolecular disulphide bond, instead the other free cysteine forms an intermolecular disulphide bond with κ-CN (Johnsen et al., 1995).
Moreover, donkey (-1.115), mare (-1.127) and human (-1.013) α_{s1}-CN have comparable GRAVY scores (hydropathicity index), which are lower than that of cow α_{s1}-CN, indicating an overall higher hydrophobicity for the latter (table 1.3).

The heterogeneity of α_{s1}-CN is due to genetic polymorphism and therefore to identification of several variants of this protein in cow milk (A, B, C, D, E, F, G, H); instead for human α_{s1}-CN two variants were found, with only silent amino acid substitution ($A^{112}\rightarrow V$). To date, no information is available on genetic polymorphism of α_{s1}-CN in equidae milk (mare and donkey).

In bovine milk, α_{s1}-CN is a major structural component of the casein micelle and plays a functional role in curd formation (Walstra and Jennes, 1984). The relatively low level of α_{s1}-CN in equid milk (mare and donkey) compared with bovine milk may be significant and, coupled with the low protein content, may be responsible for the soft curd produced in the infant stomach and the foal. Goat milk lacking α_{s1}-CN has poor coagulation properties compared with milk containing α_{s1}-casein (Clark and Sherbon, 2000).

1.5.2 alpha-s2 casein (α_{s2}-CN)

The complete amino acid sequence of equine α_{s2}-CN is unknown but Ochirkhuyag et al. (2000) published the sequence of N-terminal 15 amino acid residues (Lys-His-Lys-Met-Glu-His-Phe-Ala-Pro???-Tyr-???-Gln-Val-Leu), and only five of these amino acids (Lys-His-Asn-Met-Glu-His-Arg-Ser-Ser-Ser-Glu-Glu) were confirmed by Miranda et al. (2004). Bovine α_{s2}-CN is the most highly phosphorilation casein, usually containing eleven phosphorylated serine residues, with lesser amounts containing ten, twelve or thirteen phosphate groups (Swaisgood, 2003). There are no reports on the presence of α_{s2}-CN in human milk.
Donkey α_{s2}-CN, as cow, is encoded by a gene (CSN1S2 I) constituted by 19 exons mapped on chromosome 6 which showed an identity of about 74% with corresponding bovine sequence and encodes for a predicted protein of 221 amino acids. Cosenza et al. (2010) also sequenced a donkey α_{s2}-CN cDNA sequence (CSN1S2 II) (16 exons) which encodes for a predicted peptide of 168 amino acids. So, in donkey's milk, the cDNA deduced amino acid sequences of two α_{s2}-CNs (GenBank Acc. Nos CAV00691.1) (221 aa) (Ramunno, 2008) and (GenBank Acc. Nos CAX65660.1) (168 aa) (Ramunno, 2009) have been reported. At this regard, for the first time, the primary structure of the expressed protein corresponding to the only available donkey α_{s2}-CN cDNA sequence was determined using a proteomic approach (Chianese et al., 2010). Its structural characterization confirmed for the first time the correctness of the cDNA-derived sequence reported by Ramunno (2008). The main properties of bovine and donkey α_{s2}-CN are reported in Table 1.5

Table 1.5: Properties of Equine, Bovine, Human and donkey α_{s2}-CN. Values were calculated from the amino acid sequences of the mature protein provided on SWISS-PROT database (http://au.expasy.org/tools).

Protein	Species	Primary Accession Number	Amino acid residues	M_r (Da)	pI	GRAVY	Cys residues
α_{s2}-CN	Equine	-					
	Bovine	P02663	207	24348.5	8.34	–0.918	2
	Human	-					
	Donkey	B7VGF9	221	26030.1	5.74	–0.908	2

Saletti et al. (2012) have reported the identification and direct sequence determination of the primary structure of four α_{s2}-CN isoforms (Fig. 1.4) as internally (non-allelic) deleted donkey's α_{s2}-CN forms using the amino acid sequence of the known full-length donkey's α_{s2}-CN as reference (GenBank Acc.No. CAV00691). In detail, the protein with M_r 25429 Da (A^1) differs from the full length α_{s2}-CN (A) for the deletion of the Asn ^{176}-Gln^{180} pentapeptide, whereas in the protein with M_r 21939 Da ($A1^{\Delta4,5,6}$), in addition to the same peptide, the sequence Asp^{12}-Glu^{42} is also suppressed. In the third non-allelic deleted form (M_r 25203 Da) (A^2), the Tyr^{212}-Leu^{218} heptapeptide is absent in comparison with the full-length α_{s2}-CN, whereas in the primary structure of the component with M_r 21713 Da ($A2^{\Delta4,5,6}$), both the heptapeptide Tyr^{212}-Leu^{218} and the sequence Asp^{12}-Glu^{42} are missed. Taking into account the exon modular structure of the donkey's α_{s2}-CN (Cosenza et al., 2010), it can be observed that the sequence Asn^{176}-Gln^{180}, absent in the components with M_r 25429 (A^1) and 21939 Da ($A1^{\Delta4,5,6}$), represents the first five amino acids encoded by the exon 17; the sequence Asp^{12}-Glu^{42}, absent in the primary structure of the components with M_r 21939 ($A1^{\Delta4,5,6}$) and 21713 Da ($A2^{\Delta4,5,6}$), is encoded by exons 4, 5, and 6; the heptapeptide Tyr^{212}-Leu^{218}, absent in the primary structure of the components with M_r 25203 (A^2) and 21713 Da ($A2^{\Delta4,5,6}$), is the terminal part of the sequence encoded by the exon 17. As evidenced by these data, 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 starting five amino acids or the final seven amino acids encoded by this exon in the final products. In light of these consideration, the full length donkey's α_{s2}-CN and the four internally (non-allelic) deleted forms can be named as reported in Table 1.6.

Table 1.6: Proposed nomenclature for the full-length and the internally deleted donkey's α_{s2}-CNs (Saletti et al., 2012).

Nomenclature of the isoforms	Calculated M_r	Amino acid differences
A	26028	-
A^1	25429	-Asn ^{176}-Gln^{180}
$A1^{\Delta4,5,6}$	21939	-Asp^{12}-Glu^{42}; -Asn ^{176}-Gln^{180}
A^2	25203	-Tyr^{212}-Leu^{218}
$A2^{\Delta4,5,6}$	21713	-Asp^{12}-Glu^{42}; -Tyr^{212}-Leu^{218}

The existence of a full-length α_{s2}-CN (221 AA and M_r 26028) (Chianese et al., 2010) and its four different deleted forms resulting from differential splicing events, is analogous to what was already noted for the α_{s1}-type CNs (Cunsolo et al., 2009a). This polymorphism seems to be correlated with the complex intron/exon modular structure of the genes encoding these proteins, consisting of a large number of short exons, that may undergone differential splicing events during primary transcript processing, thus originating protein components with different amino acid lengths (Lenasi et al., 2003). Comparative analysis of the primary structure of the α_{s2}-CN from donkey and other mammalians reveals that the differential splicing events involving the exons 4,5,6 and the last seven amino acids encoded by exon 17 seem unique of donkey species, whereas the first five amino acids (N^{176}KINQ^{180}) encoded by exon 17 are also constitutively spliced in the α_{s2}-CN from mare (NCBI Acc. No. NP_001164238) (Martin et al., 2009). Moreover it is interesting to note that the amino acid sequences absent in the internally deleted α_{s2}-CN forms from donkey are traits of some IgE-binding epitopes of bovine α_{s2}-CN. In particular, the NKINQ sequence absent in the deleted forms $A^1$and $A1^{\Delta4,5,6}$ from donkey and constitutively spliced in the α_{s2}-CN from mare is a trait of two major IgE-binding epitopes of bovine α_{s2}-CN an therefore could be related to the already demonstrated low allergenic properties of donkey's milk.
Regarding α_{s2}-CN genetic polymorphism, only four variants (A, B, C, D) are known for bovine α_{s2}-CN, while to date no information is available on genetic polymorphism of α_{s2}-CN in equidae milk (mare and donkey).

1.5.3 Beta-casein (β-CN)

According to Lenasi et al. (2003), the number and approximately length of individual exons in horse (as in donkey) are most probably the same as in the majority of other species. By aligning equine β-CN cDNAs with corresponding cDNAs of other species, 9 exons mapped on chromosome 6 were found in equine β-CN, where the first and the last two exons represent 5' and 3' UTR (untranslated region, respectively) (Fig. 1.3).
β-CN's primary structure from donkey milk has been characterized completely (Cunsolo et al., 2009b), using mare's β-CN derived from cDNA as reference reported by Lenasi et al. (2003) and revised by Girardet et al. (2006). It is constituted of 226 amino acids and it has a molecular weight of 25529 Da. Human, cow and

mare β-CN contain 211, 209 and 226 amino acid residues, respectively (Table 1.7). However donkey β-CN primary structure displays polymorphic patterns, due to alternative splicing processes leading to casual exon skipping events involving exon 5, as occurring in the mare's β-CNs used as reference and reported by Miranda et al., 2004.
In fact Cunsolo et al. (2009b) also identified an deleted component of β-CN, which has a molecular weight of 24406 Da. So the two donkey β-CNs differ by the presence of the domain $E^{27}SITHINK^{34}$ in the full-length component, as occur in the mare's β-CNs used as references, which explains the mass difference of 923 Da.

Table 1.7: Properties of Equine, Bovine, Human and donkey β-CN. Values were calculated from the amino acid sequences of the mature protein provided on SWISS-PROT database (http://au.expasy.org/tools).

Protein	Species	Primary Accession Number	Amino acid residues	M_r (Da)	pI	GRAVY	Cys residues
β-CN	Equine	Q9GKK3	226	25511.4	5.78	–0.415	0
	Bovine	P02666	209	23583.2	5.13	–0.355	0
	Human	P05814	211	23857,8	5.33	–0.289	0
	Donkey	P86273	226	25529,3	5.54	–0.406	0

In view of theses analogies, the same nomenclature of mare's β-CNs was adopted to name the new donkey β-CN variants. Therefore, the full length (226 amino acids) β-CN was named variant A, whereas the β-CN lacking the region 27-34 (218 amino acids) encoded by exon 5 was termed variant $A^{\Delta 5}$ (Table 1.8).

Table 1.8: Proposed nomenclature for the full-length and the deleted donkey's β-CN (Cunsolo et al., 2009b).

Nomenclature of the isoforms	Calculated M_r	Amino acid differences
A	25529	-
$A^{\Delta 5}$	24406	-Glu ^{27}-Lys^{34}

The two donkey β-CNs differ from the homologous mare's β-CNs by the presence of nine amino acid substitutions: $L\rightarrow S^{37}$, $R\rightarrow H^{52}$, $S\rightarrow N^{81}$, $P\rightarrow V^{84}$, $L\rightarrow V^{91}$, $R\rightarrow Q^{203}$, $P\rightarrow L/I^{206}$, $L\rightarrow F^{210}$, and $A\rightarrow P^{219}$. Taken together, this substitutions account for the increase of 18 Da in the M_r of the donkey's β-CNs in comparison with the mares counterparts.
The primary structure of the full-length donkey β-CN contains seven potential phosphorilation sites (Ser^9, Ser^{15}, Ser^{17}, Ser^{18}, Ser^{23}, Ser^{24} and Ser^{25}), together with two other potential phosphorilation sites located at Thr^{12} and Thr^{207}, analogously to the homologous mare's β-CN. The Ser-Xxx-Glu/SerP and Ser-Xxx-Xxx-Glu motifs are the recognition sequences characterized for cow mammary gland casein Kinase I (Mercier, 1981) and II (Kuenzel et al., 1987), whereas studies on the specificity of mammary gland casein kinase have shown that the sequence motif Thr-Xxx-Glu is a poor substrate for the mammary gland enzyme and is usually not phosphorylated (Bingham & Groves, 1979; Sorensen & Petersen, 1994). Moreover the mRNA of donkey's β-CN, as mare's β-CN contains an additional exon (exon 4' corresponding to the 21-26 region), which is not observed in the other mammals, and seems specific to equidae (Miranda et al., 2004). This additional exon 4' encodes a region containing a phosphorilation cluster with three potential sites and, hence, is partly the cause of the high content in SerP of equine and donkey β-CN. The presence of exon 4' in equidae would largely compensate for the substitution of a SerP residue of the –SerP-SerP-SerP-Glu- cluster, that is usually found in the exon 4-encoded region (residues 12-20) of numerous mammals, by an Asn residue in position 19.
Therefore the analysis of the donkey phosphorylated β-CNs demonstrated that the full-length variant A consist of three components carrying from five to seven phosphate groups, whereas no data were obtained about the phosphorilation level of the short β-CN variant $A^{\Delta 5}$. 6P and 7P isoforms of the variant A occur with comparable relative abundances whereas the 5P protein is present as a minor component, revealing that this protein is more phosphorylated (Cunsolo et al., 2009b) than the homologous mare's β-CN, in which the predominant phosphorylated components carries five phosphate groups even if its maximum degree of phosphorilation is 7P (Girardet et al., 2006). According to Mateos et al. (2009a), in equine milk the short β-CN variant $A^{\Delta 5}$ also carries five phosphate groups even if its maximum degree of phosphorilation is 7P. Also cow β-CN contains four o five phosphorylated serine residue (Swaisgood, 2003), instead human β-CN has up to six phosphorilation level (Sood & Slattery, 2000) and the phosphate groups are located in a cluster sequence near the amino terminus, within the first ten residues of this protein (Greenberg et al., 1984).
Moreover, unique to equine milk and apparently absent from the milk of other mammals (including ruminants) is a low molecular mass β-CN protein which accounts for 4% of the casein content (Miclo et al., 2007). This short protein (94 amino acid residues) is an internally truncated form of the full length equine β-

CN (226 residues), in which a large internal peptide sequence (132 residues) is lacking. According Miclo et al. (2007), this large deletion (residues 50-181) might be due of an alternative donor splice site, identified in the terminal part of the nucleotide sequence encoding exon 7. Such a cryptic splice site usage should result in RNA forms that lacked the first 396 nucleotides of exon 7. Deletion of the basic 50-181 region conferred to this short protein a more acidic nature than corresponding full-length β-CN. Seven phosphorilation were identified with one to seven phosphate groups, even if the major isoforms carry five and six phosphate groups. Moreover, no spontaneous deamidation of this low molecular mass form of β-CN has been found, and the preferential site of hydrolysis by plasmin (Lys^{55}-Ile^{56}) in β-CN sequence was lost after truncation of exon 7, whereas the highly sensitive peptide bond toward chymosin (Leu^{198}-Tyr^{199}) was preserved (Miclo et al., 2007).
Equine β-CN is a good substrate for plasmin associated to equine caseinate, where the Lys^{47}-Ile^{48} bond of β-CN is readily hydrolysed leading to the production of γ-like caseins, whereas no cleavage of a corresponding bond Lys^{48}-Ile^{49}, in bovine β-CN occurs (Egito et al., 2003). In bovine milk, plasmin activity on β-CN results in production of γ1-, γ2-, γ3-CNs (fragments 29-209, 106-209 and 108-209 of bovine β-CN, respectively) and of their complementary amino-terminal peptides, components 5 of proteose peptone (PP5), components PP8-slow, and component PP8-fast (fragments 1-105/107, 29-105/107, and 1-28 of β-CN, respectively). In bovine β-CN, Lys^{28}-Ile^{29} is readily cleaved by plasmin but the equivalent, Lys^{28}-Ile^{29} in equine β-CN is insensitive (Egito et al., 2002). Other plasmin cleavage sites in equine β-CN are Lys^{103}-Arg^{104} Arg^{104}-Lys^{105} and Lys^{105}-Val^{106} (Egito et al., 2002). Equine β-CN is readily hydrolysed by chymosin at Leu^{190}-Tyr^{191} (Egito et al., 2001) and the two fragments generated seemed to be resistant to further hydrolysis by chymosin at pH 6.5.
Equine, bovine, human and donkey β-CN have a very hydrophilic N-terminus, followed by a relatively random hydropathy distribution in the rest of the protein, leading to an amphiphilic protein with a hydrophilic N-terminus and a hydrophobic C-terminus (Table 1.7).

Regarding β-CN genetic polymorphism, this phenomenon is quite complex in cow's milk, due to the high variability and to the presence of a large number of cases not characterized or not well clarified variants. However for cow β-CN, in addition to already identified genetic variants (A1, A2, A3, B, C, D, E), new variants have been identified (F, H1, H2 and I). According to Greenberg et al. (1984), there is also the existence of genetic polymorphism in human β-CN, but probably the substitution involving uncharged amino acids make difficult to demonstrate this phenomenon. To date no information is available on genetic polymorphism of β-CN in equidae milk (mare and donkey).

1.5.4.1 Kappa casein (κ-CN)

The presence of κ-CN in equine milk was an issue of debate for several years, with several authors (Ono et al., 1989; Visser et al., 1992; Ochirkhuyag et al., 2000) reporting its absence, even if other studies (Malacarne et al., 2000; Egito et al., 2001; Iametti et al., 2001) showed its presence, albeit at low concentration. The primary structure of equine κ-CN has been derived (Iametti et al., 2001; Lenasi et al., 2003; Miranda et al., 2004); it contains 165 amino acid residues, four less than bovine κ-CN but three more than human casein (table 1.9). The molecular mass of equine κ-CN, prior to post-translation modification is 18847.7 Da. Equine and human κ-CN have a considerably higher isoelectric pH than bovine κ-CN (table 1.9), and they have a net positive charge at physiological pH, whereas bovine κ-CN has a net negative charge. In fact, equine κ-CN contains 11 negatively charged residues and 14 positively charged residues compared with 16 and 14, respectively, in the bovine protein. This distribution is reflected in the calculated pI values (equine: 9.02; bovine: 5.93). The particular composition of equine κ-CN is similar to that found in κ-CN from monogastric species, such as human κ-CN (11 negatively charged residues; 13 positively charged residues, pI 8.68), or pig κ-CN (15 negatively charged residues; 17 positively charged residues; pI 8.68) (Iametti et al., 2001). The GRAVY score of bovine κ-CN is considerably lower than that of equine κ-CN (Table 1.9), indicating that equine κ-CN is less hydrophilic than its bovine counterpart. Bovine κ-CN is characterized by a hydrophilic C-terminus, which is very important for the stabilization of bovine casein micelles, but a comparison of the hydropathy distribution of bovine and equine κ-CNs indicates that the C-terminus of equine κ-CN is far less hydrophilic, particularly as a result of the absence of a strong hydrophilic region at residues 110-120. Equine κ-CN appears to be more like human than bovine κ-CN in terms of the distribution of hydropathy along the polypeptide chain.

Table 1.9: Properties of Equine, Bovine, Human κ-CN. Values were calculated from the amino acid sequences of the mature protein provided on SWISS-PROT database (http://au.expasy.org/tools).

Protein	Species	Primary Accession Number	Amino acid residues	M_r (Da)	pI	GRAVY	Cys residues
κ-CN	Equine	P82187	165	18844.7	8.03	–0.313	2
	Bovine	P02668	169	18974.4	5.93	–0.557	2
	Human	P07498	162	18162.6	8.68	–0.528	1

1.5.4.2 Glycosylation of κ-CN

κ-CN is the only glycosylated member of the casein family and exhibits micro-heterogeneity due to the level of glycosylation (Saito & Itoh, 1992). Oligosaccharides consisting of N-acetylneuraminic acid (NANA), galactose and N-acetylgalactosamine are attached to κ-CN via O-glycosidic linkages to threonine residues in the C-terminal portion of the molecule (glycomacropeptide, GMP, region). Human κ-CN contains approximately 5 times more sugars (around 55%) than cow. Not only galactose, NANA and N-acetylgalactosamine, but also fucose and N-acetylglucosamine were identified (Fiat et al., 1980). Glycosylation degree is higher in colostrum than in milk, and increases during mastitis infection (Dziuba & Minkiewicz, 1996). Although no direct information is available, lectin binding studies indicate that equine κ-CN is glycosylated (Iametti et al., 2001), probably at residues Thr^{123}, Thr^{127}, Thr^{131}, Thr^{149}, Thr^{153} (Lenasi et al., 2003), although these glycosylation sites are not fully in agreement with those proposed by Egito at al. (2001). In equine κ-CN, nine threonine residues were possible O-glycosylation sites Thr^{116}, Thr^{123}, Thr^{127} Thr^{131}, Thr^{150}, Thr^{153} Thr^{154}, Thr^{155} andThr^{159} (Egito et al., 2001). Equine κ-CN might thus be more glycosylated than bovine κ-CN which has six glycosylated threonine residues. In general, κ-CNs of Group II are more glycosylated than κ-CNs of Group I (as will be seen later). About two-thirds of bovine κ-CN molecules are glycosylated at one of six threonil residues, i.e., Thr^{121}, Thr^{131}, Thr^{133}, Thr^{135}, Thr^{136} (only in bovine κ-CN variant A) or Thr^{142} (Pisano et al., 1994). Human κ-CN have seven glycosylation sites Thr^{113}, Thr^{123}, Thr^{128}, Thr^{131}, Thr^{137}, Thr^{147} and Thr^{149} (Fiat et al., 1980). To date, no non-glycosylated κ-CN has been identified in equine milk (Martuzzi & Doreau, 2006).

No direct information is available on the phosphorilation state of the equine protein. However, three putative phosphorilation sites, namely Thr^{127}, Thr^{150} and Ser^{160}, may be suggested based on the sequence similarity studies with other κ-CNs and on prediction analyses (Iametti et al., 2001).

κ-CN is located mainly on the surface of the casein micelles and is responsible for their stability. The presence of a glycan moiety in the C-terminal region of κ-CN enhances its ability to stabilize the micelle, by electrostatic repulsion, and may increase the resistance by the protein to proteolytic enzymes and high temperature (Dziuba & Muzinska, 1996). Biologically, NANA residues have antibacterial properties and act as a bifidogenic factor (Dziuba & Muzinska, 1996). κ-CN is thought to play a major role in preventing adhesion of *Helicobacter pilori* to human gastric mucosa (Stromqsit et al., 1995). It is likely that heavily glycosylated κ-CN provides protection due to its carbohydrate content and breast-feeding infants is thought to provide some protection, especially *Helicobacter pilori* infection is occurring in increasingly younger age (Lonnerdal, 2003). In spite of some peculiarities, some of which also occur in other monogastric species, equine milk κ-CN appeared to have all the properties found in other κ-CNs and associated with the role of this protein family in the formation and stabilization of the micellar structure of casein in milk (Iametti et al., 2001).

1.5.4.3 Hydrolysis of κ-CN

The hydrolysis of bovine κ-CN by chymosin at Phe_{105}-Met_{106} leads to the production of the hydrophobic (non-glycosylated) N-terminal para-κ-CN and the hydrophilic, highly charged (O-glycosylated) C-terminal caseinomacropeptide (CMP) (Walstra & Jennes, 1984). Release of CMP causes destabilisation of the casein micelles, resulting in their coagulation. The insoluble para-κ-CN remains in the coagulum, whereas CMP is released into the soluble phase (Jollès et al., 1972) Calf chymosin hydrolyses the Phe_{97}-Ile_{98} bond of equine κ-CN (Egito et al., 2001) and slowly hydrolyses the Phe_{105}-Met_{106} bond of human κ-CN (Plowman et al., 1999). However as summarized in Table 1.10, the CMPs released from equine or human κ-CN are considerably less hydrophilic than bovine CMP.

Table 1.10: Properties of Equine, Bovine, Human para-κ-CN and CMP. Values were calculated from the amino acid sequences of the mature protein provided on SWISS-PROT database (http://au.expasy.org/tools).

Protein	Species	Amino acid residues	M_r (Da)	pI	GRAVY
Para-κ-CN	Equine	1-97	11693.3	8.96	–0.675
	Bovine	1-105	12285.0	9.33	–0.617
	Human	1-105	11456.9	9.63	–1.004
CMP	Equine	98-165	7169.3	4.72	0.203
	Bovine	106-169	6707.4	4.04	–0.370
	Human	106-169	6723.7	4.24	0.182

The sequence 97-116 of κ-CN is highly conserved across species, suggesting that the limited proteolysis of κ-CN and subsequent coagulation of milk are of major biological significance (Mercier at al., 1976). A grouping system for mammals based on κ-CN structure and the site of cleavage by chymosin has been suggested. Group I species (cow, goat, sheep, buffalo) have a high content of dicarboxylic amino acids and low hydrophobicity and carbohydrate content and κ-CN is cleaved at Phe_{105}-Met_{106}, while Group II species (human, mouse, pig, rat) have a high proline content, less dicarboxylic amino acids and a much higher hydrophobicity and carbohydrate content and are cleaved at Phe_{97}-Ile_{98} or Phe_{105}-Leu_{106}. Cleavage of equine milk at Phe_{97}-Ile_{98}, as well as other characteristics of its κ-CN (i.e., zebra), place the horse in Group II. The divergence between species into Groups I and II could account for differences in the clotting mechanism of ruminant and non-ruminant milk (Herskovitis, 1966). In addition to differences in cleavage sites, the grouping system also divides species based on the number of O-glycosylation sites. As equine and human κ-CNs are considerably more highly glycosylated than bovine κ-CN and non-glycosylated κ-CN has not been found in equine milk (Egito et al., 2001), equine and human κ-CN belong to the same group. The level of glycosylation does not affect micelle structure but it does affect the susceptibility of κ-CN to hydrolysis by chymosin, with susceptibility decreasing as the level of glycosylation increases (Addeo et al., 1984; Zbikwoska et al., 1992). Therefore, equine milk probably has a different chymosin-induced clotting mechanism than bovine milk.

The proportion of κ-CN in equine milk appears to be lower compared to that cow's and human milk (Egito et al., 2001). Also in donkey's milk this protein might be in minute, and therefore undetectable quantities,, making their characterization difficult (Vincenzetti et al., 2008). However Chianese et al. (2010) identified this protein in donkey milk, thanks to the recent advances in proteomic technology. The results obtained by Chianese et al. (2010) showed, also for this protein, a high heterogeneity due to its different glycosylation level. However, to date the knowledge of donkey κ-CN is scant and only the primary structure of κ-CN, deduced from corresponding cDNA, has been reported (Di Gregorio, 2011). κ-CN cDNA have been characterized in several species and its structure is very similar among them. Regarding κ-CN (CSN3) gene in horse, it consists of 5 exons mapped on chromosome 6, even if the mature κ-CN is only encoded by part of the exon 3 and the entire exon 4 (Hobor et al., 2006). The first and the last exons represent 5' and 3' UTR (untraslated region), respectively, while the signal peptide encoded by exon 2 does not also transated (Lenasi et al., 2003). Moreover, the κ-CN gene was postulated to be not evolutionary related to the genes encoding the calcium sensitive caseins, but evolutionary to fibrinogen gene family whose cleavage by thrombin results in blood clotting (Jolles et al., 1978). This hypothesis is sustained by the structural and functional similarities between the proteins, and by nucleotide sequence similarities between κ-CN and fibrinogen cDNAs.

The genetic polymorphism of κ-CN is also of interest, since some variants could be more beneficial from the point of human nutrition or be associated with milk quality, composition and technological characteristics (Yahyaoui et al., 2003). In case of cow's milk, B allelic variant of CSN3 gene is preferable and animals with genotype BB produce milk with better cheese making ability and shorter rennet time.

To date no information is available on genetic polymorphism of κ-CN in equidae milk (mare and donkey) and specifically for donkey κ-CN, its primary structure was not determined.

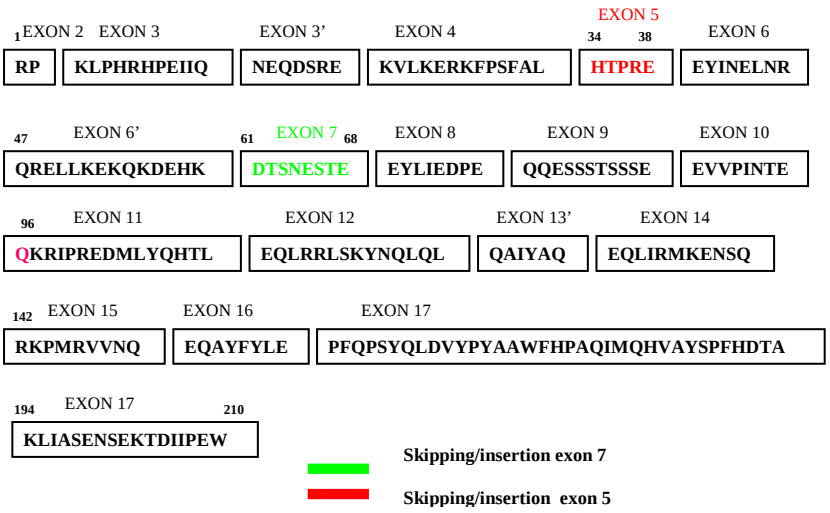

Fig. 1.1 Primary structure of donkey α_{s1}-CN (202 aa since exon 7 outspliced). IN RESULTS AND DISCUSSION, the insertion of exon 7 which encodes for octapeptide DTSNESTE as in homologous mare α_{s1}-CN, determines the formation of donkey α_{s1}-CN with 210 aa long. With the insertion of exon 7 and skipping of first codon CAG of exon 11, donkey α_{s1}-CN is 209 aa long.

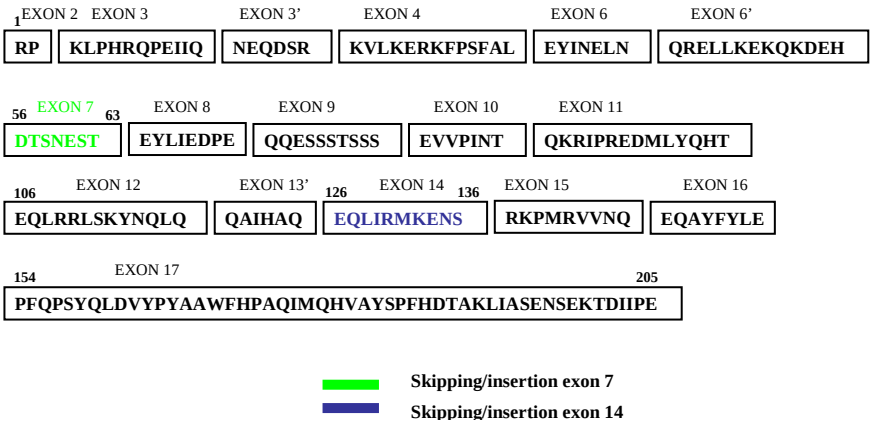

Fig. 1.2 Primary structure of mare α_{s1}-CN (exon 5 outspliced).

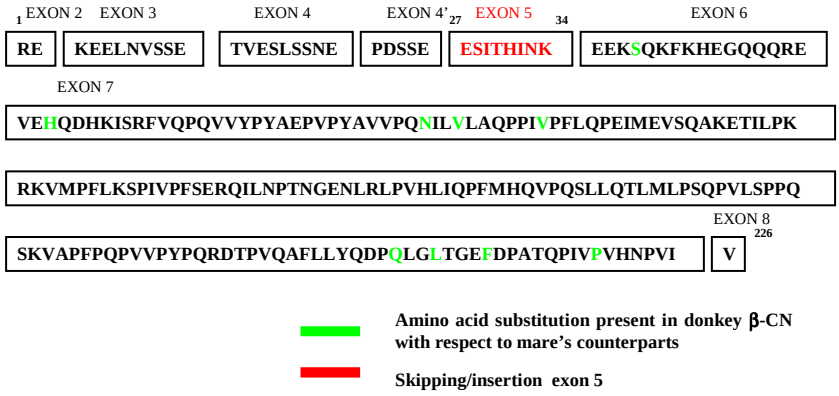

Fig. 1.3: Primary structure of donkey and mare β-CN.

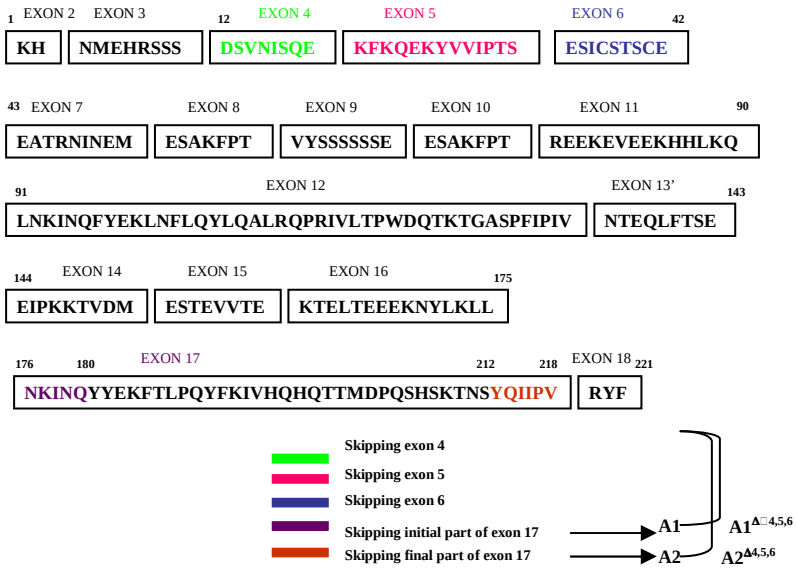

Fig. 1.4: Primary structure of donkey α_{s2}-CN.

1.6 Donkey whey proteins

Donkey's milk whey proteins represent the 35-50% of the nitrogen fraction whereas in bovine milk only the 20% (Herrouin et al., 2000). The most important whey proteins in donkey's milk are α-Lactalbumin (α-La), β-Lactoglobulin (β-Lg) and lysozyme (Lyz), as demonstrated by Fantuz et al. (2001). In Table 1.11, a comparison of the concentration of total caseins, whey proteins and non-casein proteins in human, mare's and donkey's milk is shown.

Table 1.11 : Comparison of the concentration of total caseins, total whey proteins and of the relative amounts of non-casein proteins in human, mare's and donkey's milk (Malacarne et al., 2002; Miranda et al., 2004).

	Human (mg/ml)	Equine (mg/ml)	Donkey (mg/ml)
Caseins	5.8	10.3b-14.0a	6.60
Whey proteins	2.1	8.03a-7.40b	7.50
Lysozyme	0.50a	1.10a	1.00
β-Lattoglobulin		3.00a	3.75
α-Lactalbumin	1.60a	3.30a	1.80

In contrast to bovine milk proteins, only limited data are available for genetic polymorphism of donkey whey proteins.
Similar to α-La of bovine, caprine, ovine, camelid, human and mare's milk, donkey α-La contains 123 amino acids.
In horse milk, Godovac-Zimmermann et al. (1987) showed the occurrence of three genetic variants of α-La named α-La A, B and C, which differ by only a few single amino acids, whereas only one α-La genetic variant was reported in donkey milk, even if apparent heterogeneity of the protein was observed (Giuffrida et al., 1992) for the identification of two isoforms (A and B) both of them glycosylated (Bertino et al., 2010). Donkey α-La is closely related to the horse A-type variant according to the amino acid sequence alignment. The percentage of sequence identity between the two proteins is 99.2%, whereas it is 96,7% with horse B/C type variants. Bovine α-La occurs as two, or possibly three, genetic variants (Bell et al., 1981) and human α-La has two genetic variants, one of which has been identified only recently (Chowanadisaia et al., 2005). The primary structures of mare, bovine, human and donkey α-La differ only by a few single amino acid replacements, and the proteins have similar properties (Table 1.12)

Table 1.12: Properties of equine, bovine, human and donkey α-La. Values were calculated from the amino acid sequences of the mature protein provided on SWISS-PROT database (http://au.expasy.org/tools).

Species	Variant	AA residues	Molecular Mass (Da)	pI	GRAVY score	Disulphide bridges
Equine	A	123	14223.2	4.95	-0.416	4
	B	123	14251.2	4.95	-0.503	4
	C	123	14249.3	5.11	-0.438	4
Bovine		123	14186.0	4.80	-0.453	4
Human		123	14078.1	4.70	-0.255	4
Donkey		123	14222.3	5.11	-0.416	4

Equine α-La A, B, and C have an isoelectric point at pH 4.95, 4.95 and 5.11, respectively, as donkey α-La (5.11), whereas bovine and human α-La have pIs at pH 4.80 and 4.70, respectively (Table 1.12). The GRAVY scores of equine, bovine and donkey α-La are comparable, whereas that of human α-La is distinctly higher (Table 1.12), indicating a lower hydrophobicity. The eight cysteine residues of bovine and human α-La form four intra-molecular disulphide bonds (Cys_6-Cys_{20}, Cys_{28}-Cys_{111}, Cys_{61}-Cys_{77}, Cys_{73}-Cys_{93}). Based on the very high similarity between equine, bovine human and donkey α-La, as well as the α-La from other species, it is highly likely that equine and donkey α-La also contains four intra-molecular disulphide bridges, at the aforementioned positions.
According to Vincenzetti et al. (2008), the donkey α-La content showed a marked increase three month after parturition to reach the value of 1.8 mg/mL, that remained almost stable during the remaining lactation period (Table 1.13). This value obtained for donkey's milk is in good agreement with the α-La content in human milk (1.6 mg/mL) but is very low to the mare α-La content (3.3 mg/mL), which is instead very close to the α-La content in bovine milk (Table 1.11).

However, α-La, a unique protein in the milk of mammals, is homologous with the well characterized c-type lysozyme. It is a calcium metalloprotein, in which Ca^{2+} plays a crucial role in folding and structure. The determination of calcium amount bound to the A and B isoforms showed that A α-La binds less Ca^{2+} than B, so its apparent heterogeneity is due to different ability to bind $Ca^{2+,}$ missing in the α-La A (Conti et al., 1989; Herrouin et al., 2000). Moreover, α-La is synthesized in the rough endoplasmic reticulum, from where it is transported to the Golgi apparatus, where it has a regulatory function in the synthesis of lactose. Together with β-1,4 galactosyltranferase, which is the catalytic component of the lactose synthetase, α-La enhances affinity for glucose 1000-fold in the final step of lactose synthesis, when glucose is linked to galactose (Neville, 2009).

β-Lg is the major whey protein in the milk of ruminants, and is also present in milk of monogastrics and marsupials, but not in that of humans and rodents (Hambling et al., 1992). It is a member of the superfamily of hydrophobic molecule transporters or Lipocalins which have a strong affinity for hydrophobic molecules. The physiological function of this protein is unclear, though it is suspected to be involved in retinol and fatty acids transport (Pérez & Calvo, 1995). Bovine β-Lg is very resistant to peptic digestion and can cause allergenic reaction on consumption, even if the resistance to digestion is not uniform among the species and this is important considering the potential application of donkey's milk as a hypo-allergenic dairy product (El-Zahar et al., 2005).

The donkey β-Lg content proved to be stable during the different stages of lactation with a mean value of about 3.75 mg/mL (Table 1.13) (Vincenzetti et al., 2008) which is very close to that of bovine milk (3.3 mg/mL) and mare's milk (3.0 mg/mL), whereas in human milk the β-Lg content is absent (Miranda et al., 2004) (Table 1.11).

Table 1.13: Quantitative determination of α-La, lysozyme, β-Lg in different stage of lactation (Vincenzetti et al., 2008).

Days after parturition	Lysozyme (mg/mL)	β-Lg (mg/mL)	α-La(mg/mL)
60	1.34	Non deter.	0.81
90	0.94	4.13	1.97
120	1.03	3.60	1.87
160	0.82	3.69	1.74
190	0.76	3.60	1.63

In ruminants β-Lg is encoded by a single gene, although the presence of a β-Lg pseudogene has been described in the cow (Passey & MacKinlay, 1995) and goat (Folch et al., 1996). In the milk of these species, β-Lg exists predominantly as a dimer. Bovine β-Lg occurs mainly as two genetic variants A and B, both of which contain 162 amino acids and differ only at position 63 (Asp in variant A, Gly in variant B) and 117 (Val in variant A, Ala in variant B); a further eleven, less common genetic variants of bovine β-Lg have also been reported (Sawyer, 2003). In donkeys milk as in horse, dogs and dolphins, two structurally different monomeric β-Lgs exist, called β-Lg I and β-Lg II. The presence of third protein in milk (β-Lg III) has been described only in cats, where β-Lgs exhibit a high polymorphism (Halliday et al., 1993).

In donkey's milk, β-Lg I is the major component and represents about 80% of total β-Lg, instead β-Lg II is the minor component (about 20% of the total protein) (Godovac-Zimmermann et al., 1990). β-Lg I is 162 amino acids long (4 cysteines) and two genetic variants named A (18524 Da) (Godovac-Zimmermann et al., 1988a) and B (18510 Da) (Herrouin et al., 2000) with a molecular mass difference of 14 Da due to three amino acid substitutions E^{36}→S, S^{97}→T e V^{150}→I (Table 1.14). The homology of donkey β-Lg I A with B variants accounted for 98.1% and each of them, was 98.8% and 99.4%, identical to horse counterpart, respectively. In the same way the homology of β-Lg I with donkey β-Lg II and cow counterpart accounted for 65.6% and 56.0% respectively (http://au.expasy.org).

β-Lg II presents 163 amino acids for the additional glycine residue between 116-117th aa as in mare's milk. Four genetic variants of donkey β-Lg II named A (Godovac-Zimmermann et al., 1990), B (Herrouin et al., 2000), C (Herrouin et al., 2000) and D (Cunsolo et al., 2007) have been identified and characterized (Table 1.14).

Unlike ruminants β-Lgs and donkey β-Lg II A (each having five cysteine residues), only four cysteines were found in asinine species. Specifically, donkey, equine and bovine β-Lg contain two intra-molecular disulphide bridges, linking Cys_{66}-Cys_{160} and Cys_{106}-Cys_{119} in equine and donkey β-Lg I, Cys_{66}-Cys_{161} and Cys_{106}-Cys_{120} in equine and donkey β-Lg II and Cys_{66}-Cys_{160} and Cys_{106}-Cys_{119} or Cys_{121} in bovine β-Lg A and B (Cunsolo et al., 2007). At this regard, unlike bovine β-Lgs, equine and donkey β-Lg lack a sulphydryl group, which has large implications for denaturation and aggregation of the protein. Also, unlike bovine β-Lg, equine and donkey β-Lg do not dimerize (Sawyer, 2003).

Table 1.14: Physico-chemical characteristics of donkey β-Lg I and β-Lg II genetic variants.

Protein	Genetic variant	Amino acid Substitutions	Amino acid residues	Molecular mass (Da)	Number and positions of disulphide bridges	pI
β-Lg I	A	E^{36} S^{97} V^{150}	162	18528	2 (66-160; 106-119)	4.79
	B	S^{36} T^{97} I^{150}	"	18514	"	4.85
β-Lg II	A	D^{2} R^{18} V^{25} D^{96} $^{f}C^{110}$ M^{118} G^{162}	163	18263	2 (66-161;106-120)	4.70
	B	P^{110} T^{118}	"	18227	"	4.70
	C	E^{96} P^{110} T^{118}	"	18241	"	4.72
	D	P^{110} D^{162}	"	18311	"	4.64

The two β-Lgs (β-Lg I and β-Lg II), each containing 162 and 163 amino acid respectively, were also found in equine milk (Halliday et al., 1991). The main characteristics of bovine, equine and donkey β-Lg were reported in Table 1.15, where bovine β-Lg A and B have a GRAVY score of –0.167 and–0.162, respectively, and are, therefore, considered to be less hydrophilic than equine and donkey β-Lg I and β-Lg II.

Table 1.15: Properties of equine, bovine, human and donkey β-Lg. Values were calculated from the amino acid sequences of the mature protein provided on SWISS-PROT database (http://au.expasy.org/tools).

Species	Variant	AA residues	Molecular Mass (Da)	pI	GRAVY score	Disulphide bridges
Equine	I	162	18500.1	4.85	-0.386	2
	II	163	18261.6	4.71	-0.300	2
Bovine	A	162	18367.3	4.76	-0.167	2
Bovine	B	162	18281.2	4.83	-0.162	2
Donkey	I	162	18528.2	4.79	-0.404	2
Donkey	II	163	18262.6	4.70	-0.265	2

1.6.1 The donkey lysozyme

Lysozyme (Lyz) is known to be a natural antimicrobial agent (very resistant to acid and protease gastrointestinal digestion) since it catalyses the hydrolysis of glycosidic bonds of mucopolysaccharides in bacterial cell walls (Chiavari et al., 2005), and so inhibiting many pathogens bacteria development makes the milk somewhat selective with regards to the bacteria it can host. So this enzyme, together with other factor including immunoglobulins, lactoferrin and lactoperoxidase could be one of the most important component of the immune system of children and it may function in the infant's digestive tract to reduce the incidence of gastrointestinal infections (Businco et al., 2000). According to Vincenzetti et al. (2008), the Lyz content in donkey's milk varied considerably during the different stages of lactation (Table 1.13), with a mean value of 1.0 mg/mL, and proved to be higher with respect to that in bovine (traces), human (0.12 mg/mL) and goat's milk (traces), whereas, it was very close to mare's milk (0.79 mg/mL), as observed by Stelwagen, (2003). Two genetic variants of donkey Lyz are known: A (14680) (Godovac-Zimmermann et al., 1988b) and B (14631) (Herrouin et al., 2000) with three amino acid substitutions in comparison with Lyz A $N^{49} \rightarrow D$, $Y^{52} \rightarrow S$ and $S^{61} \rightarrow N$, which explain the loss of 48 Da in the molecular mass of Lyz A. However, donkey Lys B showed 97.7% identity with donkey Lyz A. In comparison with horse Lyz, the donkey Lyz B has a greater percentage identity than donkey Lys A (98.4% vs. 97.7%) (Table 1.16)

Table 1.16: Donkey Lyz genetic variants in comparison with horse Lyz.

Lyz	49	52	61	86
A Donkey	Asn (N)	Tyr (Y)	Ser (S)	Asp (D)
B Donkey	Asp (D)	Ser (S)	Asn (N)	Asp (D)
Horse	Asn (N)	Ser (S)	Asn (N)	Glu (E)

The large amount of Lyz in donkey milk may be useful not only to prevent intestine infections in infants, but also responsible for the low bacterial count as reported by Salimei et al. (2004a), giving to the donkey's milk the peculiarity to preserve their organoleptic and microbiological characteristics unchanged for a long time. In fact it has been observed in a donkey milk sample stored at refrigeration system for over 10 days that the

organoleptic characteristics, pH and microbial flora showed no significant changes (Polidori & Vincenzetti, 2007). At this regard, Zhang et al. (2008) showed that the absence of *Salmonella* and *Shighella* strains and a growth reduction of *S. choleraus* and *S. disenteriae* in donkey's milk can be attributed to the activity of Lyz and other antimicrobial molecules as LPS, lactoferrin, immunoglobulin and free fatty acids. Also Tidona et al. (2009) showed antimicrobial effect of donkey's milk on selected pathogenic bacteria with a significant reduction growth of *E. coli* during its stationary phase and inhibition of *L. monocytogenes* 2230/92 in a dose dependent way.
The interest for Lyz is also motivated by its role in mitigating the inflammation of the epidermis and scalp, which justifies the dermatological use of donkey's milk carried out since ancient Rome (Cotte, 1991).
In addition, Lyz has also other physiological functions, including inactivation of certain virus, immunoregulatory activity, anti-inflammatory and anti-tumor activity (Ibrahm & Aoki, 2003). In fact, in Mao et al. (2009) study, an high content of Lyz in donkey's milk may contribute to its anti-proliferative and anti tumor effects on A549 human long cancer cells *in vitro*.
Donkey milk active components, as Lyz, reduced the viability of A549 cells in dose-dependent and time dependent manners either directly by apoptosis or indirectly through the secretion of molecules responsible of lymphocytes and macrophages activation.
Considering the numerous benefits of donkey milk, including its healthy-promoting characteristic and probiotic effect, Chiavari et al. (2005) and Coppola et al. (2002) suggested the possibility of using donkey's milk for probiotic purposes. Donkey's milk could be valorised as a very good base for a fermented milk beverage, since it proved to be a good growth medium for probiotic lactobacilli (*L. rhamnosus* and *L. casei*) because of its initial low microbial count, high lactose content and mainly high Lyz content (Coppola et al., 2002). In fact it must be noted that Lyz can be considered an indirect "bifidogenic factor" and so a vehicle for the consumption of probiotic bacteria. However the high Lyz content in donkey's milk seems did not affect the probiotic strains viability during the storage and so, only partially it influenced the growth of the strains tested without also any significant effect on their acidifying activity (Coppola et al., 2002). According to Chiavari et al. (2005), in a fermented beverage with lactobacilli, a Lyz activity unchanged in comparison with initial values, also after pasteurization carried out at 63°C for 30 min and after 30 days shelf life.
These results confirmed the possibility of producing a probiotic infant formula with favorable beneficial properties using donkey's milk as a raw material (Chiavari et al., 2005).

1.7 The mineral content

The intense neonatal pace of foal growth also requires an adequate mineral content in donkey's milk which is unaffected by breed (Mariani et al., 2001), even though it varies, with a continuous decline, during lactation. In fact, the milk produced in the first month of lactation, when milk is the only nutritional source for the foal, contained the highest levels of mineral elements, which may be related to the considerable requirements of the young foal at the first fast growth stage. Afterward, the mineral supply decreased considerably (Doreu,1994).
The mean ash content in donkey's milk (0.39g/100mL), as in mare's milk, is resulted higher than that of human milk and lower than that of ruminants milk (Table 1.17) (Polidori & Vincenzetti, 2007). The values of mineral composition are closer to human milk than other milks except for the higher absolute levels of calcium and phosphorous. In fact, in donkey's milk, Ca/P ratio, ranging averaged 1.48, lies between the lower values of cow's milk and the higher values of human milk (Salimei et al., 2004a; Polidori & Vincenzetti, 2007). The mean content of potassium in donkey's milk is slightly lower compared to that of human milk. As regards chlorine and magnesium, the mean concentrations in donkey's milk are resulted similar to that of human milk, while the sodium content is detected slightly higher than that of human milk. The calcium, chlorine, potassium, sodium and magnesium contents in cow's and goat's milk, appear to be about three times higher than in the human milk, and for phosphorous, even six times higher than human milk. Also in goat's milk, the content of chloride is very high. However it is considered that, in close connection with the feeding, even the ash content decreases during autumn (Polidori & Vincenzetti, 2007; Salimei et al., 2004a).
From a nutritional point of view, it must be noted that the renal load of solutes, mainly determined by the amount of inorganic substances and proteins in the diet, is very similar in both breast-fed infants and those fed donkey's milk, excluding the absolute calcium and phosphorous contributions (Iacono et al., 1992). From a dermatological point of view, the minerals contained in donkey's milk are useful for cleaning the skin; they, in fact, allow to sweep away dead skin cells, leaving on skin surface only live cells. They may also influence cell growth.

Table 1.17: The mean content of minerals in donkey's milk compared than that of other species (Belli Blanes, 2001).

	Minerals (g/100mL)	Ca (mg/Kg)	P (mg/kg)	Ca/P	K (mg/Kg)	Cl (mg/Kg)	Na (mg/Kg)	Mg (mg/Kg)
Donkey	0.39	676.7	487	1.48	497	336.7	218.3	37.3
Human	0.21	340	140	2.4	530	379	133.8	38.8
Mare	0.35	900	700	1.29	550	450	135	
Cow	0.71	1170	900	1.3	1448	999.5	491	121
Goat	0.80	1260	970	1.3	1844	1600	380	130

1.8 Lipid Composition

The mean fat content of donkey's milk (0,3-1,8%) is similar to values observed in mare's milk (0,3-0,5%) and it is much lower than in the other mammal milks (human 3,5-4%; cow 3,5-3,9%) (Guo et al., 2007; Polidori et al., 2009). The lower total donkey lipid content than other mammals reflects in its lower caloric content (408 Kcal/L donkey milk, vs 690 Kcal/L human milk and 660 Kcal/L cow milk) (Gastaldi et al., 2010) and there are different factors that influence fat content, such as, breed, breeding area and forage and milking technique (Fox, 2003).
Milk lipids consists mainly of triglycerides (98%). Other constituents are mono- and diglycerides, phospholipids, sterols and free fatty acids (FAs) (Fox & McSweeney, 1998). The triglycerides are composed of FAs of different length and saturation. The diameter of donkey milk fat globules varies from 1 to 10μm, showing a dimensional distribution consistent with that reported by cow's milk (Salimei, 2011). With regard to milk FAs composition, its profile in mammal milks is indicative and can also vary, because influenced by factors like breed, diet, state of lactation, individual factors, health issue and environmental factors (Chiofalo et al., 2004).

Table 1.18: Average composition of fatty acid classes (% of the total) in the lipid fraction of the donkey milk (mean ± standard deviation SD) (Chiofalo et al., 2003).

Fatty acids	Mean (g/100g) ± SD
Saurated	67.57±2.78
Monounsaturated	15.82±0.95
Polyunsaturated ω3	7.45±1.15
Polyunsaturated ω6	8.65±1.07
Polyunsaturated	16.60±2.33

In donkey milk FAs identified are (Table 1.19): 22 saturated linear FAs from C4 to C22, 9 monounsaturated FAs from C10 to C20 and 13 polyunsaturated FAs from C18 to C22, of which 3 of ω6 series and 8 of ω3 series, containing from 2 to 6 double bonds (Table 1.19) (Chiofalo et al., 2003; 2004).
Compared to monounsaturated and polyunsatured classes (table 1.18), saturated milk fatty acids SFA are the most represented (67,57%): these results, consistent with those reported for mare's milk (Martuzzi et al., 1998), were found lower than those in ovine (73%) (Chiofalo et al., 1994) and caprine (77%) (Chiofalo et al., 1996). SFAs found in greater quantity were: caprilic (C8:0), caprinic (C10:0) and palmitic (C16:0). Among the FAs of nutritional interest and found in modest amounts, the high concentrations were observed for the miristic acid (C14:0), while the lowest levels for the stearic acid (C18:0) was similar to those (1,55%) in mare's milk, as observed by Chiofalo & Salimei 2001 and by Chiofalo et al., 2003. The relatively high content of FAs with 16 and less carbons could be due to a synthesis of these FAs from acetate and 3-hydroxybutirate, as in ruminants, and not from glucose, as monogostrics (Duroe & Boulot, 1989). Among SFAs, medium chain fatty acids (MCFAs) together with palmitic acid represent about the totality of SFAs. From the nutritional standpoint, (MCFAs) undergo preferential intestinal absorption vs long chain fatty acids LCFAs, which are present in very small quantitaties in donkey's milk and in much higher quantitaties in cow's milk (Beermann et al., 2003).
Monounsaturated fatty acid MUFA found in greater quantity was oleic acid (C18:1) (Chiofalo et al., 2003; 2004) with anti-atherogenic and protective effects on endothelial cells (Massaro et al., 2002) as seen also in the milk of Martina Franca donkeys (Pinto, 1998). The caproleic (C10:1) and palmitoleic (C16:1ω7) acids, showed lower quantity (2,20% and 2,37% respectively) (table 1.19) than milks of different breeds, however these acids reach higher levels in comparison with those of the different asinine races (Pinto, 1998), and the highest levels in comparison with those of other animal species (Chiofalo et al., 1996). The sum of the MUFAs percentages was 15,82% (Table 1.18), lower than that of each other species both non ruminant and ruminant (Chiofalo, 2001).

Table 1.19: Fatty acid composition (%) of donkey milk (mean ± SD) (Chiofalo & Salimei, 2001).

Fatty Acids	Mean	SD	Fatty Acids	Mean	SD
Saturated			**Monounsaturated**		
C4:0	0.60	0.29	C10:1	2.20	0.16
C6.0	1.22	0.22	C12:1	0.25	0.10
C7:0	Tracce		C14:1	0.22	0.05
C8:0	12.80	0.59	C16:1 ω7	2.37	0.57
C10:0	18.65	0.91	C17:1	0.27	0.05
C12:0	10.67	0.49	C18:1 ω9	9.65	0.70
C13:0r	0.22	0.05	C20:1 ω11	0.35	0.10
C13:0	3.92	0.90	**Polyunsaturated ω3**		
C14:0r	0.12	0.05	C18:3:3	6.32	1.02
C14:0	5.77	0.33	C18.4:3	0.22	0.10
C15.0r	0.07	0.01	C20:3:3	0.12	0.05
C15:0	0.32	0.05	C20:4:3	0.07	0.01
C16:0r	0.12	0.05	C20:5:3	0.27	0.05
C16:0	11.47	0.59	C22:5:3	0.07	0.01
C17:0r	0.20	0.08	C22:6:3	0.30	0.08
C17:0	0.22	0.05	**Polyunsaturated ω6**		
C18:0	1.12	0.24	C18:2:6	8.15	0.94
C20:0	0.12	0.05	C18:3:6	0.15	0.03
C22:0	0.05	0.01	C20:2:6	0.35	0.10

Among polyunsaturated fatty acids PUFAs, in absolute quantitaties, ω3 PUFAs contained in donkey's milk (746 mg/L) are slightly higher than human milk (689 mg/L) and much higher than cow's milk (280 mg/L); this is one of the chief point of interest in this milk. However, in terms of percentage FA composition, PUFAs content of donkey's milk is higher than that of human milk and, particularly, of cow's milk (Gastaldi et al., 2010). In donkey's milk PUFAs showed a similar distribution of the ω3 (7,45%) and ω6 PUFAs (8,65%). Among these acids, the linolenic (C18:1ω3 = 6,32%) and the linoleic (C18:2ω6 = 8,15%) acids (Table 1.19) are the most represented components of the polyunsaturated class, which reached the value of 16,60% (Table 1.18) (Chiofalo et al., 2003; 2004). FA levels of this class in comparison with those of other animal species, except mare, showed the higher values. Among the essential FAs, the linolenic and linoleic acids show higher values than those observed in the ruminants (C18:1ω3 = 0,7-1%) (C18:2ω6 = 1,8-2,1%) (Chiofalo et al., 1996), but are comparable with those (C18:1ω3 = 5-19%) (C18:2ω6 = 5-10%) found in mare's milk (Duroe & Boulot, 1989). These data confirm that in monogastric herbivora, like equidae, the amount of unsaturated long chain FAs in milk is related to the amount consumed with forages: the absence of saturation and hydrogenation of FAs in the digestive tract before absorption, as occurring in ruminants, can in fact explain the high content in linoleic and linolenic acids (Duroe & Boulot, 1989). In donkey's milk, the PUFAs class showed small amounts of eicosapentanoic (EPA) and docosahesaenoic (DHA) acids, whose involvement is necessary in myelination process (Chiofalo et al., 2003). However the unsaturated fatty acids UFAs content was 32,42% (Chiofalo et al., 2003; 2004), lower than that reported by Pinto (1998) in Martina franca donkey's milk (50,69%) but higher than that observed in ruminant's milk (19-26%) (Chiofalo et al., 1994; 1996). In donkey milk, no significant difference was observed for SFAs and MUFAs, while PUFAs showed a significant variability during the lactation, probably to changes between ω6 PUFAs than ω3 PUFAs, which instead showed stable values throughout the lactation period (Chiofalo et al., 2004).
The ω3/ω6 PUFAs was 0,86 (Table 1.20) and it is similar to that observed in the equine species (0,84) (Salimei et al., 2006); however these species showed higher ratio than that found in ruminants' (0,44-0,45) (Chiofalo et al., 1996) and human's milk (0,07) (Pinto, 1998). It is interesting to note that in lactating donkeys, the transfer from blood to milk of ω3 PUFAs was found to be more efficient than that of ω6 PUFAs; this could probably be related to the other destination of ω6 PUFAs during the metabolic cycle (constituents of adipose tissue, of tryglicerides as well as of membranes phospholipids), and from a dietetic point of view, this transfer enhances the nutritional characteristics of the milk (Chiofalo et al., 2007). The unsaturated/saturated ratio in donkey's milk (0,48) (Table 1.20) is similar to mare's milk (0,65) (Martuzzi et al., 1998) and higher than that found in the ruminants' milk (0,26-0,38) (Chiofalo et al., 1994; 1996).

Table 1.20: Fatty acid ratio of donkey milk (Mean± SD) (Chiofalo & Salimei, 2001).

	Mean	SD
Ratio ω3/ ω6	0.86	0.07
Ratio UFA/SFA	0.48	0.06

Donkey milk FAs profile also reflects dietary pattern and with the fresh forage vs hay administration the level of ω3 PUFAs are significantly higher, while ω6 PUFAs showed no significant differences between treatment (Chiofalo et al., 2006b). This phenomenon can be attributed that, in some polygastric animals occur anaerobic fermentation involving the rumen hydrogenation of UFAs, resulting in saturation and formation of SFAs; instead, in monogastric herbivora, like equidae, the amount of unsaturatated long chain FAs in milk is related to the amount consumed with forages: the absence of saturation and hydrogenation of FAs in the digestive tract before absorption, as occurring in ruminants, can in fact explain the high content in linoleic and linolenic acids (Duroe & Boulot, 1989).
Consequently, the quality indices, Atherogenic IA (0,86-0,81) and Thrombogenic IT (0,64-0,79), are correlated to pathological phenomena such as the formation of atheroma and/or thrombosis, therefore expression of animal welfare, despite being higher than in the vegetable oils, appeared significantly lower than those of cow's milk. For these reasons, donkey's milk can be considered a "functional food" (Chiofalo et al., 2003; 2004). In fact, compared with ruminants' milk, a low content of SFAs which together with an high UFAs content found in donkey's milk, make it extremely useful in prevention of cardiovascular, autoimmune and inflammatory diseases (Chiofalo & Salimei, 2001). The significant content of the ω3 PUFAs can counteract the above-mentioned pathologies through the synthesis of anti-inflammatory, antiaggregant and non immunosuppressant substances, like lipidic mediators (eicosanoids), prostaglandins (PGE_3) and leukotrienes (LTB_5), cytokines, interleukins and tumor necrosis factors. These ω3 PUFAs characteristic constituents of the fish oils, could also have an interesting role on the outcome of the grafts, on some kinds of tumor, on the bodily and neuropsychic development (William, 2000). Besides, the considerable percentage of MCFAs influences the vasodilation phenomena and, together with the SCFAs, determine an increase of the anti-oxidative defences of the organism. In this direction, some Authors pointed out the role of donkey's milk in the osteogenesis process and in the atherosclerosis dietotherapy, in the rehabilitation of the patients with coronary hearth disease (Chiofalo, 2001). However donkey's milk has limits which, if supplemented by appropriate nutrients, could optimize its role of "functional food". The major limit is represented by its low fat content (Salimei & Chiofalo, 2006), and it is also characterized by the low presence of some LCFAs as coniugated linoleic acids (CLA) (Doreau et al., 2002), responsible for the wide variety of beneficial effects (anticarcenogenic, antiatherosclerotic, antidiabetic effects) as well as arachidonic (C20:4ω6) and DHA (C22:6ω3) acids, particularly represented in the membranes of nervous cells, in the external segments of the retinal photoreceptor and in the acrosome of the spermatozoa, therefore essential during the neonatal development of the nervous tissues as well as of retinal tissue and reproductive apparatus (Cocchi, 2000). It must be noted that dietary supplementation with vegetable or marine oils changes the FAs profile of donkey milk, even thought it has a negative o null effect on the milk fat percentage (Salimei et al., 1996). Increased intake of soluble fibre also affect the donkey milk FAs percentage and reducing the total ω6 PUFAs content (Chiofalo et al., 2006a;b). So, levels of ω6 and ω3 PUFAs should be in the same range as those found in human milk to ensure the best effects on growth, cognitive development and visual acuity and to limit the side effect due to the lack of balance between ω6 and ω3 (Gastaldi et al., 2010).

1.8.1 Donkey milk Triglycerides

The knowledge of the composition of milk lipids, in terms of molecular species of triglycerides (TAGs) is fundamental in order to explain the physical properties, nutritive and organoleptic characteristics and the biosynthetic pathways of milk fat (Morera Pons et al., 1998). The little literature at disposal (Dugo et al., 2005) underline some quali-quantitative differences in the FAs typology that form the donkey's milk TAGs in comparison with those of human and cow milk.
The peculiar TAGs distribution of donkey's milk as well as mare's milk is largely due to the high presence of capric acid that is recovered in 18 TAGs out of 55 TAGs identified. Moreover, the milk fat of donkey shows a TAG pattern with 30-54 acyl carbon atoms, as well as mare's milk, in relation to the high content of medium and long chain FAs. The most frequent TAG components found in donkey milk are: palmitic-oleic-linoleic (6.69%), palmitic-oleic-oleic (6.22%), palmitic-palmitic-oleic (5.25%), capric-palmitic-oleic (4.63%), palmitic-oleic-linoleic (4.48%) and palmitic-palmitoleic-oleic (4.18%) (Dugo et al., 2005). The composition of the TAG fraction of human milk shows that the main components are: Palmitic-oleic-oleic (24%) and palmitic-oleic-linoleic (19%), followed by palmitic-palmitic-oleic, palmitic-palmitoleic-oleic and linoleic-oleic-oleic present in amounts ranging from 3 to 8% (Morera Pons et al., 2000). These results show a certain degree of qualitative similarity in the TAG fraction of these two products, both very dissimilar from the composition of cow's milk, where the main components are butyric-palmitic-oleic, butyric-palmitic-palmitic and butyric-myristic-palmitic and where TAGs containing PUFAs (linoleic and linolenic acids) are not found (Gresti et al., 1993). This observation could be explained by the absence in donkey small intestine of isomerisation and hydrogenation processes of FAs prior to absorption and esterification in the blood; it also could confirm the nutritional value of donkey milk fat composition in relation to human health.

Moreover, according to Morera Pons et al. (2003), the trend of TAGs in donkey's milk during the lactation (Chiofalo et al., 2006a) show a certain degree of similarity with that of TAGs profile in human milks; in fact during the lactation, some TAG species (caprylic-oleic-linolenic, caprylic-palmitic-oleic, oleic-linoleic-linolenic and capric-palmitic-oleic) also recovered in human milk, show relatively constant levels and could thus be considered as markers of donkey milk TAG profile. It has also to be noted that concentrations of other TAG species (capric-caprylic-palmitic, capric-palmitic-palmitic, and palmitic-palmitoleic-oleic) vary considerable (Chiofalo et al., 2006a) and consequently these may be understood to be especially affected by external factors such as dietary intake, nutritional status etc. (Morera Pons et al., 2003) which could have changed the availability of some FAs involved in the TAG synthesis in the mammary gland.
In donkey's milk, the position of FAs on glycerol backbone is very similar to that of human milk: this fact, in conjunction with the relatively high content of medium chain triglycerides (MCT), makes this lipids in donkey's milk, through quantitatively modest, highly bioavailable. Palmitic acid is present in donkey's milk in position 2 of the glycerol (72.9% of the total), as observed in human milk (74.8%), whereas for the cow's milk the position 2 of the glycerol is esterified by the oleic acid (Gastaldi et al., 2010). It has been reported that an high 2-palmitoyl TAG content in the lipid fraction increases fat absorption. For the specificity of the pancreatic lipase towards the primary ethereal bonds, the digestion of donkey and human milks permits a greater absorption of the palmitic acid like monoacylglycerides, while, during the digestion of the cow's milk, the palmitic acid, set free from the glycerol, bind itself to the calcium present in the intestinal lumen, precipitate as calcium soap, and therefore is excreted through the faeces with consequent a twofold nutritional damage: the loss of palmitic acid and of calcium (Fidanza & Liguori, 1988) from the body.
However, the low lipid content, together with a low percentages of EPA, DHA and AA make donkey milk inadequate as an exclusive food in infants for the first year of life; nevertheless, the better quality of the lipid pattern in donkey's milk versus cow's milk might make this milk a better substrate to produce formulas (starting or follow on) to be used for feeding of newborns and infants in the early months of life. In this case, the defattening process usually applied for cow's milk, would not be necessary and a formulation would just require a slight increases of DHA, EPA and AA, which are especially necessary for adequate growth, neurological development and cardiovascular health (Aldamiz-Echevarria et al., 2008).

1.9.1 Use of donkey's milk in pediatric diet: the role of donkey's milk in the treatment of allergy to cow's milk protein

The term allergy or hypersensitivity is used for immune-mediated reactions, and the term intolerance for non immune-mediated reactions (Pelto et al., 2003). Immune-mediated reactions may be immunoglobulin (IgE)-mediated or non-IgE-mediated, whereas intolerance may be enzymatic, pharmacologic, metabolic deficiencies and food poisoning. The terms allergy and hypersensitivity are used variably in literature: normally the term allergy is used in infants and children, while hypersensitivity is used in adults for adverse clinical reactions based on any type of abnormal immune response to milk as an allergen. The ubiquity of fresh dairy products and the availability of milk powder resulted in significant increase in protein cow's milk intake and this has probably increased the number of patients allergic or intolerant to these proteins. The protein content of cow's milk is higher than human milk (3.2% *vs* 1%) and the proteins are mainly represented by caseins (80%) and in lesser amount by whey proteins (20%) (against 40% and 60% respectively in human milk). Cow' milk proteins are the first source of foreign proteins given in large quantities to an infant, and in the immature intestine it may provoke allergic reactions (Sampson, 1999). Cow's milk contains several proteins known to be potential antigens: they can be generally classified in two major groups: caseins and whey proteins (Restani et al., 2002). Because β-Lg is absent from human milk, it has long been considered the most important of cow's milk allergens (Savilahti & Kuitunen, 1992), but several studies have demonstrated that the casein fractions, too, have an important allergic potential (Muraro et al., 2002). Moreover, the heat treatments, as pasteurization, and the homogenization do not eliminate the allergenicity of cow's milk proteins (CMP) (Host, 1994; Chiarelli & Di Michele, 2007).
Cow's milk protein allergy (CMPA) is a common disease occurs in infancy and childhood involving approximately 3% of children below the age of three years (Host & Halken, 2004; Sampson, 2004); the incidence of CMPA ranges from 0.3 to 7.5% in population based studies in different countries. However, in most cases this allergy does not last a lifetime, since during the years the child can acquire the tolerance to milk and its active ingredients. Although the majority of children (80-85%) allergic to CMP acquires tolerance by fifth year of life, approximately 15% of patients with IgE-mediated CMPA keep this allergy also in the second decade of life, and 35% of patients will show allergic reactions also to other foods. In these patients APLV, from infancy to schooling years, becomes a serious problem of food security which (for 10% of cases) may evolve into a state of multiple food allergy (Sampson, 2004).
Symptoms of CMPA can appear immediately or start seven hours or even days after the intake of moderate to large amount of cow milk. CMPA can cause symptoms of variable severity which can affect the skin (atopic dermatitis, urticaria, angiodema), gastro-intestinal tract (food refusal, status-weight growth retardation,

gastro-esophageal reflux, diarrhea, vomit, persistent constipation, gastritis, eosinophilic gastroenteritis, enterocolitis). All this may determine histopathological lesions of the intestinal mucosa which are very similar to those found in celiac disease (intestinal villous atrophy) with repercussions on health until the malabsoprtion. Other symptoms concerning the respiratory system (chronic rhinitis, pneumonia, asthma, hypotension and anaphylactic shock) (Murch & Walker-Smith, 1988; Carroccio et al., 2000a; Iacono & D'Amico, 2001).

The therapy for CMPA consisting in eliminating CMP from the child's diet, which has to be replaced with appropriate substitute. It is very important to remove from the diet of children allergic to CMP also foods containing hidden sources of these proteins which are often used as additive in many foods such as biscuits, ham, soups. The elimination of allergenic protein from nurse diet is important in breast feeding children because traces of CMP were found in human milk if the mother follows a free diet (Cant et al., 1985; Host et al., 1990). Since the nutritional needs of the child in the first two years of life (and especially in the first year), when human milk is unavailable or insufficient, are satisfied by cow's milk, pasteurized or adapted formula, and by its derivates, since the child satisfies the 50% of the nutritional and energy requirements (especially with regard to the calcium requirement which is very high in this age) with these foods, it becomes necessary to choose an alternative formula, which should ideally have the characteristics of:

- hypoallergenicity (protein contained in the milk substitute should not be recognised as "enemy" by immune system which would not cause any type of allergenic reaction after ingestion;
- non cross-reactive with cow's milk (the proteins in milk substitute should not be structurally similar to those of cow milk, otherwise the immune system react against them);
- nutritional adequacy;
- palatability (pleasant taste is an important requirement in view of the young age of patients);
- low cost.

At present the ideal formula, meeting all these requirement at once, does not exist, and nor is there an international consensus for which formula should be considered first choice in treating CMPA when mother's milk is not available.

Hypoallergenic alternative to breast milk have been proposed through the years and include extensively hydrolysed formulas (eHF), amino acid formulas (AAF) and soy formulas.

eHF, industrial milk protein – based products treated with proteolitic enzymes, are recommended worldwide as first choice for CMPA treatment (or for the dietotherapy of CMPA patients) by the European Society for Paediatric Allergology and clinical Immunology (ESPACI), the European Society for Paediatric Gastroenterology, Hepatology and nutrition (ESPGHAN) (Host et al., 1999) and the American Academy of Pediatrics (AAP) (American Academy of Pediatrics Committee on Nutrition, 2000).

These formulas are currently the most used substitutes of CMP. They derived from hydrolysis of casein and whey protein of cow's milk but are not tolerated by all patients with CMPA because they may contain residual allergenic epitopes (Hill et al., 1995). As regard their nutritional adequacy, even thought they are able to ensure an appropriate height-weight growth, their palatability is low, being inversely proportional to the hydrolysis degree. In the first months of life, this does not represent generally a problem but in later life unpleasant and bitter taste can cause a complication in taking these formulas. Furthermore, their costs are high, even if lower than the amino-acid formulas (Carvalho et al., 2001; Host & Halken 2004).

According to ESPACI-ESPGHAN guidelines, only amino acid formulas (**AAF**) can be considered completely hypoallergenic, because the immune system can not recognize the protein from which the amino acids are derived. They are therefore indicated in several clinical manifestation of CMPA and of multiple food allergy, including soy and eHF. As regards the nutritional value, the AAF ensure a normal height-weight growth; their palatability is similar to that of eHF, because their flavour is unpleasant, that children especially the older ones, but sometimes even infants over six months refuse to assume them even though sweetened or mixed with fruit. Another problem is the cost, which is significantly higher than eHF (Host et al., 1999; Sampson, 2004; Host & Halken, 2004).

Soy-protein-based formulas (SF) have been subject to several discussion during the years, especially for the risk of allergy to their proteins, which occurs with a rate ranging from 8% to 14% of children with IgE-mediated CMPA. ESPACI-ESPGHAN does not generally recommend its use for the initial treatment of infant and young children with CMPA, in whom soy protein allergy has been reported with frequencies between 17% and 47% (Host et al., 1999; Host & Halken 2004). SF are also contraindicated for the treatment of infants with some forms of non IgE-mediated CMPA with gastrointestinal sympthoms as in enterocolitis, enteropathy and in proctolite (AAP, 2000). According to AAP, if tolerated, these SF represent an effective alternative to cow's milk in infants older than six month. In fact, several studies have shown its nutritional adequacy, as well as a normal height-weight growth and a regular bone mineralization in term infants, even if they are used for long periods. On the other hand, the SF are contraindicated in preterm infants, in whom they can cause poor growth; the high aluminium content contraindicates its use in both preterm subjects and in patients with renal disease. These formulas also contain from two to six times the concentration of manganese compared to cow's milk which may have potential long-term effects on the central nervous

system; in these formulas some physiologically active substances are also present, as phytoestrogens, whose long term effects are not yet clear. Palatability and discrete costs, are instead in favour of SF (Muraro et al., 2002).
Today, the continuing danger of allergic reactions also even to those foods considered apparently safe, their high costs and unpleasant taste have made necessary the search for alternative foods.
For these reasons, in the last years other non-bovine mammalian milks (goat, sheep, mare, camel, donkey) has been studied to identify the best natural substitute for human milk (Belloni-Businco, 1999; ; El-Agamy et al., 1997; Businco et al., 2000; Muraro et al., 2002; Restani et al., 2002).
As regards **goat's milk**, it has been shown that this milk with a lower alpha casein content is less allergenic than cow's milk. This protein could act as a carrier for other allergenic proteins in cow's milk, as β-Lg, which closely linked to casein micelles, is more difficult to digest. The low content of alpha casein in goat's milk ensures, therefore, a better digestion of β-Lg and other allergens (Bevilacqua et al., 2001; Di Pillo et al., 2011). However many studies (Muraro et al., 2002; Infante et al., 2003; Pessler et al., 2004) have shown that goat's and sheep milk are contraindicated as their proteins have shown, for common epitopes presence, extensive cross-reactivity (>90%) with CMP both in *vitro* and in *vivo*. In goat milk, the high content of protein and minerals, particularly calcium, phosphorus, sodium and potassium than human milk could result in excessive solute load for the infant's kidney. In addition, goat milk is nutritionally inadequate, because deficient in folic acid, B6 and B12 vitamins and iron (with a consequent risk of anemia for extensive use) (Restani, 2004). No data is available on the palatability, but this is better than hydrolysates. The cost varies, because there is no a global market for this milk (D'auria et al., 2010).
In many part of the world (North-East Africa, Middle East, Arabian Peninsula and China) **camel milk** is used as a substitute of human milk in bottle fed babies. Camel's milk contains only 2% of fat, especially polyunsatured fatty acids and it is rich in micronutrients. As human milk, camel's milk does not contain β-Lg, allergenic whey protein present in ruminant milk and for low or no *in vitro* cross-reactivity with CMP represents a valid alternative to cow's milk for children CMPA above the two years of age. No data is available on the palatability, but it is reasonably more pleasant than hydrolysates. However, this milk has main disadvantage, the difficult of supply in Italy (Di Pillo et al., 2011).
Mare's milk appears to be more promising as in composition it is much closer to human milk than cow milk (Muraro et al., 2002; D'Auria et al., 2005) and it has been found to be tolerated by some children (24/25 patients - 96% tolerability) with severe IgE-mediated CMPA (Businco et al., 2000). However there are few clinical studies concerning the allergenicity and tolerability of goat's and mare's milk and their results are controversial (Iacono et al., 1992; Belloni-Businco et al., 1999; Businco et al., 2000) and for mare's milk its availability is limited and collection is difficult.
Donkey's milk, as mare's milk, has a chemical composition and organoleptic characteristics more similar to human milk than cow's milk (Guo et al., 2007; Salimei et al., 2004a; Vincenzetti et al., 2008). So, it was found to be a valid alternative to both IgE and non IgE-mediated CMPA and to multiple food allergy also at soy and eHF formulas, with favourable effects on palatability and weight-height gain in an increasing number of clinical trials (Iacono et al., 1992; Carroccio et al., 2000a; Monti et al., 2007; Vita et al., 2007; Tesse et al., 2009), although the mechanism of this tolerance likely related to the protein composition, has not yet been fully clarified.
Donkey's milk is more available than mare's milk in Italy; it was evaluated initially in 1992 by the group of Prof. Iacono, in a clinical study performed on 9 infants with multiple food allergies, for whom human milk was not available. This food was well tolerated by all patients (9/9- 100% tolerability), who have continued to tolerate donkey's milk for the entire duration of follow-up (15-20 months).The authors have also reported in this study the good palatability and nutritional adequacy of the milk (Iacono et al., 1992). In a second study, donkey's milk was evaluated in 2000 by the group of Prof. Carroccio, in a clinical study performed on 21 children with severe IgE-mediated CMPA and multiple food allergies also to eHF and amino acid formulas. This food was tolerated by 18-21 patients (86% tolerability), who have continued to tolerate this milk for the entire duration of follow-up (1-8 years) without any allergic reaction and resumption of growth parameters. Moreover, in this clinical study, donkey's milk was well tolerated by patients with intolerance to goat's milk and sheep's milk, confirming the possibility of lack of cross-reactivity between natural milks of different origin (Carroccio et al., 2000a). In 2007 the group of Prof. Vita published a study on the use of goat's milk and donkey's milk in 26 children (age 6 months-3 years) with atopic dermatitis (AD) and CMPA. The children were randomized to goat and donkey's milk for 6 months, then switched to the other milk for further 3 months. At the end of the study, 23 of 26 children did not tolerate goat's milk, while only one of 26 children did not tolerate donkey's milk with 88% of tolerability for this milk. So this study report that Equidae milk could be an appropriate alternative to cow's milk, but is the first demonstration that donkey's milk is better tolerated than goat's milk, which is in turn still largely used (Vita et al., 2007). Similarly, Monti et al. (2007) demonstrated the tolerance of donkey's milk (82.6%) in a selected population of children (38/46 patients, age 12-149 months) with CMPA, for whom it was not possible to use any cow's milk substitute and because of their concomitant multiple food allergies, required a substitute food that was palatable and

tolerated, as well as being nutritionally valid. In Monti's study, the efficacy of donkey's milk in treating highly problematic children with cow's milk allergic was valuated also in *vivo* and the degree of cross-reactivity with donkey's milk proteins was very weak and aspecific. Recently in 2009, the group of Prof. Tesse also investigated the tolerance and nutritional adequacy of donkey's milk in 30 children (age 6 months-11 years) with IgE and non IgE mediated CMPA. 24/25 patients (96% tolerability) tolerated this milk and showed an adequate increase in weight and length stature. Auxological data also resulted improved by the end of the study in all patients, while biochemical and metabolic parameters did not vary during the follow-up (4-6 months) (Tesse et al., 2009). These authors, as Monti et al. (2007), concluded that the effect on growth of donkey's milk is related to its ability to fill some nutritional gaps present in the diet of the subjects treated.
Other authors, as D'Auria et al. (2011) have found a case of growth impairment and nutritional deficiences in a five-month infant fed by unmodified donkey's milk, for its low lipid content and a low caloric value in addition to a very low iron content. However, there are no clinical studies with an adequate statistical design to evaluate the nutritional efficacy of donkey's milk, at least in the first year of life.
These clinical studies evaluated donkey's milk tolerability that seems quite good, even if it did not achieve the 90% tolerability value required to define a hypoallergenic formula. If in a multicenter study, donkey's milk was tolerated by at least 90% of children with CMPA, it could be considered a natural "hypoallergenic" food for CMPA therapy, in accordance with the international guidelines. In this way, donkey's milk may be a valid alternative, especially for children with multiple food allergies, with clear advantages in terms of cost and palatability in relation to the only alternative available for these patients, represented by amino acid formulas. If it were possible to optimize the production, the milk's cost could be lower: in fact, the use of this milk is today limited by the high cost, intermediate between eHF and AAF cost, and by difficulty of finding. The use of donkey's milk for therapeutic purposes is also hampered by other factors, such as the extinction of asinine species, reluctance for use by parents, medical risk related to the administration of unconventional therapy, risk of infection related to hygiene of the food (animals not controlled for human consumption).

1.9.2 Use of donkey's milk in the prevention of cardio-vascular and autoimmune diseases

The donkey's milk is characterized by a low fat content, where the lipid fraction contains elevated amounts of essential fatty acids (EFAs). These fatty acids are found in donkey's milk in concentrations higher than those ruminants, perhaps due to the absence of hydrogenation, a biochemical process of the rumen activity (Chiofalo et al., 2003). Polyunsatured fatty acids entering into the composition of cell membranes, influence the oxidation and transport of cholesterol, reducing its concentration. They facilitate the enzymatic activity and the production of biologically active substances, eicosanoids and cytokines, which in turn regulate interactions among cells. The EFAs are involved in the synthesis of prostaglandins, which have a key role in various bodily functions such as hormone synthesis, vasoconstriction, regulation of pain and inflammation. Moreover, these EFAs, in particular those belonging to ω3 and ω6 classes have a fundamental role in preventing and inhibiting either cardiovascular and autoimmune diseases because:

- they avoid the accumulation of more unsafe lipids (cholesterol and triglycerides) on the arteries, blocking the hardening of blood vessels;
- they protect the cardiovascular system: the blood made more fluid, better circulates, removing the risk of coronary heart disease, hypertension, atherosclerosis and thrombosis;
- they attenuate inflammatory reactions such as rheumatoid arthritis and other inflammation and asthma;
- they promote the vitality of central nervous system's cells, with antidepressant functions, but also of the retina and of the reproductive functions;
- they enhance the immune system and reinforce the skin's defences;
- they aid in the treatment of psoriasis and other skin diseases;
- they act on the microcirculation (useful for cellulite and edema);
- they facilitate the anti-inflammatory response for injuries or infections.

Several studies have also shown that lysozyme in donkey milk has many physiological functions, including inactivation of certain virus, immuno-regulatory activity, anti-inflammatory and anti-tumour activity (Ibrahim & Aoki, 2003; Salimei et al., 2004a; Zhang et al., 2008). In fact, Ye et al. (2008) investigated the anti-tumour and anti-angiogenesis effects of newly isolated marine lysozyme both in *vitro* and in *vivo*, and showed that marine-derived lysozyme specifically inhibited the proliferation of endothelial cells (EVC304), and markedly inhibited tumour growth in mice bearing either sarcoma 180 and hepatoma 22. In Mao et al. (2009) study, an high content of lysozyme in donkey milk may contribute to its anti-proliferative and anti-tumour effects on A549 human lung cancer cells in *vitro*. Donkey milk active components, (whey protein fractions with a Molecular Mass > 10KDa), as lysozyme, reduced the viability of A549 cells in dose-dependent and time-dependent manners either directly by apoptosis or indirectly through the secretion of molecules (with anti-tumour activity), responsible of lymphocytes and macrophages activation. Activated

macrophages are considered as the pivotal immunocytes of host defence that inhibit tumour growth. The tumoricidal activity of macrophages is mediated mainly through nitric oxide, cytokines, interleukins and tumour necrosis factors.
Mao et al. (2009) study has shown that donkey milk active fractions could stimulate cytokine production from lymphocytes and macrophages in *vitro*. These cytokines are contributory to an immune response and are influential upon the maturation, anti-proliferation and differentiation of A549 tumour cells. In fact, the significant suppressive effect of donkey milk on the proliferation of A549 tumour cells is partly attributed to the promotion of differentiation of A549 cells into normal cells. However, donkey milk whey protein, which possesses potent anti-proliferative activity, may have potential in the treatment of lung cancer, although *in vivo* evidence would be required to determine any such possible actions.

1.9.3 Digestibility of donkey milk protein and its use for gut regulation, particularly for the defence of the low immune system of children, elderly and the convalescent

In *vivo* digestion of milk protein is initiated in the stomach by pepsin and, in young animals, by chymosin. Coagulation of milk in the stomach delays the degradation of proteins and allows for their better assimilation by the body. Degradation of casein is slow but extensive and while β-Lg is relatively resistant to gastric proteolysis, α-La is readily hydrolysed when the gastric pH is 3.5 (Savalle et al., 1988). The structure of the coagulum formed in the stomach depends on the casein content of ingested milk; high casein milk yields firm, tough clots. Species that nurse their young at frequent intervals (horses, humans, donkeys) produce dilute milk with a casein content <60% of the total protein and the coagulum formed in the stomach is soft and disintegrates quickly (Oftedal, 1980). The physico-chemical differences between human and bovine caseins result in the formation of different types of curd in the stomach and because the protein profile of equidae milk (horse and donkey) is quite similar to that human milk, equidae milk may be more appropriate in human nutrition than bovine milk. Turner (1945) compared the digestibility of equine, human and bovine milk based on the average percentage conversion of acid-insoluble protein to acid-soluble protein during digestion. Equine and human milk have a much lower buffering capacity than bovine milk and, while equine milk is very digestible, it was slightly less so than human milk but significantly better than bovine milk. Turner (1945) concluded that both equine and human milk form soft curds in the stomach which pass through the digestive tract more quickly than bovine milk curd. Kalliala et al. (1951) also reported that the overall digestibility of equine and human milk (by in-vitro experiments) appeared quite similar and both were easier to digest compared to bovine milk. Human milk forms fine, soft flocs in the stomach with an evacuation time of 2-2.5h, whereas bovine milk forms compact hard curds with a digestion time of 3-5 h.
Digestion of milk proteins from different species, as donkey, has been also studied in *vitro* through a two step simulation using human gastric and duodenal enzymes (Ingligstad et al., 2010; Tidona et al., 2011). These studies showed that the overall digestibility of casein is high and comparable for horse, donkey, cow, goat and human milk, the percentage of undigested caseins after gastric and duodenal steps being 4%, 7%, 4%, 6% and 5% respectively. However, after gastric digestion the percentage of undigested caseins was almost double for cow and goat caseins compared with horse and human milk (Ingligstad et al., 2010). The low protein and casein content of donkeys and mares' milk might be the reason for the last digestible caseins compared with high casein predominant milk of other species such as cow and goat. In human milk, the casein degradation during the gastric step was also fast at low pH (Iinglingstad et al., 2010). Lactoferrin in milk from the same specie is also highly digested; however, similarly to cow and goat β-Lg, horse and donkey β-Lg is resistant to gastric enzymes although after digestion with human duodenal enzymes only 25% and 30% of β-Lg remain intact respectively in horse and donkey milk, compared with the high concentration observed for cow (64%) and goat (62%) milk. The reason of major digestibility of this protein in horse and donkey milk is that, β-Lgs are present as monomers in equidae at normal milk pH compared with the corresponding dimeric form in ruminant milk (Ingligstad et al., 2010; Tidona et al., 2011). In Equid, as well as cow and human, α-La were very resistant to gastric and duodenal digestion, with a percentage of intact protein at the end of the process ranging from 91% to 95% (Ingligstad et al., 2010; Tidona et al., 2011). Lysozyme from equid milk is also relatively resistant to digestion with 64% and 75% of undigested protein in horse and donkey milk, respectively (Ingligstad et al., 2010; Tidona et al., 2011). There is a close correlation between the vivo digestion of human milk proteins and in vitro digestion with human neonatal gastric juice. Differences in digestibility of potential milk allergens (Ingligstad et al., 2010; Tidona et al., 2011), in amount and distribution of nitrogen components (Uniacke-Lowe et al., 2010; Salimei, 2011), and in primary structure of milk proteins (Cunsolo et al., 2011) may help explain the hypoallergenic properties of equid milk compared with milk from conventional dairy species.

Donkey milk provides an array of defence factors (proteins), such as lactoferrin, lactoperoxidase, immunoglobulins and lysozyme that confer high hygienic qualities (Coppola et al., 2002; Zhang et al., 2008) to the milk, contributing to its low bacterial concentration (10^4 CFU/ml). In the same time they also have the

capability to kill or inhibit a large spectrum of pathogen microorganism (*Salmonella cholerous, Scighella dysenteriae*) (Zhangh et al., 2008), (*Bacillus cereus, Staphilococcus aureus, Enterococcus faecalis* and *Escherichia coli* pathogenic strains) (Nazzaro et al., 2010; Tidona et al., 2011), providing the neonates an immune protection against infections (Baldi et al., 2005), mainly diarrhea.
These occurrences are generally much lower in breast-fed infants that in formula fed infants. However, according to Tidona et al. (2011) the antimicrobial activity of donkey milk might result from a synergistic effect of substances, possibly peptides released by gastrointestinal enzymes together with undigested proteins, as lysozyme. Lysozyme in donkey milk ranges from 100mg/100mL (Vincenzetti et al., 2008), which is consistent with data on horse milk (Doreau & Martin Rosset, 2011), whereas only traces were found in bovine milk (Salimei et al., 2004a; Guo et al., 2007). Lysozyme in equid milk is highly thermo-stable (Coppola et al., 2002; Di Cagno et al., 2004) and its activity is intense against Gram-positive bacteria and much weaker on Gram-negative bacteria, as described by Kato (2003). Moreover, donkey milk lysozyme is very resistant to acid and protease digestion, playing in this way a significant role in the intestinal immune response (Tidona et al., 2011). According to Nazzaro et al. (2010) and Tidona et al. (2011), donkey milk contains many antimicrobial components further than lysozyme. They are essentially functional peptides, released from caseins (β- and α_{s1}-CN) and whey proteins (β-Lgs) during a simulated digestion process or fermentation in *vitro*. These bio-molecules released during the digestive proteolysis could have important nutritional values and physiological roles (antihypertensive, immuno-modulating, oppioide, antithrombotic) as well as contributing to the antimicrobial activity of donkey milk (Nazzaro et al., 2010; Tidona et al., 2011).
In a vitro study simulating gastrointestinal digestion of donkey milk, Bidasolo et al. (2011) identified one β-CN derived peptide with potent angiotensin converting enzyme (ACE)-inhibitory activity. Small proteolytic fragments of both bovine and human lactoferrin have been shown to exhibit antimicrobial (bactericidal) and bacteriostatic activities, the latter of which was thought to deprive microorganism of iron (Tomita et al., 1994).
In conclusion, donkey milk contains different protective antimicrobial factors, including peptides released during the digestion process, that can exert a beneficial impact on gut health, particularly for the low immune defence system of children, elderly and the convalescent, which cannot be fed either with the human milk and are also allergic to other milks (Nazzaro et al., 2010; Tidona et al., 2011).

1.9.4 Use of donkey's milk in geriatric diet

The low fat content of donkey's milk, the highest whey protein content (easily assimilated by the immune system), minerals, probiotics, growth factors, hormones and the high lactose content (important in facilitating the calcium absorption) have led to postulate its use in the defined "healing" feeding of the elderly.
It found that the consumption of cow's milk and its derivates in adulthood is greatly reduced, partly because of gastrointestinal disturbances, and although the most common cause is considered to be the deficit amount of the enzyme lactase, which hydrolyzes lactose, the presence of allergy in elderly patients should not be excluded. The milk and derivatives have an important role in human nutrition for the prevention and correction of osteoporosis. Atherosclerosis and osteoporosis, which have a high incidence in the elderly, perhaps seen in bad nutrition (diet poor in products containing calcium and rich in saturated fats), the more important reason.
The inability to digest lactose, the main milk sugar, by the involved enzymes (for lactase deficiency, which can affect 10-60% of the population), especially in adulthood, leads to limit or abolish the use of bovine milk and derivatives. The patients complain of abdominal pain and tension, flatulence and diarrhea when the milk is consumed. However, recent studies have found that many patients who do not consume milk and dairy products for these disorders, have a normal ability to digest lactose (Carroccio et al., 2000a; D'Amico et al., 2007).
It was assumed therefore, that the protein content of cow milk could be responsible for the symptoms experienced by patients who self-define intolerant to milk. So it should be considered in the elderly, the possibility that there is an allergy to CMP, a frequent condition in children, who, with increasing age, generally acquired immunological tolerance towards allergenic foods. However, many studies suggested that this is not true and the symptoms of hypersensitivity may persist in childhood and adulthood (Carroccio et al., 2000b). Any study, to date, evaluated the possible presence of CMPA in subjects who reported an intolerance to milk but that, normally adsorb lactose. So in subjects auto-defined milk intolerant, the abolition of the product assumption results in a reduced daily intake of calcium, leading in predisposition to osteoporosis. The use of donkey's milk is therefore an important defence therapy in children with CMPA and can, consequently, be offered to adults (also with osteoporosis) whose symptoms may be due to the same disease.
In the elderly, the donkey's milk carries positive effects in the treatments of atherosclerosis, in the recovery of myocardial infracted, in cases of premature aging and in hypo-cholesterolemic diets. Therefore, a regular

intake of donkey's milk, for its low content of saturated fatty acids and high content of polyunsaturated fatty acids, especially ω3 repairers of nerve cell membranes, plays a preventive action in respect of the cardio-circulatory districts, preventing the formation of atherosclerotic plaques and reducing the risk of heart diseases due to increases in blood flow and pressure to the onset of heart attack (Carroccio et al., 2000a;b; Chiofalo, 2001; Salimei et al., 2004a; D'Amico et al., 2007).

1.9.5 Use of donkey's milk in Dermocosmetics

The pool of substances contained naturally (as lysine) in donkey's milk are used for combating and preventing the process of skin aging through:

- a stimulation of metabolic activity of fibroblasts, by reactivating the process of endogenous production of collagen, elastin and hyaluronic acid, which form the support structure of the dermis;
- an increase in skin hydration to give tone and firmness to the skin;
- a protective action against free radicals which are formed continuously.

Therefore in cosmetics, donkey's milk is used for its cleaning and hydrating action (characteristics of any milk): fat's micelles dispersed into the aqueous component of the milk reach easily the dirt particles; the aqueous part leaves dirt from the micelles and hydrates the treated part) combined with an antioxidant action (due to the presence of polyunsatured fatty acids, vitamins A, B and E, lysozyme and other active composts) that prevents the aging. In fact, the fat content in donkey's milk nourishes the skin and give it softness and comfort, also because the essential fatty acids, such as omega 6, help the skin in the vitamin absorption. So, they leave the skin elastic and are useful in the prevention of cutaneous diseases, as eczema and psoriasis, because these fatty acids have anti-inflammatory action and are able to restore and protect the skin cellular membranes. The multivitamin complex A, B, C and E blocks and removes the catabolites of the skin cellular metabolism and begins a protector action of skin. More specifically, Vitamin C is an important antioxidant in cosmetics and in this way helps the slow of the skin aging and accelerates the mechanisms of healing. Vitamin E is known to be essentially an antioxidant which slows the skin aging and contributes to stability of cell structures, reducing the risk of skin diseases. The minerals contained in donkey's milk are useful in the cleaning of skin, because they move away the dead cells, leaving on the skin surface only the living cells. They may also influence cell growth, improve the problems of capillary fragility (with anticouperose and anti-irritation effect) and they limit and reduce the signs of dark circles. Regarding the lysozyme, it shows antiviral, anti-inflammatory and healing properties and it is ideal for treatment of skin lesions, to care delicate skin and lips and to calm irritation symptoms (burning, itching). Lactoferrin and lactoperoxidase have instead bactericidal and fungicidal action useful to purify acne and oily skin, high antioxidant power which protects the skin against free radical attack or stress (due to chemical damage and UV radiation in the atmosphere), as well as having healing and lenitive properties (help skin healing and stimulate the renewal of tissues). The whey proteins complex contained in donkey's milk has the ability to be absorbed and deposited gradually on the skin surface to form a film with a effect filler which acting on the relaxation and contraction of muscles, facilitates the prevention of classic skin wrinkles. The effect is developed through the formation of a very thin elastic film which retreating because of water evaporation exerts a tension effect on the skin, with visible and durable results. Even the high lactose content in donkey milk has a soothing and hydrating effect and draws by osmosis an increased inflow of blood to the skin tissue, improving nutrition and elasticity.
However, in order to enhance the cleaning and moisturizing abilities of donkey's milk, cosmetics has developed formulation such as soaps, creams and bubble baths where the milk is not used fresh but freeze-dried. The freeze-drying of donkey milk for cosmetics is preferred, because it is only process which ensures 100% stability and preservation over time, a hygienic safety and a true integrity of active compounds in the finished product (Orsingher, 2011).

1.9.6 Use of donkey's milk in the daily diet

The donkey's milk is used in the common daily diet, especially in subjects at dietary regime, in sports and in the elderly, given its reduced lipid content. It is also used to replace the common cow's milk in the production of ice creams and creams in the confectionery industry required by people with allergy or subjected to diet.
The donkey's milk not only may represent a precious source of nutrients but also of probiotic microorganism, able to make beneficial effects to the body and thus demonstrating the functional properties of milk. The probiotic microorganism isolated in donkey milk essentially belong to genus of Lactobacillus and Bifidobacterium (Shah, 2001), which according to the official report of FAO/WHO 2001, are living organism which administered in adequate amounts (10^6-10^8 UFC/ml product), are able to make a health benefit to the host organism". Probiotics are microorganism resistant to the action of gastric acids and bile salts, which they meet along the gastrointestinal tract; thus they have the ability to adhere to the intestinal mucosa and colonize it, remaining so numerically dominant in relation to pathogenic or undesirable microorganisms. They are also

able to enhance the host body's immune defence, at systemic and mucosal level, and produce metabolites with trophic effect on intestinal epithelium (butyrate) or inhibiting the growth of pathogenic microorganism (bacteriocins); finally, among other beneficial properties of probiotics, it is worthy mentioning the ability to improve the cholesterol metabolism, and to grow in milk or other fermented foods.
For this reason, today, donkey milk (raw) is designed with the aim to isolate and identify microbial species with probiotic activity. In particular, a study was carried out, isolating lactic acid bacteria strains (essentially *Lactobacillus brevis* strains and *Lactobacillus plantarum* strains), which possess a good ability to survive in conditions such as the resistance to bile salts (about 70%) and simulated gastric juice (about 75%). The presence of probiotics in donkey milk may be, therefore, an interesting prerequisite both from healthy point of view and from an applicative point of view. In fact, in addition to using the isolated probiotics as fermentation starter for different industrial productions, it is possible to suppose the use of these starter for the formulation of yogurt and dairy products made with donkey milk (Kefir, koumiss). The fermentation with selected probiotic strains, naturally occurred in the raw food, could have positive effects on the organoleptic properties and texture of the final product (Nazzaro et al., 2007).

1.9.7 Use of donkey milk for a fermented beverage production with lactobacilli

Equid milk, as donkey milk, can be considered a good substrate for probiotic beverage production. The use of fermented horse milk is an ancient tradition in central Asia (Mongolia, Siberia, and Kazakhstan), where Koumiss or airag are considered beverages with health-promoting properties (Uniake-Lowe, 2011). A novel *Bifidobacterium* species, *Bifidobacterium mongoliense* sp. nov. has been isolated from airag (Watanabe et al., 2009), while a novel *Lactobacillus casei* strain, *Lb. casei* Zhang, is under investigation for its probiotic potential (Guo et al., 2009).
Bacteriocins produced by lactic acid bacteria in airag have recently been isolated and characterised by Batdorj et al. (2006).
Koumiss, a lactic-alcholic beverage derived from mare's milk, is an effective combination of raw milk and indigenous microbial populations, mainly Lactic Acid Bacteria LAB and yeasts, whose diversity is of increasing interest (Di Cagno et al., 2004).
Koumiss is the national drink of the people in central Asia and is also quite popular in countries bordering the Russian federation. It has been suggested that Koumiss has probiotic properties and has even prescribed as a cure for patients with various diseases in Russian hospitals. It is practically unknown in the rest of Europe and the Western world (Chiavari et al., 2005). Koumiss is also rich in ACE-inhibitory peptides, supporting the clain of its beneficial effects on cardio-vascular health (Chen et al., 2010).
The chemical-physical and microbiological properties of the raw donkey milk demonstrated that it has a high lactose content, a low microbial load (10^4 CFU/mL essentially represented by LAB which constituted about 80% of the milk flora) and an elevated content of lysozyme which makes it somewhat selective with regards to the bacteria it can host (Chiavari et al., 2005). In fact, the growth of LAB (and also fungi) at 20°C suggested that the natural antimicrobial substances in donkey milk could not control these kinds of microorganism and thus this milk (for its hygienic and probiotic effects) could be used as a very good base ingredient for preparation of fermented probiotic and therapeutic milk beverage as suggested by Coppola et al. (2002).
However, effective measures should be taken to avoid undesirable fermentative acidification of raw milk caused by LAB and fungi. According to Zhang et al. (2008), the refrigeration temperature (+4°C) might be an effective measure to control the microorganisms, mainly fungi which are not influenced either by the natural antimicrobial substances and by low temperature.
Chiavari et al. (2005) and Coppola et al. (2002) underlined the possibility of producing a fermented beverage from donkey's milk using the probiotic bacterial strains of *Lactobacillus rhamnosus* naturally present in donkey milk or isolated from other dairy products. According these authors, the raw milk used for the fermented beverage, was subjected to pasteurization (63°C for 30 min) as an additional safety measure which did not have any effect on the anti-microbial activity, confirming the elevated thermostability of the lysozyme. Pasteurized milk (after cooling at 37°C) has been inoculated with some strains of *Lactobacillus rhamnosus*, with probiotic properties. The probiotic strains of LAB used for fermented beverage production, after an initial adaptation phase, must have a high growth degree (10^8 CFU/ml), even after fermentation and storage, representing almost all of bacterial content in the final beverage, even after 30 days of shelf life and despite the high concentration of lysozyme either in the raw milk and in the thermically treated milk.
The fermentation provides good results, either in terms of cellular viability and in terms of presence of some metabolites in the final product. From a microbial growth point of view, the product is a probiotic and gives beneficial effects on consumers' health, if it ensures a microbial count of about 10^6 -10^8 (Shah, 2001).
The presence of 10^8 CFU/ml of probiotic lactic bacteria is sufficient to ensure the daily intake suggested by Vanderhoof & Young (1998), even with limited consumption of the beverage.
It is interesting to note that *Lactobacillus rhamnosus* strains remained highly viable after 15 days of storage at 4°C and at low pH (3.7-3.8), thus demonstrating to be a good candidate for probiotic foods. The high

lysozyme content only partially influenced the growth of the strains tested without any significant effect on their acidifying activity (Coppola et al., 2002; Chiavari et al., 2005).
Moreover, strains of Lactic Bacteria that confer pleasant sensorial traits to the fermented product are used. Texture and flavour of fermented horse and donkey milk, on the other hand, may be a constraint to the acceptability of the products, so that fortification with Na-caseinate, pectin and threonine or the addition of flavours can enhance the rheological and sensory quality (Di Cagno et al., 2004; Chiavari et al., 2005). The production of fermented milks by means of a standardised manufacturing protocol should be considered crucial for consumers and markets, according to Di Cagno et al. (2004). However, good quality Koumiss is produced when lactic and alcoholic fermentation proceed simultaneously so that the products of fermentation occur in definite proportion (Berlin, 1962). During fermentation, 0.7-1.8% lactic acid, 0.6-2.5% ethanol and 0,5%-0.9% CO2, volatile acids and other compounds are formed that are important for aroma and taste. Up to 10% of the equine milk proteins are hydrolysed after 96h but the far remains unchanged (Berlin, 1962; Tamine & Marshall, 1984). Koumiss is a milky, grey, fizzy liquid with a sharp alcohol and acid taste (Berlin, 1962; Tamine & Marshall, 1984). Products with varying amount of lactic acid and ethanol are produced and generally 3 categories of Koumiss, mild, medium and strong, are known, and it is thought to be more effective than raw milk in the treatment of various illness due to probiotics and peptides and bactericidal substances from microbial metabolism (Doreu & Martin-Rosset, 2002).
Finally, donkey milk is a possible basis for a fermented beverage, as it contains several advantageous qualities, such as a low microbial counts and high lysozyme content, as well as being a vehicle for the consumption of probiotic bacteria. Further studies may be warranted in order to select other bacterial strains with probiotic properties that are even better adapted to donkey's milk.

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2 AIM of THE WORK

In Europe, especially in Italy, the worry of a definitive loss of asinine species has drawn attention of the scientific world and local authorities, not only for its maintenance but also for productive enhancement, as incisive tool to ensure the "survival" of the donkey over time. So, the Community Agricultural Policy has allowed the revival of small donkey farms to retrieve the original strains and promote the reproduction of different animals avoiding, in this way, the gradual extinction of asinine species. In the last years, the loss of asinine genetic patrimony and the demand of a hypoallergenic and nutritionally adequate milk, have stimulated the interest of many breeders, mainly in the inner areas of the Italy, which have rediscovered the donkey breeding, as important for environment and biodiversity.
The increasing of the donkey breeding made possible a good availability of this milk arousing the scientific world's interest. In order to have a greater spread of donkey's milk, the research is essential to orient the breeder towards a correct diet of animal and breeds more suitable for the milk production in respect of animal welfare to achieve a donkey's milk with excellent nutritional characteristics. At the same time, it is therefore important the collaboration of the scientific and medical world, in order to begin an information's campaign on the properties of this food.
The preservation of asinine breeds is due to donkey's reintegration in programs of social and economic interest, such as, onotherapy, onodidactics, onotourism, onocosmetic and onomilk (milk production). In fact, donkey's milk, is a natural product attributable to category of pharmafood/nutraceuticals useful for children and adults (Gatti, 2007).
In addition, in the production of milks for infants, the cow's milk is humanized by decreasing the protein content (3.2% in cow's milk *vs* 1% in human milk) and inverting the CN:WP ratio (80/20 in cow's milk *vs* 40/60 in human milk). This type of technology requires heat treatments which, nevertheless, can cause changes to the basic components of the milk (lactose, proteins, vitamins, minerals) resulting in alteration of its organoleptic characteristics and reduction of its biological and nutritional value.
For this reason, in recent years, the interest of the scientific research was aimed at enhancing the milk of other species (donkey), as substitute for breast milk and bio-functional food.
Since the knowledge about the genetic profile of donkey proteins (casein and whey proteins) of animals reared in Italy is very scarce, as well as there are very few experimental evidences of the expression level of the individual milk proteins, the present research was aimed to filling this gap and to relating it to the milk nutritional quality.
The aim of this PhD thesis is placed in this context, namely the quali-quantitative characterization of protein fraction of donkey milk important for its use in infant feeding and in patients with cow's milk protein allergy.
As a potential experimental methodology for the identification and characterization of complex mixture of proteins, such as caseins and whey proteins extracted from individual samples of donkey milk, was proposed the proteomic approach. This methodology is based on the combination of analytical and instrumental techniques highly efficient for the separation of asinine proteome's components, in particular:

- Electrophoretic techniques which separate the proteins according to different principles (net charge, isoelectric point, molecular mass) either one-dimensional (PAGE pH 8.6, SDS-PAGE, UTLIEF) and two dimensional 2-DE (PAGE→UTLIEF; IPG→SDS-PAGE). The proteins separated with various electrophoretic system can be identified with the classic Blue Coomassie staining or by immunostaining with specific polyclonal antibodies produced against each protein fraction and/or by mass spectrometry analysis of spots excised from electrophoretic plates;
- Chromatographic techniques with high resolution, as RP-HPLC also interfaced to mass spectrometry (LC-ESI-MS, MALDI-TOF-MS, ESI q-TOF/MS) for the identification of the protein components.

On the basis of the advantages recognised in infant nutrition of the use of donkey milk, dietary and therapeutic properties of which have been known since ancient time (Oftedal & Jennes, 1988) the present study was carried out to examine not only qualitative but also quantitative characteristics of donkey milk. Thus, in the present work, the milk protein content was determined by Kjeldahl method and the CN:WP ratio was determined by electrophoretic technique (SDS-PAGE) coupled with densitometry, while the relative percentage of each individual casein and whey protein component isolated from individual milk samples was determined by integration of HPLC peaks.

2.1 References

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3. RESULTS AND DISCUSSIONS

3.1 Topic/theme 1: characterization of donkey milk caseins

At present, compared with bovine milk, the characterization of donkey milk caseins is at relatively early stage of progress, and only limited data are related to its genetic polymorphism. In this work the heterogeneity of donkey "caseome" was investigated using proteomic approach. This study carried out on milk proteins, as caseins, is aimed to the characterization of their molecular composition (primary structure, disulphide bridges and other post-translational modifications as phosphorylation and glycosylation), which can justify both their functional properties (solubility, clotting aptitude, thermal denaturation) and nutritional quality (amino acid composition, digestibility, bioactivity).

3.2 Materials and methods 1

All chemicals were of the highest purity commercially available and were used without further purification. Tetra-methyl-ethylene-diamine (TEMED), 2-mercaptoethanol, ammonium persulphate, glycine, glycerol, TRIS (hidroxymethyl-amino methane), SDS (sodium dodecyl sulfate), acetic acid, methanol, urea, ammonium bicarbonate (AMBIC), HPLC grade H2O, formic acid (FA) and acetonitrile (CH3CN) were purchased from Carlo Erba (Milan, Italy). Trichloroacetic acid (TCA), Trifluoroacetic acid (TFA), sinapinic acid (SA), α-cyano-4-hydroxycinnamic acid (CHCA), iodoacetamide (IA), dithiothreitol (DTT), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) were obtained from Aldrich (St. Louis, MO, USA). Modified trypsin, sequencing grade was from Promega (Madison, WI, USA), alkaline phosphatase (AP) was from Roche (Mannheim, Germany). Dialysis tubing with molecular weight cut off (MWCO) 12–14.000 Da was from Medicell International Ltd (London, UK). Amicon® Ultra centrifugal filters 3.000 MWCO and ZipTip™ C18 micro columns were obtained from Millipore (Bedford, MA, USA). Acrylamide, bis acrylamide, Ampholine buffers were from GE Healthcare Amersham Biosciences (Buckinghamshire, UK). Coomaassie Brilliant Blue (CBB) R250 and G250 were purchased from Bio-Rad (Richmond, CA, USA).

3.2.1 Donkey milk sampling (sample Casein preparation)

Milk samples collected from 77 donkeys reared in middle and southern Italy (Abruzzo and Sicily) were analysed individually. The milk was defatted by centrifugation, at 4000 rpm for 30 min. The fat layer was solidified at −24°C and drawn up. The casein fraction was precipitated from skimmed milk at pH 4.6 with sodium acetate/acid acetic buffer and separated from the supernatant by centrifugation at 4000 rpm for 30 min), as described by Aschaffenburg & Drewry, 1959. The sodium caseinate precipitate was washed twice with the buffer, dialyzed against water and finally freeze-dried and stored at −20°C before use. The supernatant, containing the whey proteins, after being recovered by centrifugation (4000 rpm for 30 min) at room temperature, was dialysed in tubing with molecular cut off 12,000–14,000 Da and subsequently freeze-dried and stored at −20 °C before use.

3.2.2 Polyacrylamide gel electrophoresis (PAGE) at pH 8.6

Sample Preparation: Casein samples (20 g/L) for electrophoretic analysis were dissolved in 9 M urea solution 9 M, containing 2-mercaptoetanol (1 ml/L). At 100µl of this protein solution, 100µl glycerol 87% and 5µl bromophenol blue were added. 10 µl of this casein solution were loaded in the gel for the electrophoretic run.

PAGE analysis at pH 8.6: polyacrylamide gel electrophoresis (PAGE) at pH 8.6 was carried out with a vertical electrophoretic apparatus (Protean II, Bio-Rad, Richmond, CA, USA) at 200 V and 6°C for 7h according to the procedure described by Chianese et al. (2010). Briefly, this electrophoretic technique provides a polyacrylamide gel named "stacking gel" (3% T and 1% C) containing 3.6 M Urea, 75% (w/v) glycerol in 0.5 M Tris-HCl at pH 6.8. The "stacking gel" is stratified on another polyacrylamide gel named "running gel" (7.5 % T and 2.5% C) containing 6.1M Urea and 1.5 M Tris-HCl at pH 8.6. So, the parameters which define the gel composition are T, sum of acrylamide and bis-acrylamide (g/100 ml solution) and C, the percentage ratio of the bis-acrylamide compared to T. The size of the pores is determined by T, while the crosslinking degree of the gel is expressed by C. Specifically, a T increase determines a reduction of the pores size and the separation is influenced by the size of the molecules. The polyacrylamide gels are obtained by co-polymerizing acrylamide and bis-acrylamide with N,N,N',N'-tetrametiletilenediammina (TEMED) and ammonium persulphate (PER) added in a final concentration of 0.04% (v/v) and 0.07% (w/v), respectively, as activator and catalyst of polymerization reaction. The migration buffer consists of 0.19 M glycine and 0.025 Tris with a pH 8.6. The gel staining was performed with 0.05% (w/v) CBB R-250 dissolved in a

mixture of 50% (v/v) methanol and 7% (v/v) acetic acid, followed by destaining in a solution containing 30% (v/v) methanol and 10% (v/v) glacial acetic acid.
This technique allows to separate proteins according to their net charge, negative or positive, depending on the used pH.

3.2.3 Ultra-thin layer isoelectric focusing UTLIEF (on polyacrylamide gels) analysis

Sample Preparation: Casein samples (20 g/L) for electrophoretic analysis were dissolved in 9M urea solution, containing 2-mercaptoetanol (1 ml/L). 10 µl of these solutions were loaded on the gel and used for UTLIEF analysis.
UTLIEF analysis: Ultra-thin layer isoelectric focusing (UTLIEF) on polyacrylamide gels (124×258×0,25 mm) was carried out according to the procedure of Chianese et al. (2010). Briefly, the polyacrylamide gel with the pH gradient in the range 4.2-6.0 was obtained by mixing 1% (v/v) Ampholine buffers 4.2-4.9 (400 µl), 4.5-5.4 (400 µl), and 4-6 (250 µl). UTLIEF analysis was performed with LKB Multiphore II instrument (Pharmacia LKB, Bromma, Sweeden) at 10°C and the electrophoretic analysis consists of three steps: pre-focusing (2000 V, 15 mA, 4 W, 30 min), focusing with the sample (2000 V, 15 mA, 4 W, 60 min), final focusing (3000 V, 5 mA, 20 W, 130 min). After the electrophoretic run, the fixation of the protein bands on the gel was carried out by gel's immersion in 20% (v/v) trichloroacetic acid solution. The gel was stained with CBB G-250 as described by Krause et al. (1988). This technique allows to separate proteins according to their isoelectric point.

3.2.4 Two-dimensional (2-DE) electrophoresis analysis (PAGE→UTLIEF) and (IPG→PAGE-SDS)

The 2-DE (PAGE→UTLIEF) procedure was achieved by combining the first dimension PAGE gel with the second dimension UTLIEF gel; in particular, an unstained strip of gel PAGE, rinsed twice in distilled water and in 9M urea with mercaptoethanol 0.01% (v/v), was applied along the cathode of prefocused UTLIEF plate.
For 2-DE analysis (IPG→PAGE-SDS), the first step is the rehydration phase where 330µg of casein sample was dissolved in 450 µL of solution containing 9M urea, 2% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 65 mM dithiothreitol (DTT), ampholytes 3.5-10, 0.001% (w/v) bromophenol blue.
A pre-cast immobilized pH gradient gel strip (IPG-strip) with a linear pH gradient range of 4-7 (IPG ReadyStrip pH 4-7, length 18 cm; Bio-Rad, Richmond, CA, USA) was placed in the Protean IEF Cell (Bio-Rad, Richmond, CA, USA) and was rehydrated with the protein solution for 16 h at 20°C without voltage. For the first dimension, the focusing was performed at 20°C in three steps: in the first step a voltage of 500 V for 1 h was applied to remove excess salts; in the second step a voltage of 1000 V was applied for another hour and in the third step, a voltage of 8000 V was applied for 5 h. The current limit per IPG-strip was 50 µA. Subsequently, IPG strips were equilibrated for 15 min in Equilibration Buffer I (0.375 M Tris-HCl pH 8.8, containing 6M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 130 mM DTT). After 15 min, the strips were alkylated in Equilibration Buffer II (0.375 M Tris-HCl pH 8.8, containing 6M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 135 mM IA) and applied on 12% SDS-PAGE gels using a Protean II apparatus (Bio-Rad, Richmond, CA, USA) with the procedure described by Laemmli, (1970). For this second dimension, the gels were run at 120 Volt for 20 min and then at 200 Volt for 6-7 hours. When the gels were not submitted to western blotting, proteins were stained with CBB R 250. The gels were destained until the protein spots became evident and the gel background transparent.

3.2.5 Immunoblotting analysis

This analysis is based on cross-reaction between specific polyclonal antibodies produced against the individual casein fractions αs1-, αs2-, β- and κ-CN (or casein peptides) and their respective antigens. Specifically, this technique allows to identify the individual casein fractions according to the specificity shown by specific polyclonal antibodies produced against these casein fractions after immunization of rabbits with the individual caseins αs1-, αs2-, β- and κ-CN or their casein peptides.
The reagents for immunoblotting were: PBS buffer (4.5% NaCl, 3.9% NaH_2PO_4, pH 7), wash buffer solution and saturating the membrane "blocking solution" (equine serum at 10% v/v in PBS), development buffer (0.5 mg/ml diaminobenzidine in 10 mM Tris-HCl 0.1 M pH 7.5, 0.6 mg/ml $NiCl_2$ 1%).
For immunoblotting analysis, the casein fraction separated either by electrophoretic analysis were transferred by capillary diffusion from the gel into a nitrocellulose membrane (0,45µm, Trans-Blot, Bio-Rad, Richmond, CA, USA). Immunodetection was carried out according to the procedure already described by Chianese et al. (2010), using rabbit polyclonal antibodies against donkey synthetic peptides reproducing the C-terminal sequence of α_{s1}-CN (187-202), α_{s2}-CN (206-221), β-CN (211-226) and porcine κ-CN as primary antibodies.

The synthetic peptides and the porcine κ-CN, the latter fractionated by RP-HPLC, were united at ovalbumin through the sulphydryl group of the C-terminal or N-terminal cysteine residue, according to the procedure described by Mattson et al. (1993). The conjugates of ovalbumin were used as antigens by Primm (Milan, Italy) to immune two rabbits and thus to obtain rabbit polyclonal antisera against these casein fractions. Finally, the antisera obtained after the animals' death were filtered using 0.45 mm filters (Millipore, Bedford, MA), divided into aliquots of 1ml and stored at −20°C.
For immunodetection, nitrocellulose membrane was incubated for 1h with specific primary antibody diluted in Wash Buffer. This step was followed by incubation with second antibody, preceded by washing of the membrane with the same wash buffer solution " blocking solution". As secondary antibody, the polyclonal antiserum produced by goats against the rabbit IgG and peroxidase-conjugated (Goat-Antirabbit antibody conjugated to peroxidase, Bio-Rad, Hercules, CA) was used. After 1 h of incubation with the second antibody and washing with "wash buffer", the membrane is immersed in the solution for the development of the colorimetric reaction. This phase was carried out by adding hydrogen peroxide.

3.2.6 Reversed-phase high performance liquid chromatography (RP-HPLC) analysis

The HPLC system was provided by Kontron Instrument (Milan, Italy) and is constituted by two pumps model 420, an injector of the sample (loop 50 μL) and a DATA SYSTEM 450 for the chromatography's management and for the integration of the peak areas of the chromatograms.
The casein samples were fractionated by RP-HPLC on a 214TP54 5μm Vydac C_4, 250 mm × 4.6 mm internal diameter column (Vydac, Hesperia, CA, USA) and the detection was at a wavelength of 220 or 280 nm with a UV detector Kontron (Mod. 430). The solvent used were: solvent A and solvent B. Solvent A was 0.1% (v/v) TFA in ultra pure water and solvent B 0.1% (v/v) TFA in acetonitrile. 50 μL of a solution containing 4 mg (casein sample)/ mL (solvent A) were loaded into a C4 column, equilibrated with solvent A. The elution program involved a gradient from 34 to 42% solvent B in 35 min, then from 42% to 100% B in 2 min, with a flow rate of 1 $mLmin^{-1}$. Each eluted casein fraction was manually collected, freeze-dried and stored at −20°C.
This technique allows to separate proteins according to their hydrophobicity.

3.2.7 LC-ESI-MS analysis

The molecular mass determination of native or dephosphorylated caseins (as donkey β-CNs) was carried out using a single quadrupole instrument LC-ESI-MS. Specifically, liquid chromatography/electrospray mass spectrometry (LC-ESI-MS) was performed using an HPLC modular system (HP1100-MSD, Agilent Technologies, Santa Clara, CA, USA) with UV detector, interfaced with a single quadrupole mass spectrometer equipped with a electrospray source (ESI) and monitored by HP Chem Station software for the acquisition, analysis and processing of the mass spectra.
The casein samples were fractionated by RP-HPLC on a 213TP54 5μm Vydac C_3, 250 mm × 4.6 mm internal diameter column (Vydac, Hesperia, CA, USA) and the UV detection was at a wavelength of 220 nm. Solvent A was 0.1% (v/v) TFA in ultra pure water and solvent B 0.1% (v/v) TFA in acetonitrile. 20 μL of a solution containing 10 mg (casein sample)/ mL (solvent A) were loaded into a C_3 column, equilibrated with solvent A. The elution program involved a gradient from 34 to 42% solvent B in 30 min, then from 42 to 55% B in 15 min, at a flow rate of 0.4 $mLmin^{-1}$.
The ESI mass spectra were scanned in the positive ion mode, from 800-2200 *m/z* at a scan cycle of 5 sec/scan. The source temperature was 120°C and the orifice voltage was 40. Mass values are reported as average masses. Signals recorded in the mass spectra were associated with the corresponding casein proteins on the basis of the molecular mass, taking into the account the reported amino acid sequences of donkey caseins available on database Swiss-prot (ExPASy proteomics server).

3.2.8 Dephosphorylation of the casein fraction

An aliquot of the casein fraction was dephosphorylated using alkaline phosphatase from bovine intestinal mucosa (Mamone et al., 2003). In detail, the casein fraction (casein sample or casein fraction) was dissolved in 0.4% NH_4HCO_3 aqueous solution, pH 8.5, at a concentration of 10 mg/mL and then boiled at 100°C for 3 min to deactivate the plasmin. The enzyme was also solubilised in 0.4% NH_4HCO_3 aqueous solution, pH 8.5, at a concentration of 1 mg/mL and added at a molar enzyme/substrate ratio 1:25 to casein solution. The enzymatic reaction was started by addition of 60 μL of enzyme solution (1 mg/mL) to 90 μL casein solution (10 mg/mL). This final solution was incubated at 37°C overnight and the digestion was stopped by freezing.

3.2.9 Trypsin enzymatic digestion

The digestion of the caseins from electrophoretic spots was carried out following the procedure reported by Mamone et al. (2003). Briefly, the spots from stained gel were manually excised with a clean scapel, placed in a Eppendorf tube and twice washed with 50 μL Milli-Q water. Each gel piece was completely destained (spots become white) by immersion into a solution 50 mM NH_4HCO_3 in 50 % (v/v) aqueous acetonitrile. The destained spot was dehydrated by submersion into acetonitrile, and dried under vacuum after acetonitrile removal. The dried gel was covered by 30 μL 50 mM NH_4HCO_3 containing 12 ng/μL trypsin maintained in an ice-cold tube. After 45 min action, the supernatant was removed and incubated overnight at 37°C. The resulting tryptic digest was extracted in 40 μL of acetonitrile/5% formic acid solution (1:1 v:v) and then the volume concentrated to a tenth in a vacuum centrifuge for the mass spectrometric analysis. Careful precautions are important to avoid gel contamination with foreign proteins. As regards the tryptic digestion of each isolated casein fraction, collected by RP-HPLC, it was dried using a Savant concentrator (Speed-Vac, Milan, Italy), lyophilized, denatured and alkylated before trypsin hydrolysis. Briefly, each isolated casein fraction (for example donkey α_{s1}- or β-CN) was denatured at room temperature, in a guanidine buffer (6M guanidine in 0.5 M Tris-HCl with 1 mM EDTA) at pH 8. Subsequently, disulfide bridges were reduced by of 10 mM/L DTT for 60 min at 56°C. SH groups were subsequently alkylated with 55 mM/L IA in the above guanidine buffer, and maintained for 30 min in the dark. With the aim to remove the reagents, the alkylated casein was ultra-filtered on Amicon® Ultra filters 3000 MWCO, centrifugated at 4000 g for 25 min and washed with 50 mM NH_4HCO_3, pH 7.4. The retentate solution containing the purified alkylated casein (as α_{s1}- or β-CN), was added of trypsin (dissolved in the same solution of 50 mM NH_4HCO_3, pH 7.4) at 1:50 (w/w) and incubated overnight (approximately 14 h) at 37°C. The digestion was stopped by frozen (–20°C).

3.2.10 Matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS) analysis

MALDI-TOF-MS experiments were carried out on a Voyager DE-PRO mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a N_2 laser (337 nm, 3 ns pulse width) operating both in linear or in reflector positive ion mode, using the Delay Extraction technology. Mass spectra were acquired both in positive linear or in reflectron mode and using SA and CHCA dissolved in aqueous solution of 50% (v/v) ACN containing 0.1% (v/v) TFA, at a concentration of 10 mg/mL, as matrices for the analysis of proteins and peptides, respectively. MALDI-TOF-MS analysis of intact proteins were obtained in linear positive ion mode over the *m/z* range 10000-30000 and were averaged from about 150 laser shots.
As regards peptides analysis, the mixtures of tryptic peptides were subjected to desalting/concentration step with Zip-Tip C_{18} pre-packed micro-columns (Millipore, Bedford, MA, USA) previously equilibrated with aqueous 0.1% TFA (v/v), prior to analysis by MALDI-TOF. Each spectrum was taken with the following procedure: 1μL of sample is directly loaded on a stainless steel plate together with 1μ of the matrice. Mass Spectra were obtained in reflectron positive ion mode over an *m/z* range 600-4000 and were averaged from about 150 laser shots. External calibration was performed by acquiring separate spectra of a mixture of standard peptides (PerSeptive Biosystems).
The identification of donkey caseins and their derived peptides was carried out using the known caseins available on Swiss-prot database (ExPASy proteomic server) of Swiss Institute of Bioinformatics (SIB) and with the online software FINDPEPT (website: http://www.expasy.org/tools/findpept.html).

3.2.11 Electrospray quadrupole-time of flight-mass spectrometry (ESI-Q-TOF-MS/MS) analysis

Tandem MS (MS/MS) data were obtained using a hybrid quadrupole-orthogonal acceleration time of flight Q-STAR instrument (Applied Biosystem, Foster City, CA) equipped with a nanospray source (Protana, Odense, Denmark), operating in positive ion mode. Dried samples were resuspended in 0.1% TFA, purified from residual salts by loading into ZipTip C_{18} Reversed Phase pre-packed micro-columns (Millipore Bedford MA, USA), and introduced in the source through borosilicate needles, gold coated (Protana Odense, Denmark). The capillary voltage used was 800 V. Double-charged ion isotopic cluster were selected by using the quadrupole mass filter and the induced to fragment by collision. The collision energy was 20 to 40 eV, depending on the size of the peptide. The collision-induced dissociation was processed by using Analyst 5 software (Appied Biosystems). The deconvoluted MS/MS spectrum was manually interpreted with the help of Analyst 5 software.

3.2.12 Chymosin Hydrolysis

For chymosin hydrolysis, caseins (20 mg/mL) were dissolved in 0.1 M sodium citrate buffer, pH 6.0. For hydrolysis reaction, the rennet's solution was prepared adding 100 μL of HANSEN rennet in 5 mL sodium citrate solution 0.1M, pH 6.0.

30 μL of this chymosin solution was added to 1 mL protein solution, and the hydrolysis was performed at 37°C for 2 h. To stop the reaction, the pH was decreased by adding 1 mL of 24 % (v/v) TCA. The precipitate obtained after adding TCA solution and centrifugation was used for the preparation of electrophoretic sample.

3.2.13 Polyacrylamide gel electrophoresis-Sodium Dodecyl Sulphate (PAGE-SDS) analysis with Fluorescent Glycoprotein detection and immunoblotting

Sample Preparation: donkey casein samples (before and after chymosin hydrolysis) or whey protein samples (20 g/L) were dissolved in 9M urea solution containing 2-mercaptoetanol (1 ml/L). 200 μL of a denaturing solution (0.062 M Tris-HCl, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, pH 6.8) and 5μl bromophenol blue were added at 100μl of the protein solution. The protein samples, after adding the denaturing solution and bromophenol, were heated at 100°C for 5 min. Volumes of 10 μl of this final sample were loaded in the gels.
PAGE-SDS analysis: PAGE-SDS was carried out with a vertical electrophoretic apparatus (Protean II, Bio-Rad, Richmond, CA, USA) according to the procedure described by Laemmli, (1970) using a 4% polyacrylamide stacking gel in 0.5 M Tris-HCl buffer (pH 6.8) and 18% or 15% polyacrylamide resolving or running gel in 1.5 M Tris-HCl buffer (pH 8.6) in the presence of 10% SDS. The polyacrylamide gels were obtained by co-polymerizing acrylamide and bis-acrylamide with N,N,N',N'-tetrametiletilenediammina (TEMED) and ammonium persulphate (PER) added, respectively, as activator and catalyst of polymerization reaction. As molecular standard, a low molecular weight standard kit (LMW Calibration kit, Amersham, GE Healthcare, UK) was used. Electrophoresis was performed at constant voltage 200 V (for about 6 h) in the migration buffer containing 0.2M glycine, 0.025 M Tris and 0.1% SDS. The gel staining was performed with 0.1% (w/v) Coomassie Brilliant Blue CBB R-250 dissolved in a fixative solution (50% (v/v) methanol and 7% (v/v) acetic acid), followed by destaining in a aqueous solution containing 30% (v/v) methanol and 10% (v/v) glacial acetic acid.
Staining of glycoproteins fixed in the gel with an acetic/methanol solution was carried out with Fluorescent Glycoprotein Detection Kit (GlycoProfile III, Sigma-Aldrich, St. Louis, MO, USA), where the carbohydrates on the proteins are oxidized to aldehydes with periodic acid. A hydrazide dye is reacted with the aldehydes, forming a stable fluorescent conjugate. This allows for the specific, sensitive detection of the glycoproteins directly in gels.
The immunoblotting with specific polyclonal antibodies against each donkey casein fraction was carried out according to the procedures described in paragraph 3.2.5.

3.3 Results and discussion 1

3.3.1 Characterization of donkey α_{s1}-CN by PAGE pH 8.6 and immunoblotting analysis

The most representative and individual donkey casein samples Coomassie Brilliant Blue stained (Fig. 3.1A) were compared to cow counterparts on the basis of their relative net charge at alkaline pH by PAGE analysis. The results obtained after specific immunostaining with the polyclonal antibody against α_{s1}-CN (Fig. 3.1B) allowed to detect this casein fraction.

By comparing the two species each other, donkey α_{s1}-CN exhibited a lower and reversed anodic mobility than its cow counterpart. The PAGE analysis of the representative donkey caseins, either with Blue Comassie (Fig. 3.1A) or specific polyclonal antibody staining (Fig. 3.1B) also showed a complex heterogeneity of donkey α_{s1}-CN due to the detection of several immunostained components, where the main α_{s1}-CN components (4-6) essentially exhibited an intermediate anodic mobility between donkey β- and α_{s2}-CN. These immuno-electrophoretic results also showed an overlap between α_{s1}-CN and α_{s2}-CN, although minor α_{s1}-CN components were essentially found at anode.

However, the compositional heterogeneity of donkey α_{s1}-CN could be due to different phosphorylation degree of its components and to the presence of deleted forms, as in mare counterparts (Milenkovic et al., 2002; Lenasi et al., 2003; Miranda et al., 2004; Mateos et al., 2009b).

At this regard, Cunsolo et al. (2009a) found four donkey α_{s1}-CN components with 202, 201, 197 and 196 amino acids (aa), whose sequences were completely characterized, using the known mare α_{s1}-CN as reference (Lenasi et al., 2003). Moreover, the immuno-electrophoretic results also showed α_{s1}-CN's quantitative differences among analyzed donkey caseins, probably due to a three different expression degrees of the protein; at this regard, three expression levels can be detected, which allowed to classify the containing phenotypes as full (lanes 1, 2, 3, 4), intermediate (lane 6) and null (lane 5).

This phenomenon of "quantitative polymorphism" was found for the first time in goat milk (Grosclaude & Martin, 1997), where alleles, at goat α_{s1}-CN *locus*, were identified as defective mutants in terms of functional activity of the gene and the type of mutations is quite variable: single nucleotide substitution or deletion, or large insertion or deletions. So, a low α_{s1}-CN content in donkey milk may represent an important attribute for its use in human allergology as reported by Bevilacqua et al. (2001) in goat milk.

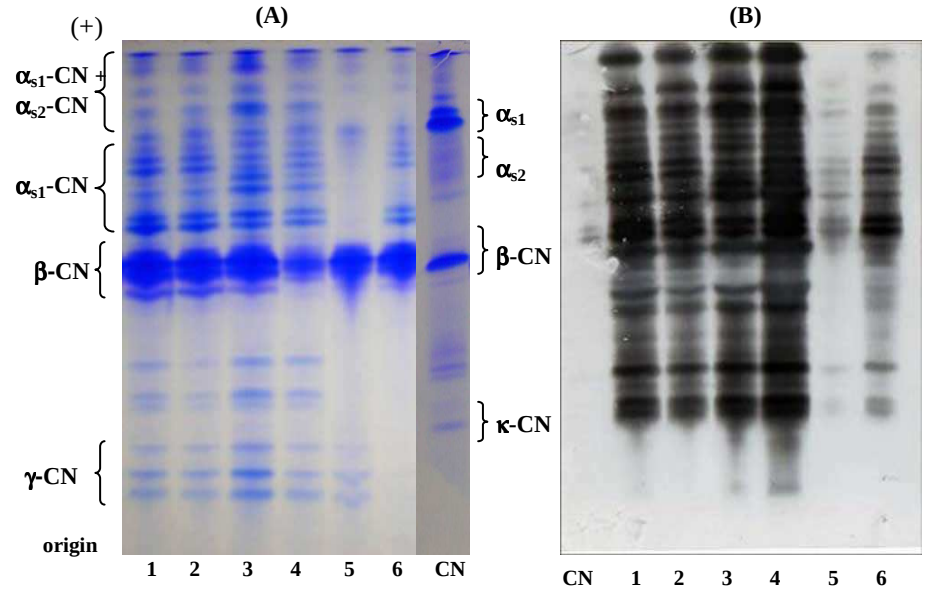

Fig 3.1: PAGE at pH 8.6 analysis of representative donkey caseins and bovine as reference (CN) after Coomassie Brilliant Blue staining (A) and immunospecific staining with polyclonal antibody against α_{s1}-CN (B).

3.3.2 Characterization of donkey α_{s1}-CN by UTLIEF and immunoblotting analysis

The UTLIEF analysis of representative donkey caseins, either with Blue Comassie (Fig. 3.2A) or with specific polyclonal antibody staining (Fig. 3.2B) also showed the complexity of donkey α_{s1}-CN. This phenomenon was due to the different quali-quantitative composition of donkey α_{s1}-CN than cow counterpart,

together with the major resolution of this electrophoretic technique with respect to the above PAGE analysis. In fact, the results underlined a more complex overlapping phenomenon of α_{s1}-CN components with other casein fractions due to their similar pI values.
The detection of several α_{s1}-CN components may be due to presence of non allelic deleted forms with different phosphorylation degrees, as in mare counterpart, and to proteolysis by action of endogenous proteases.
However, the immunoelectrophoretic techniques did not allow to detect qualitative, but only quantitative differences for donkey α_{s1}-CN, due to the identification of casein samples (5 and 6) characterized by a lower expression level of the allergenic protein. From a nutritional point of view, the minor content of α_{s1}-CN in donkey milk can be related to the tolerance mechanism of donkey milk in the allergic patients to bovine milk.

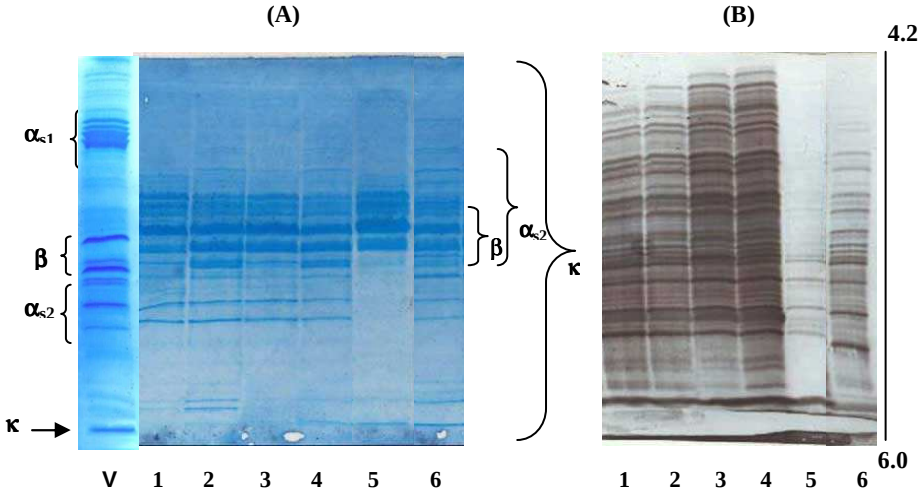

Fig 3.2: UTLIEF analysis of representative donkey caseins and bovine as reference (CN) after Coomassie Brilliant Blue staining (A) and immunospecific staining with polyclonal antibody against α_{s1}-CN (B).

3.3.3 Characterization of donkey α_{s1}-CN by two dimensional (2-DE) electrophoresis analysis (PAGE *vs* UTLIEF) and (IPG *vs* PAGE-SDS)

One dimensional electrophoresis did not provide efficient separation of caseins, as α_{s1}-CN, because of their partial overlapping. For this reason, the two dimensional electrophoresis analysis was more resolutive than one-dimensional electrophoresis. Specifically, in PAGE→UTLIEF analysis of an individual donkey casein, either after Blue Coomassie staining (Fig. 3.3A) and mainly after immunoblotting (Fig. 3.3B), the α_{s1}-CN composition consisted of two doublet bands, having decreasing pI values towards the anode. According to Chianese et al. (2010) the compositional heterogeneity was assigned to either discrete phosphorylation (5, 6, 7P/mol) or non allelic deleted forms generated by incorrect RNA splicing as already shown in the homologous goat and sheep casein.
Instead, in IPG→PAGE-SDS analysis of individual donkey casein sample, either with Blue Coomassie staining (Fig. 3.4A) and immunoblotting analysis (Fig. 3.4B), α_{s1}-CN is arranged in three level of the gel, having decreasing pI and molecular mass values toward the anode. These results also confirmed the data obtained from other authors (Cunsolo et al., 2009a; Vincenzetti et al., 2012), since of the four major levels, two of them C and D (197 aa with a different phosphorylation degree) would show the pentapeptide HTPRE deletion that decreased the molecular mass of 620 Da with respect to the other major levels A and B (202 aa with a different phosphorylation degree). Moreover, the absence of pentapeptide confers to deleted forms C and D, a more acidic nature. In our study, an additional level corresponding to α_{s1}-CN was visible for the first time and showed a higher molecular mass with respect to the previous levels. Specifically, as reported in next paragraph 3.3.4, they could correspond to the full length α_{s1}-CN components (210 aa for the insertion of octapeptide DTSNESTE encoded by exon 7) with a different phosphorylation degree. Some of these α_{s1}-CN spots with a major molecular weight were never observed, probably because they are present in donkey milk in very small amount.
The complexity of 2-DE pattern of donkey α_{s1}-CN supported the hypothesis of simultaneous presence of potential non allelic deleted forms (leading to a variability in molecular mass) with a different phosphorylation degree (leading to a variability in isoelectric point). In addition, α_{s1}-CN immunostained spots with a very low molecular weight could be peptide derived from α_{s1}-CN proteolysis

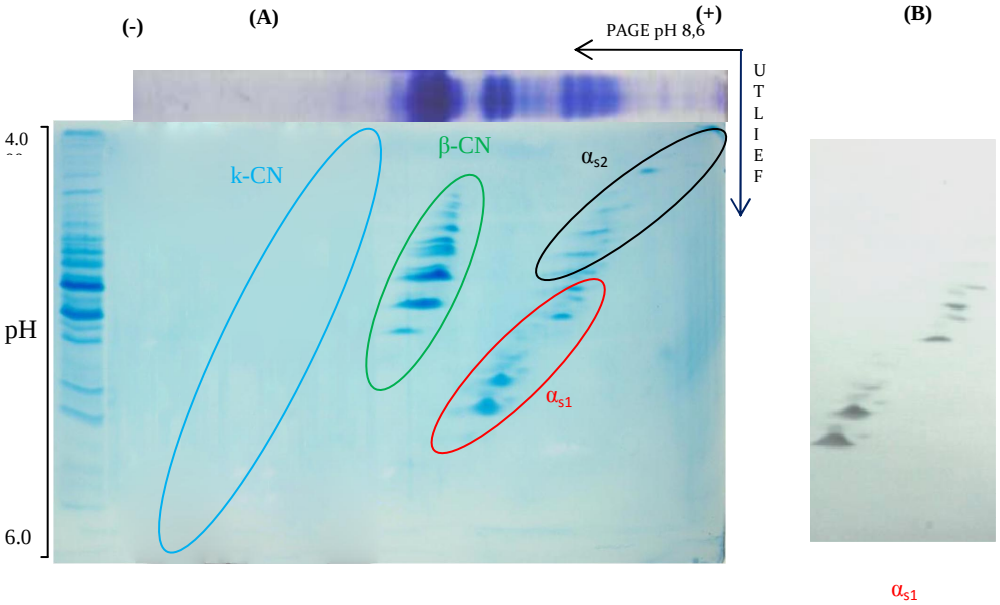

Fig 3.3: 2-DE (PAGE→UTLIEF) analysis of individual donkey casein sample after Coomassie Brilliant Blue staining (A) and immunospecific staining with polyclonal antibody against α_{s1}-CN (B).

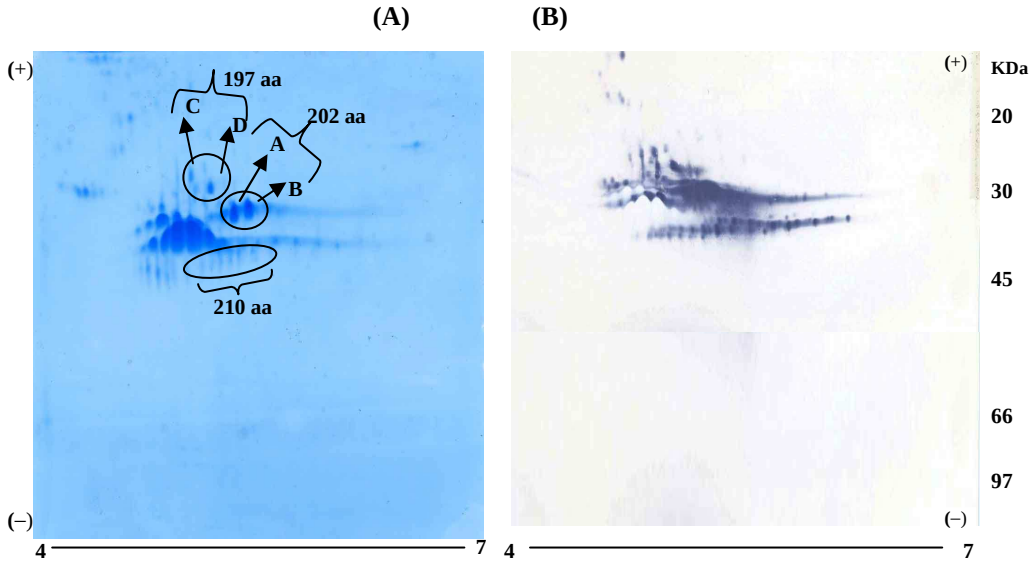

Fig 3.4: 2-DE (IPG→PAGE-SDS) analysis of representative donkey casein sample after Coomassie Brilliant Blue staining (A) and immunospecific staining with polyclonal antibody against α_{s1}-CN (B).

3.3.4 Evidences of microheterogeneity and genetic polymorphism at donkey α_{s1}-CN locus by RP-HPLC/MS analysis

Since the majority of the mutations does not only concern charged (acid or alkaline) aa, but also neutral aa undetectable with respect to the above electrophoretic analysis, the individual donkey casein samples were also analyzed by RP-HPLC analysis.

After a preliminary screening of individual donkey caseins, two characteristic HPLC profiles (A and B) were selected in (Fig. 3.5). Compositional differences between α_{s1}-CN and β-CN fractions in the two most common HPLC profiles (A and B) of donkey casein samples can be deduced in (Fig. 3.5). In particular, α_{s1}-CN eluted in two peaks (1 and 2) characterized by a different area percentage only in sample A (peak 1 > peak 2), while in sample B the peaks 1 and 2 had the same area (peak 1 = peak 2).

In order to identify the containing α_{s1}-CN components, each eluted peak was analyzed by MS analysis before and after alkaline phosphatase AP action and the identified components were reported in Table 3.1

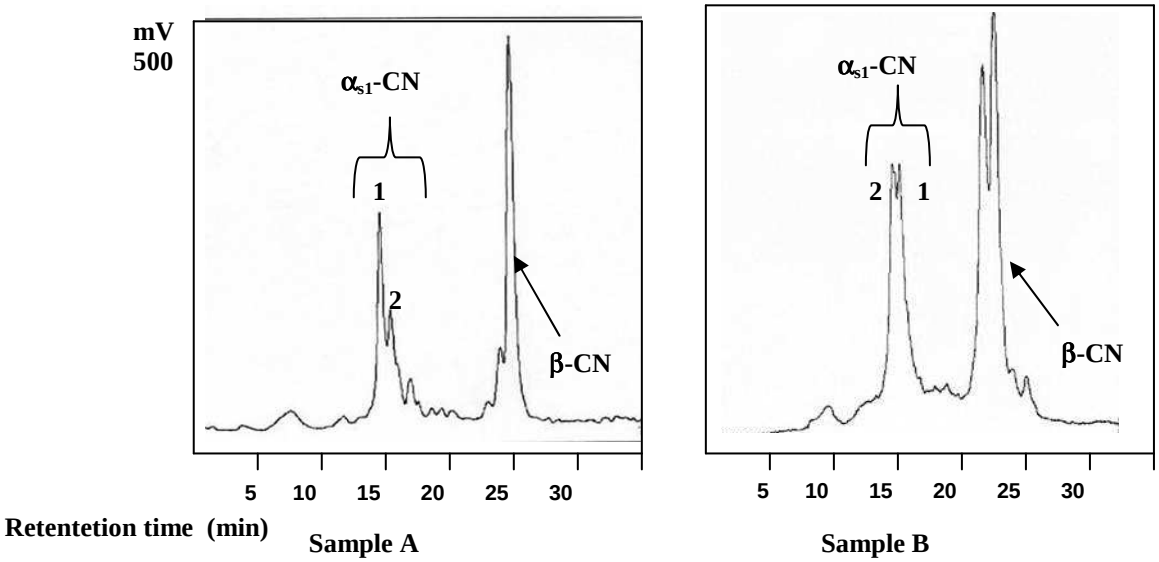

Fig 3.5: RP-HPLC profile of two representative donkey casein sample.

Table 3.1: Molecular Masses (M_r) of α_{s1}-CN components in the casein samples A and B determined by LC-ESI-MS analysis.

		α_{s1}-CN components		
Sample	*Peak*	M_r *Native (Da)*	M_r *Dephosphorylated (Da)*	*Lenght aa*
A	1	24888 6P	24408	202
		24808 5P		202
		24760 6P	24280	201 (Gln^{88})
		24680 5P		201 (Gln^{88})
		25833 7P	25273	210
		25753 6P		210
		25704 7P	25144	209 (Gln^{96})
		25624 6P		209 (Gln^{96})
	2	24265 6P	23785	197
		24185 5P		197
		24138 6P	23658	196 (Gln^{83})
		24058 5P		196 (Gln^{83})
B	1	24888 6P	24408	202
		24808 5P		202
		24760 6P	24280	201 (Gln^{88})
		24680 5P		201 (Gln^{88})
		25833 7P	25273	210
		25753 6P		210
		25704 7P	25144	209 (Gln^{96})
		25624 6P		209 (Gln^{96})
		24265 6P	23785	197
		24185 5P		197
		24138 6P	23658	196 (Gln^{83})
		24058 5P		196 (Gln^{83})
	2	24874 6P	24394	202
		24794 5P		202
		24745 6P	24265	201 (Gln^{88})
		24665 5P		201 (Gln^{88})
		25819 7P	25259	210
		25739 6P		210
		25689 7P	25129	209 (Gln^{96})
		25609 6P		209 (Gln^{96})
		24250 6P	23770	197
		24170 5P		197
		24126 6P	23646	196 (Gln^{83})
		24046 5P		196 (Gln^{83})

The results indicated that from peak 1 of sample A, in decreasing quantitative order, two proteins eluted: the first, the main component is 202 aa in length and the second, minor component, is 210 aa in length. In peak 2 of sample A, only one protein with 197 aa in length eluted, since the skipping of exon 5 results in the pentapeptide HTPRE deletion between L^{33} and E^{34}, as in mare α_{s1}-CN (Lenasi et al., 2003), which accounts for the decrease of 620 Da in its molecular mass with respect to the protein with 202 aa in length.
Each of these three protein components different in the aa length (202, 210 and 197 aa), showed a complex microheterogeneity either for the phosphorylation degree (5, 6 and 7P) or for a glutamine (Q) residue deletion (Table 3.1).
Taking into the account the exon modular structure of α_{s1}-CN in the other species and in the mare (Lenasi et al., 2003; Miranda et al., 2004), it is reasonable to assume that the existence of forms lacking a glutamine residue is probably related to a cryptic splice site occurring at the first codon (CAG) of exon 11, which explains the difference of 128 Da in their molecular masses. This phenomenon is a rather frequent, and it was already reported for ewe (Ferranti et al., 1995), goat (Ferranti et al., 1997), cow and water buffalo α_{s1}-CN.
On the other hand, the main protein component with 202 aa in length, was the same donkey α_{s1}-CN firstly reported by Cunsolo et al. (2009a), while the occurrence of α_{s1}-CN with 210 aa in length was a new finding. This "super long" component showed the insertion of an octapeptide DTSNESTE between K^{60} and E^{61}, whose synthesis is encoded by exon 7 insertion as in the homologous mare's α_{s1}-CN (Milenkovic et al., 2002). Moreover, the insertion of octapeptide DTSNESTE confers to α_{s1}-CN 210 aa in length a more acidic nature (theoretical pI of 5.57 0P) with respect to α_{s1}-CN 202 aa in length (theoretical pI of 5.95 0P) and α_{s1}-CN 197 aa in length (theoretical pI of 5.86 0P). As regarding the phosphorylation degree, the maximum number of phosphate groups of donkey α_{s1}-CN agreed well with the presence of 6 potential phosphorylation sites involving serine (Ser) residues (Ser^{18}, Ser^{73}, Ser^{74}, Ser^{76}, Ser^{77} and Ser^{78}; numbered according to the α_{s1}-CN with 202 aa in length) located in SerXxxGlu/SerP motifs, which are recognition sequences for bovine mammary gland casein kinase (Mercier, 1981). The MS analysis of the native α_{s1}-CN isoforms revealed that α_{s1}-CN was phosphorylated mainly at high level (6P or 7P) depending on the loss or conservation of exon 7. This suggested that the 61-68 sequence encoded by exon 7 would be systematically phosphorylated on its 2 potential sites (Ser^{63} and Ser^{66}), numbered according to the full length sequence with 210 aa (Fig. 1.1-1.2).

However, all these components (202, 210 and 197 aa) with a different phosphorylation degree were also eluted in peak 1 of sample B (Table 3.1); in peak 2 of sample B (Fig. 3.5), instead a new series of α_{s1}-"proteins" each with 14 Da lower in their molecular masses with respect to the α_{s1}-CN component eluted in peak 1, were identified (Table 3.1). This molecular mass difference is due to a single point mutation that is $Ala^{32}\rightarrow Gly^{32}$ in the novel variant found in this sample (Fig 3.6). Therefore, these results showed the occurrence of possible genetic polymorphism at donkey α_{s1}-CN *locus*, which was confirmed by a new variant's finding in the sample B.

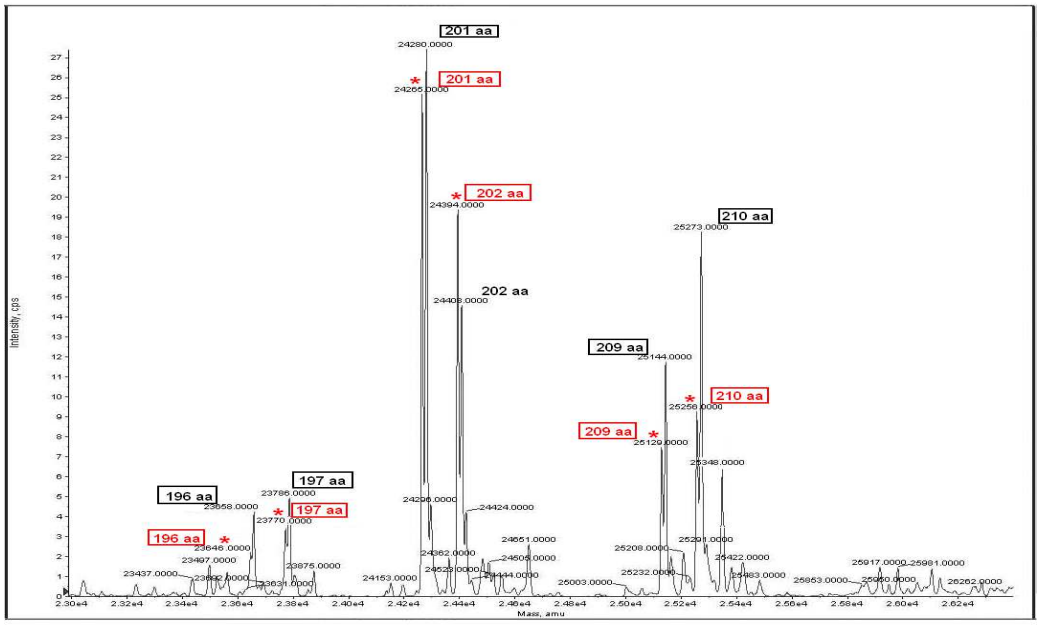

Fig 3.6: ESI-MS/MS spectrum of RP-HPLC profile of α_{s1}-CN in the heterozygous sample with the new genetic variant. The amino acid substitution in the new α_{s1}-CN variant is $Ala^{32}\rightarrow Gly$.

3.3.5 PAGE and immunoblotting analysis of donkey β-casein

The casein samples from 77 donkeys were analysed individually. In Fig. 3.7, the most common individual donkey casein samples Coomassie Brilliant Blue stained (Fig. 3.7A) were compared to the cow counterparts on the basis of their relative net charge at alkaline pH by PAGE analysis. The results obtained after specific immunstaining (Fig. 3.7B) with polyclonal antibody against β-CN, allowed to detect donkey β-CN fraction. By comparing the two species each other, cow and donkey β-CN exhibited a very similar anodic mobility, although donkey β-CN could have a major negative net charge at pH 8.6 than cow counterparts, because donkey β-CN has a major number of phosphate groups and acid aminoacids than cow protein (on SWISS-PROT database http://au.expasy.org/tools-P86273 donkey and P02666 cow). However the immunostained profile showed that donkey β-CN is the most abundant protein than other caseins and it was constituted at least by two main bands even if in some samples also a third β-CN's band was identified. In analysed samples, the different number of identified bands and their different electrophoretic mobility showed that donkey's β-CN heterogeneity is due to different phosphorilation level and to the presence of deleted forms of protein as mare's β-CN (Girardet et al., 2006), but also to genetic polymorphism.
Moreover the immunostained profiles showed also a proteolysis of β-CN, giving rise to peptides having a lower anodic mobility (β-CN derived fragments) than the parent protein. In fact considering the NNP peptide composition of donkey milk, consisting mainly in β-CN and α_{s2}-CN derived peptides generated by plasmin-like specific cleavages, it can be supposed that the same enzymes produced the above β-CN and α_{s2}-CN derived insoluble peptides at pH 4.6. So the components visible to the anode after immunostaining was attributable to the family of donkey γ-CN, C-terminal fragment generated by plasmin enzyme on β-CN (Chianese et al., 2010).

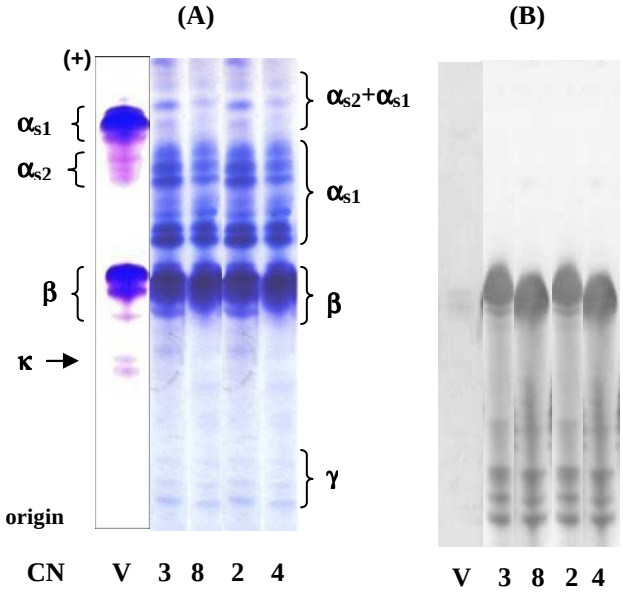

Fig. 3.7: PAGE at pH 8.6 analysis of representative donkey casein samples and bovine as reference (V) after Coomassie Brilliant Blue staining (A) and immunospecific staining with polyclonal antibody against β-CN (B).

3.3.6 UTLIEF analysis and immunoblotting of donkey β-casein

The individual donkey casein samples were subjected by UTLIEF with pH gradient 4.2-6 and separated by isoelectric point (Fig. 3.8A). After Coomassie Brilliant Blue staining, the electrophoretic profiles of donkey β-CN showed a high complexity due to the highest resolution power of this technique than the above PAGE analysis, but also to a different quali-quantitative composition than its cow counterparts.
The results obtained after specific immunostaining (Fig. 3.8B) with polyclonal antibody against β-CN allowed to detect donkey β-CN fraction. In particular, donkey and cow β-CN exhibited a very similar pI value, but a different compositional heterogeneity (8-9 components in donkey *vs.* 2-3 in cow), also responsible of overlapping phenomenon with other caseins, with respect to the above PAGE analysis.
However, these donkey β-CN components focused in the central area of pH gradient and their presence is due to the different phosphorilation degrees of each donkey β-CN component and to the presence of deleted forms, as in mare counterparts (Miranda et al., 2004). At this regard, Cunsolo et al. (2009b) found three

phosphorylated components (5P, 6P and 7P) by MS analysis only in the full-length protein, while no information on the phosphorilation degree has been provided for the shorter β-CN form lacking the β-CN fragment (27-34) (Cunsolo et al., 2009b). According to Chianese et al. (2010) both the full-length β-CN and its deleted form lacking of fragment β(27-34) have 5, 6 and 7P/mole. However, the absence of the domain $E^{27}SITHINK^{34}$ confers to deleted form a more acidic nature (theoretical pI value 5.40 0P) with respect to the full-length component (theoretical pI value 5.54 0P) and also a major number of phosphate groups both on the full-length β-CN and on its deleted form lacking of fragment β(27-34) gives them a more acidic nature than the corresponding less phosphorylated forms. This result is very important and regarding donkey β-CN has been reported for the first time.
Concordantly with PAGE analysis, even the hydrolysis products of donkey β-CN, probably generated by plasmin-like enzymatic activities, are immunostained and focused at higher pI values than their parent casein, respectively (Chianese et al., 2010).

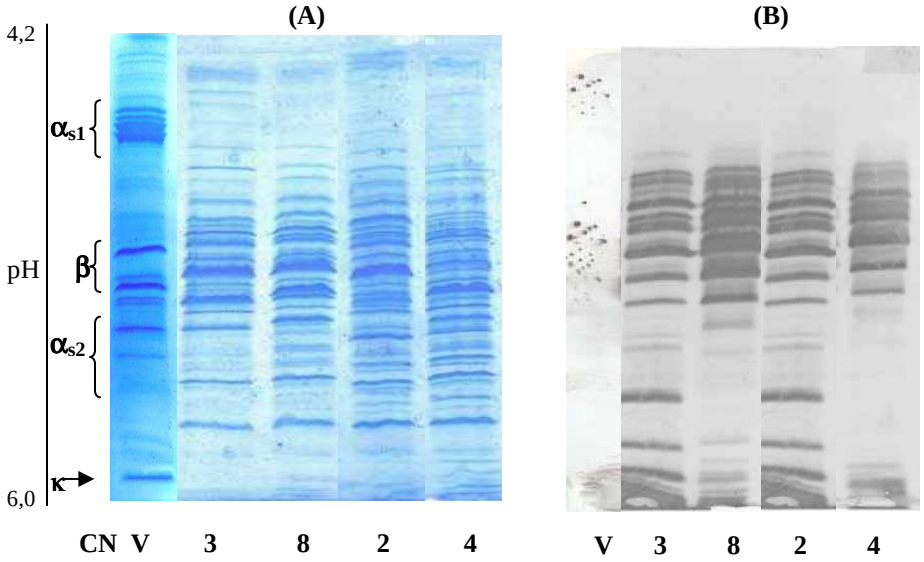

Fig. 3.8: UTLIEF analysis of representative donkey casein samples and bovine as reference (V) after Coomassie Brilliant Blue staining (A) and immunospecific staining with polyclonal antibody against β-CN (B).

3.3.7 Characterization of donkey β-CN by two dimensional (2-DE) electrophoresis analysis (PAGE→UTLIEF) and (IPG→SDS PAGE)

The complex overlapping phenomenon of the full-length β-CN and its deleted form lacking of fragment β(27-34) with other caseins can take place in one dimensional electrophoretic analysis, as UTLIEF, because of their very similar pI values.
This overlapping was partially resolved in 2-DE electrophoretic analysis. In fact, in PAGE→UTLIEF analysis of an individual donkey casein, after Coomassie Brilliant Blue staining (Fig. 3.9A) and mainly after immunoblotting with specific polyclonal antibody against β-CN (Fig. 3.9B), the number of β-CN components was about 6, and according Cunsolo et al. (2009b) and Chianese et al. (2010) they would correspond to the full-length protein, carrying from 5 to 7P as well as its shorter form, always carrying from 5 to 7P.
In IPG→PAGE-SDS analysis of individual donkey casein sample, either after Coomassie Brilliant Blue staining (Fig. 3.10A) and immunoblotting analysis (Fig. 3.10B), the complexity of β-CN was even more resolved than PAGE-UTLIEF analysis. In fact, in this 2-DE map β-CN components are arranged in two levels. A major level is associated to a higher apparent molecular mass and would correspond to the full-length protein with 226 aa, while a minor level is associated to a lower apparent molecular mass and would correspond to its deleted component with 218 aa characterized by a splicing of exon 5. Cunsolo et al. (2009b) and Chianese et al. (2010) found that two donkey β-CN components showed a molecular mass difference of 923 Da and differ for the presence of the domain $^{27}SITHINK^{34}$ in the full length component, that, as occurring in the mare's β-CN is absent in the deleted component (Fig. 1.3).

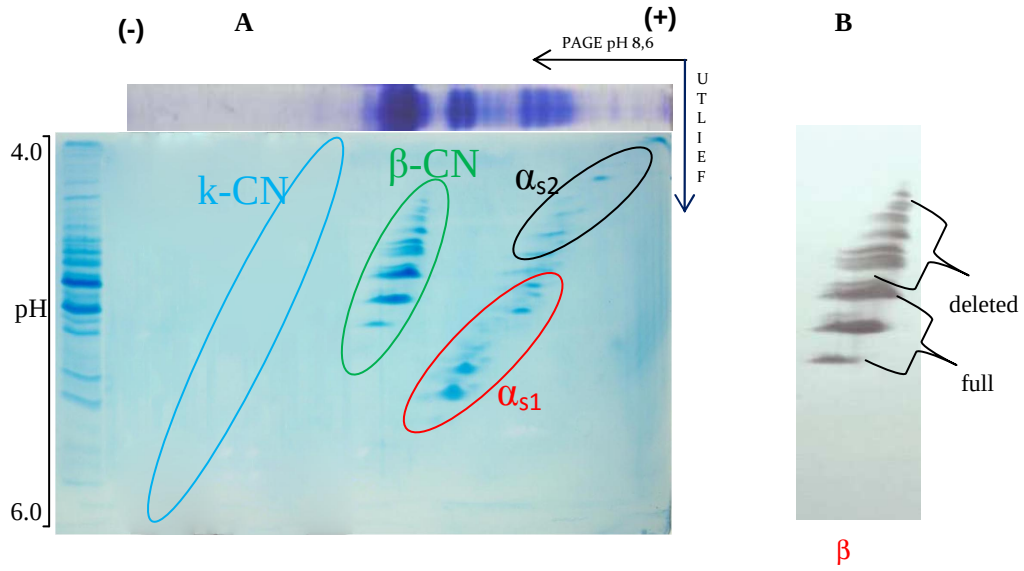

Fig 3.9: 2-DE (PAGE→UTLIEF) analysis of representative donkey casein sample after Coomassie Brilliant Blue staining (A) and immunospecific staining with polyclonal antibody against β-CN (B).

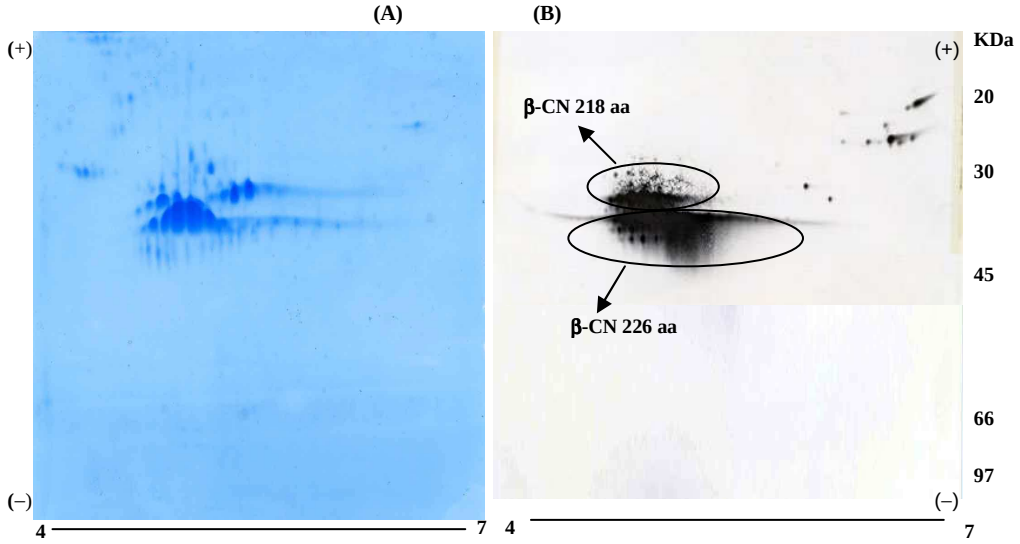

Fig 3.10: 2-DE (IPG→PAGE-SDS) analysis of representative donkey casein sample after Coomassie Brilliant Blue staining (A) and immunospecific staining with polyclonal antibody against β-CN (B).

3.3.8 Evidences of genetic polymorphism at donkey β-CN locus by UTLIEF analysis

The events of overlapping can be amplified by the heterogeneity of donkey β-CN due to post-translational phenomena (phosphorilation), non-allelic deleted forms, proteolysis by action of endogenous proteases but also polymorphism (one or two variants in individual samples, much more in bulk milk).
In fact, the UTLIEF analysis of representative casein samples, either after Coomassie Brilliant Blue and immunoblotting (Fig. 3.11A-B) showed an high heterogeneity of donkey β-CN due to genetic polymorphism at this *locus* for the finding at least of two genetic variants, as we can see in the following individual casein samples: 3, 2, 4 (probably homozygous for donkey β-CN) and 8 (probably heterozygous for donkey β-CN).
In fact, donkey β-CN focused in the central area of pH gradient and specifically in the sample 2 and 3 donkey β-CN variant focused in the same band X of pH gradient (calculated pI 4,82); in sample 4 and 8, donkey β-CN focused always in the same central area of pH gradient, but specifically in this sample donkey β-CN variant focused in the band Y of pH gradient. The pI value of the band Y is more alkaline (calculated pI 4,91) than the corresponding value of band X and therefore the β-CN variant (sample 4 or 8) which focused in bands Y showed a lowest movement towards the anode (and could be more alkaline) than β-CN variant (sample 2 or 3) which focused in bands X. Moreover, the individual sample of casein 8 could be

heterozygous for donkey β-CN, because it contain both bands (X and Y), and thus may contain two genetic variants of the protein with different pI values.

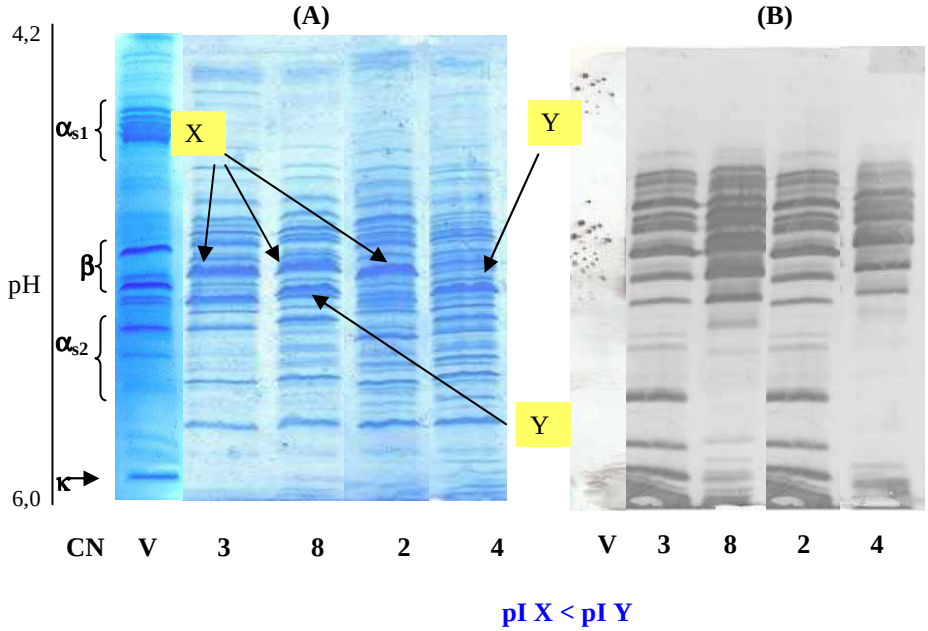

Fig. 3.11: UTLIEF analysis of representative donkey casein samples and bovine as reference (V) after Coomassie Brilliant Blue staining (A) and immunospecific staining with polyclonal antibody against β-CN (B).

3.3.9 Evidences of genetic polymorphism at donkey β-CN locus by RP-HPLC/MS analysis

The RP-HPLC UV chromatograms (Fig. 3.12) of three representative and individual donkey casein samples showed the presence of some main peaks. According to Chianese et al. (2010) in peak 1 β-CN fragments, originated by hydrolysis of both full protein length (226 aa) and from its deleted form lacking peptide (27-34) (Cunsolo et al., 2009b) were identified by LC/ESI/MS (Chianese et al., 2010). In peaks 2 and 3 donkey α_{s1}-CN components at different phosphorilation degrees (5P and 6P) as well as their non-allelic deleted forms generated by RNA incorrect splicing (Cunsolo et al., 2009), as in sheep and goat caseins (Ferranti et al., 1995; 1997), eluted and were identified by Chianese et al. (2010). In peak 4 the full-length β-CN and its deleted form lacking of fragment (27-34), both having 5, 6 and 7P/mole, eluted (Chianese et al., 2010). These forms have been already detected by Cunsolo et al. (2009b) only in full-length protein.
Regarding donkey β-CN, the comparison of the HPLC profiles of samples 4, 2 and 8 showed that in sample 4, only one β-CN peak eluted (elution time of this peak which we named D is 20.46 min) as in sample 2, only one β-CN peak eluted (elution time of this peak which we named B is 21.59 min). The casein sample 8 was more heterogeneous than the formers, for the presence of two β-CN peaks, where the peak 4* (elution time of this peak which we named C is 19.94 min) eluted at shorter elution time than peak 4 (elution time of this peak which we named A is 20.97 min). So, in analysed casein samples, in addition to two different pI theoretical values of donkey β-CNs, also the different elution time of donkey β-CNs (elution time C<D<A<B) suggested for the first time the occurrence of genetic polymorphism at this casein *locus*.
Moreover in peak 5, with higher elution time than the previous peaks, considering the known amino acid sequence of donkey β-CN A (Cunsolo et al., 2009b), a C-terminal β-CN fragment (56-226 with theoretical molecular weight of 19149 Da) probably originated by plasmin hydrolysis on Lys-X bond of full protein length (226 aa) was identified for the first time by LC/ESI/MS. Thus, in third casein sample with two β-CN peaks, in addition to the peak 5, also peak 5* eluted because this casein could be β-CN heterozygous.

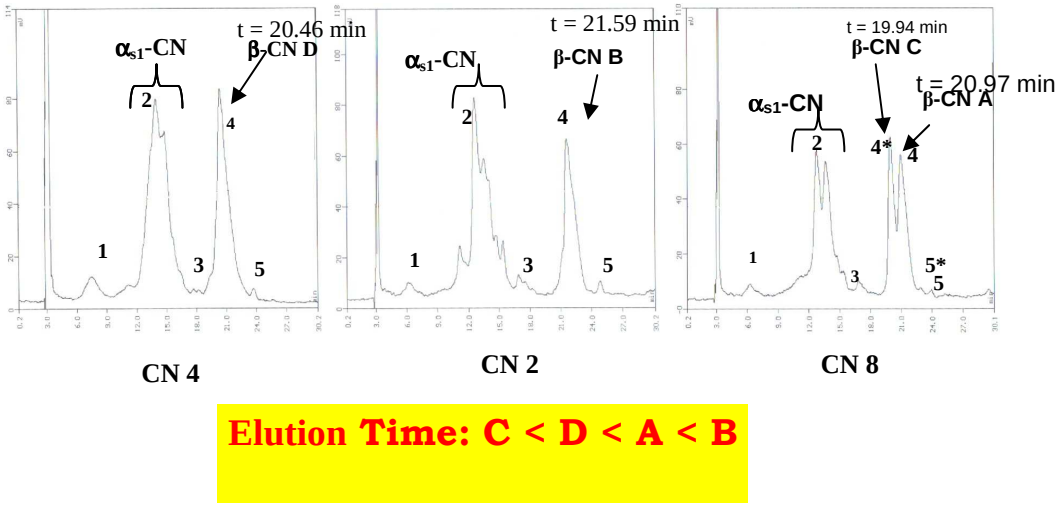

Fig. 3.12: RP-HPLC profiles of donkey β-CN variants.

3.3.10 RP-HPLC/MALDI-TOF/MS of donkey β-CN variants

To evidence the possible genetic polymorphism at donkey β-CN *locus*, after dephosphorylation, the β-CN fraction of each sample was separated from casein mixture by RP-HPLC and its corresponding peaks 4 and 4* were manually collected, freeze-dried and analysed by MALDI TOF-MS analysis.
Fig. 3.13 shows the MALDI-TOF (MS) mass spectra of the chromatographic peaks (4 and 4*) of the dephosphorylated β-CN fractions. The spectra were acquired in the *m/z* range 10000-30000, obtained operating in linear positive ion mode and using SA as matrix.
Considering the known amino acid sequence of donkey β-CN A (Cunsolo et al., 2009b), each of four MALDI-TOF-MS spectrum also showed, in addition to a main signal, a minor signal with an experimentally measured M_r of about 12760 Da, which could correspond to C-terminal β-CN fragment (114-226 2P) probably originated by plasmin hydrolysis on Lys-X bond of full protein length (226 aa) and was for the first time identified by MALDI-TOF/MS.
Regarding donkey β-CN, the molecular masses (M_r) of a full-length (226 aa) β-CN (25526 Da) and its deleted form (218 aa) lacking the region 27-34 (24606 Da) (Cunsolo et al., 2009b) together with cDNA-derived sequence of β-CN (226 aa) with a M_r 25539 Da (Di Gregorio, 2009) are known. By comparison with M_r of these donkey β-CNs known in literature, the MALDI-TOF MS analysis of the collected chromatographic peak 4 allowed to detect β-CN fraction in this peak. Specifically, in peak 4 (A) of sample 8, a measured M_r 25521 Da corresponded to theoretical M_r (25526 Da) of donkey β-CN known in literature named A variant (Cunsolo et al., 2009b), while in peak 4 (B) of sample 2, a measured M_r 25535 Da corresponded to theoretical M_r (25539 Da) of donkey β-CN only deduced from cDNA (Di Gregorio, 2009), which we named B variant.
The MALDI-TOF MS analysis of the collected chromatographic peaks 4*(C) and 4 (D) of samples 8 and 4 allowed even to detect two unknown components, having an experimentally measured M_r of 25546 and 25556 Da respectively, which could correspond to two new donkey β-CN genetic variants (which we named C and D respectively) not yet known in literature. In this way, the occurrence of genetic polymorphism at donkey β-CN *locus* hypothesized by UTLIEF and RP-HPLC analysis of representative donkey casein samples was for the first time confirmed by MS methods as MALDI-TOF/MS analysis.

3.3.11 Characterization of the primary structure of the new donkey β-CN variants

In order to obtain additional information about the amino acid sequences of new donkey β-CN (B, C, D) variants, the tryptic mixture of each chromatographic peak corresponding to dephosphorylated protein was analyzed by MALDI-TOF and the results interpreted using the known donkey β-CN A variant as reference. In Table 3.2, the position, peptide sequence, theoretical and experimentally measured monoisotopic MH^+ of selected fragments identified in the tryptic digestion mixture of donkey β-CN variants were reported. The sequence of the known donkey β-CN A (Cunsolo et al., 2009b) was taken as reference. The amino acid substitutions with respect to the donkey's reference β-CN A variant were red stained.
Specifically, regarding donkey β-CN B variant, the mass spectrum signals of four peptides with experimentally deduced MH^+ 3792.9, 4404.6, 3268.0, 4032.3 allowed the identification of two amino acid

substitutions in donkey β-CN B variant with respect to the corresponding known β-CN A variant. In detail, in comparison with β-CN A variant, the identification and characterization of peptides at m/z 3792.9 and 4032.3 allowed us to deduce β-CN sequences which corresponded to the theoretical N-terminal (1-34) and C-terminal (190-226) tryptic fragments, containing the same amino acids with respect to donkey β-CN A variant. The interpretation of mass spectra signals of the peptides at m/z 4404.06 and 3260.0, allowed use to deduce β-CN sequences which corresponded to the domains (1-39) and (76-105) respectively, carrying the amino acid substitutions Ser37→Val and Val84→Pro with respect to the donkey β-CN A variant.
Regarding donkey β-CN D variant, the identification and characterization of peptides (signals) at m/z 3267.81, 3806.9, 4418.78 and 4046.07 allowed use to deduce β-CN sequences which corresponded to N-terminal (1-34), (1-39), (76-105) and C-terminal (190-226) domains respectively, carrying the amino acid substitutions Asn33→Lys, Ser37→Val, Val84→Pro and Ala213→Ser, with respect to the donkey β-CN A variant.
Finally, regarding donkey β-CN C variant, the identification and characterization of peptides (signals) at m/z 4392.89 and 3268 allowed us to deduce β-CN sequences which corresponded to the theoretical tryptic fragments (1-39) and (76-105) respectively, also containing the same amino acid substitutions than donkey β-CN A variant. The interpretation of mass spectra of the peptides (signals) at m/z 3806.6 and 4045.9 allowed us to deduce β-CN sequences corresponding to the N-terminal (1-34) and C-terminal (190-226) domains respectively, which contained, as β-CN D variant, the amino acid substitution Asn33→Lys and Ala213→Ser with respect to the donkey β-CN A variant.
In summary, all these proteomic methods allowed the characterization of three new genetic variants of donkey β-CN, whose identified amino acid substitutions were reported in Table 3.3. In this table 3.3, the amino substitutions, pI and theoretical M_r values were reported for each donkey β-CN variant (A, B, C, D). These values were calculated, theoretically, on the basis of the amino acid substitutions with respect to the reference donkey β-CN A variant (Cunsolo et al., 2009b). Moreover, these data confirmed that donkey β-CN A and B variants, on the basis of their amino acid substitutions, have the same pI value (4.82). Donkey β-CN C and D also have the same calculated pI value (4.91), which are for the presence of Lys 33 in their amino acid sequences more alkaline than the corresponding value in β-CN A and B variant (4.82).
However, these theoretical pI values of the four β-CN variants allowed us to identify them on the UTLIEF profile, where β-CN A and B variants focused at the same lower pH gradient than C and D variants (Fig. 3.14).

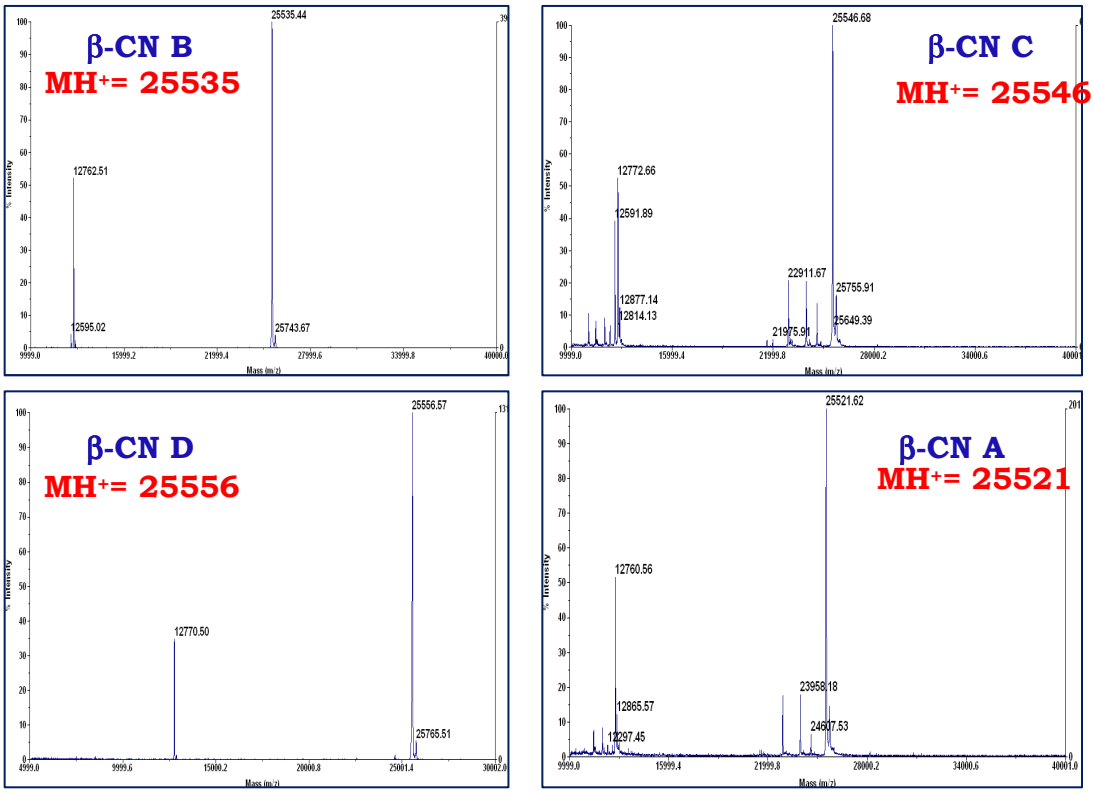

Fig. 3.13: MALDI-TOF-MS spectra of four chromatographic peaks containing the corresponding dephosphorylated β-CN variants.

Table 3.2: β-CN tryptic peptides identified by MALDI TOF MS analysis

Peptide	Theoretical Mass (Da)	Reference sequence β-CN A	B	D	C	A
1-34	3790.77	REEKELNVSSETVESLSSNEPDSSSEESITHINK	3792.90	3806.90 (+14)	3806.66 (+14)	3792.75
1-39	4392.64	REEKELNVSSETVESLSSNEPDSSSEESITHINKEKSQK	4404.12 (+12)	4418.78 (+14) (+12)	4392.89	4392.54
76-105	3269.96	AVVPQNILVLAQPPIVPFLQPEIMEVSQAQ	3268.01	3267.81	-	-
190-226	4029.10	DTPVQAFLLYQDPQLGLTGEFDPATQPIVPVHNPVIV	4032.33	4046.07 (+14)	4045.99	4032.55

Table 3.3: Amino acid substitutions, theoretical molecular mass and pI values of different β-CN variants

β-CN	33	37	84	213	Theoretical Molecular Mass (Da)	pI
B	Asn	Val	Pro	Ala	25539	4.82
D	Lys	Val	Pro	Ser	25570	4.91
C	Lys	Ser	Val	Ser	25558	4.91
A	Asn	Ser	Val	Ala	25526	4.82

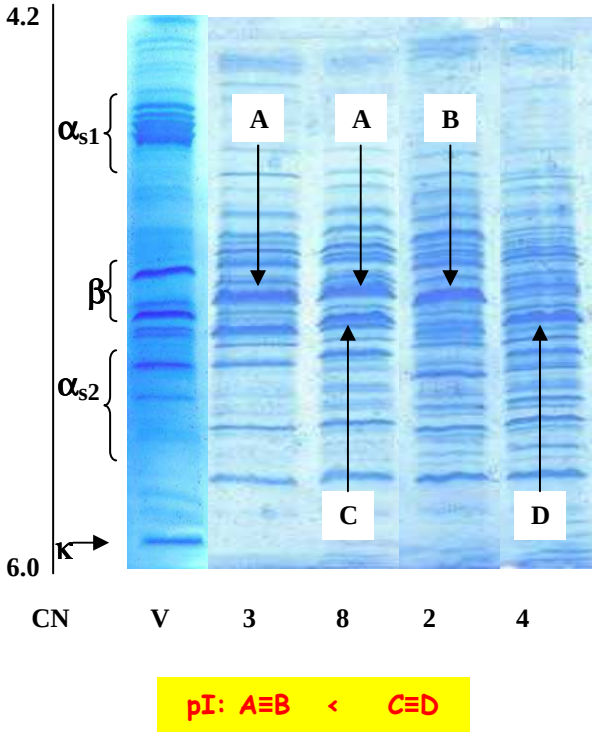

Fig. 3.14: UTLIEF analysis of representative donkey casein samples and bovine as reference (V) after Coomassie Brilliant Blue staining (A) and immunospecific staining with polyclonal antibody against β-CN (B).

3.3.12 Characterization of the phosphorylation level of the new donkey β-CN variants

The molecular mass determination for the phosphate-free form of the β-CN variants and their deleted forms Δ5 enabled a precise determination of the maximum degree of phosphorylation to be made. It corresponded to 7P (Chianese et al., 2010) per molecule and specifically, analysis of the new phosphorylated β-CNs demonstrated that each full length variant (B, C, D), as donkey β-CN A variant (Cunsolo et al., 2009b) consists of three isoforms carrying from 5 to 7P. This same phosphorylation degree was also found for each corresponding deleted isoform (B Δ5, C Δ5, D Δ5) as for β-CN A Δ5 Chianese et al. (2010).
This result is important because emphasizes that amino acid deletions or substitutions between the different donkey β-CN variants do not affect serine residue which can phosphorylated. Moreover, for each new β-CN variants (B, C, D) as well as for their corresponding β-CNs Δ5 (lacking the region encoded by exon 5), the

isoform carrying 6P and 7P occur with comparable abundances, whereas the 5P protein is present as a minor component. This is in agreement with the results obtained for donkey β-CN A (Cunsolo et al., 2009b) and donkey β-CN Δ5 (Chianese et al., 2010).
These results, however, reveals that donkey β-CN is more phosphorylated than the homologous mare's β-CN, in which the predominant phosphorylated isoforms carries 5P, despite 7 phosphorylation sites are available. Instead, the human protein, which is also phosphorylated at different levels, carries up to 5P. Thus, the donkey β-CNs could also be more phosphorylated than both bovine (a fully phosphorylated protein with 5P) and the human β-CN, the major isoform of which are β-CN 2P and 4P (Sood & Slattery, 2000).
It is difficult to explain the high phosphorylation level of donkey β-CN, but this phenomenon, as mare's β-CN, could be due to the presence of an additional exon 4',only observed in equidae, which encodes a region (21-26) containing a phosphorylation cluster (Miranda et al., 2004). Thus, the presence of at most seven group bond to donkey β-CN agrees with the protein primary structure which contains six potential phosphorilation sites (Ser^{9}, Ser^{15}, Ser^{18}, Ser^{23}, Ser^{24} and Ser^{25}), located in a Ser-Xxx-Glu/SerP motif, and one Ser (Ser^{17}) in a Ser-Xxx-Xxx-Glu motif, together with two other potential phosphorilation sites located at Thr^{12} and Thr^{207}, located in a Thr-Xxx-Glu motif, analogously to the homologous mare's β-CN. The Ser-Xxx-Glu/SerP and Ser-Xxx-Xxx-Glu motifs are the recognition sequences characterized for cow mammary gland casein Kinase I (Mercier, 1981) and II (Kuenzel et al., 1987), whereas studies on the specificity of mammary gland casein kinase have shown that the sequence motif Thr-Xxx-Glu is a poor substrate for the mammary gland enzyme and is usually not phosphorylated (Bingham & Groves, 1979; Sorensen & Petersen, 1994).

3.3.13 Primary structure comparison of the donkey's and cow's β-CNs

Studies of large population of patients allergic to CMA (cow's milk allergy) show that β-CN, the second most abundant protein in cow's milk, where it constitutes about 28% of the total milk proteins, represents one of the major cow's milk allergens (Monaci et al., 1998). In human milk, the casein fraction is essentially composed by β-CN.
β-CN is a phosphoprotein with a distributed proline content but with a lack of cysteine amino acids. So, in bovine β-CN disulphide bonds are absent and therefore it possesses virtually no secondary structure and shows a reduced rigidity of the tertiary structure (Kumosinski et al., 1991). As a consequence, these factors increase the likelihood that the important allergenic epitopes of this protein are linear rather than conformational. As results of several investigations, six major (AA 1-16, AA 45-54, AA 55-70, AA 83-92, AA 107-120 and AA 135-144) and three minor (AA149-164, AA167-178 and AA 185-208) IgE binding regions on bovine β-CN were identified. Among these, three epitopes (AA 1-16, AA 83-92 and AA 135-144) were the most frequently recognised epitopes while two minor epitopes (AA 149-164, and AA 167-178) are recognised only by older patients but not by the younger age. Instead, eight major epitopes (AA 1-14, AA 23-34, AA 55-68, AA79-92, AA 107-120, AA 135-144, AA 149-160 and AA 183-208) and one minor (AA 169-184) IgG binding regions on bovine β-CN were identified. Among these, two epitopes (AA 135-144 and AA 183-208) were recognised by all patients (Chatchatee et al., 2001).
Therefore, the knowledge about the allergenic epitopes of bovine β-CN, together with the determination of the primary structure of different donkey β-CN variants and their internally deleted isoforms (here reported) provide the basis for some comparison of their sequences. According to Cocco (2007), the substitutions or deletions of one or more amino acids in a protein as donkey β-CN could result in a partial reduction or elimination of IgE binding regions in majority of patients.
However, the best alignment of the bovine and donkey's β-CN variant A reveals that these two proteins share a low sequence homology (58% of identity). In particular, alignment reveals that the IgE- and IgG- binding linear epitopes of cow's β-CN and the corresponding domains present in donkey β-CN have remarkable differences in their amino acid sequences. Moreover, it is interesting to note that the amino acid sequence E^{27}SITHINK^{34} absent in the internally deleted β-CNΔ5 isoform from donkey is a trait of an important IgE- and IgG-binding epitopes of bovine β-CN; thus, its deletion from the full-length β-CN sequence together with the considerable differences between the primary structure of donkey and bovine β-CNs could be related to the already demonstrated low allergenic properties of donkey's milk and could contribute to explain its better tolerance also with respect to goat's milk for children suffering CMPA (Vita et al., 2007). In fact, it should also be noted that goat's and cow's β-CNs show 91% of identity and that anaphylactic reactions to goat's milk in children with CMPA have been reported (Pessler, 2004).
Although these considerations seem reasonable, further biochemical studies and clinical experiences are needed to establish precise relationship between donkey's milk protein structures and its low allergenic properties.

3.3.14 PAGE Analysis and Immunoblotting of donkey α_{s2}-CN

Regarding donkey α_{s2}-CN, the results of PAGE analysis at pH 8.6 on representative individual casein samples, obtained after CBB (Fig. 3.15A) staining and mainly specific immunostaining with polyclonal antibody against α_{s2}-CN (Fig. 3.15B), allowed to detect this casein fraction, which seemed to be one of the less abundant casein in donkey milk. By comparing cow and donkey casein, each other, donkey α_{s2}-CN exhibited a higher and reversed anodic mobility than its cow counterpart. The immunostained profiles also showed an overlap between α_{s1}- and α_{s2}-CN components, although the latter showed an higher anodic mobility compared to some of the major α_{s1}-CN components. In donkey caseins analyzed, this immunoelectrophoretic technique revealed a variability in the protein's migration, suggesting also a genetic polymorphism at donkey α_{s2}-CN l*ocus*.
Moreover, the presence of more immunostained α_{s2}-CN components could be due to its different phosphorylation degree (10-12 P), as reported by Chianese et al. (2010) and to the presence of its deleted forms, as reported by Saletti et al. (2012). In fact, at this regard Saletti et al. (2012), using the aa sequence of the known full length α_{s2}-CN as reference (Chianese et al., 2010), detected four α_{s2}-CN deleted forms which were produced from differential splicing events involving exon 4, 5, 6 and part of exon 17 in the gene encoding for the asinine protein (Fig.1.4).

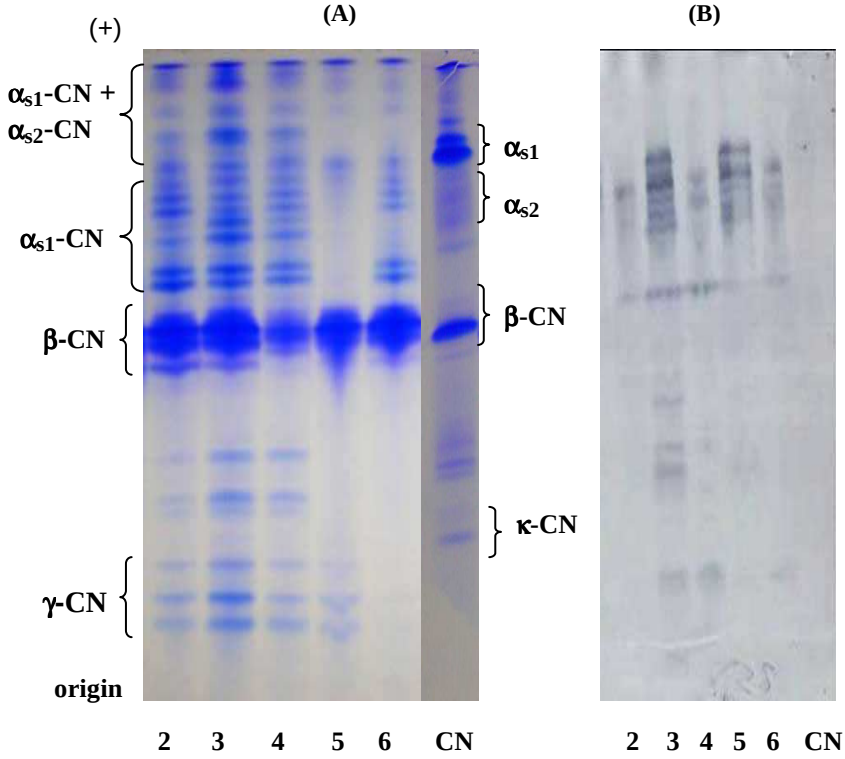

Fig 3.15: PAGE at pH 8.6 analysis of representative donkey casein samples and bovine as reference (CN) after Coomassie Brilliant Blue staining (A) and immunospecific staining with polyclonal antibody against α_{s2}-CN (B).

3.3.15 UTLIEF Analysis and Immunoblotting of donkey α_{s2}-CN

UTLIEF profiles of representative donkey caseins, either with Blue Coomassie (Fig. 3.16A) and mainly with polyclonal antibodies against α_{s2}-CN (Fig. 3.16B) showed the compositional heterogeneity of the asinine α_{s2}-CN.
For donkey α_{s2}-CN this phenomenon may be due to qualitative and, above all, quantitative polymorphism, because there are casein samples characterized by α_{s2}-CN content much more lower than the others. Moreover, the detection of different α_{s2}-CN components may be due to the presence of non allelic deleted forms (Saletti et al., 2012) with a different phosphorylation degree, and to the proteolysis by action of endogenous proteolytic enzymes (Chianese et al., 2010).
Specifically, regarding the phosphorylation degree, the high number of phosphate groups of donkey α_{s2}-CN, agreed well with the presence of 11 phosphorylation sites involving serine (Ser) residues (Ser^{8}, Ser^{9}, Ser^{10}, Ser^{17}, Ser^{32}, Ser^{65}, Ser^{66}, Ser^{67}, Ser^{70}, Ser^{142}, Ser^{154}) and Thr^{141} or Thr^{165}, numbered according to the full length α_{s2}-CN with 221 aa located in SerXxxGlu/SerP and ThrXxxGlu motifs, which are recognition

sequences for mammary gland casein kinase. The phosphorylation sites were determined using the program NetPhos 2.0, available at www.expasy.ch proteomic server in donkey α_{s2}-CN (Accession No. B7VGF9) However, these results underlined a more complex overlapping phenomenon, due to the similar pI values of casein components, with respect to the above PAGE analysis. In particular, α_{s2}-CN components (5-6) for the presence of many acid amino acids and phosphate groups (10-12) focused at more acid pH gradient than the main α_{s1}-CN components, even if they partially overlapped β-CN and other minor α_{s1}-CN components.

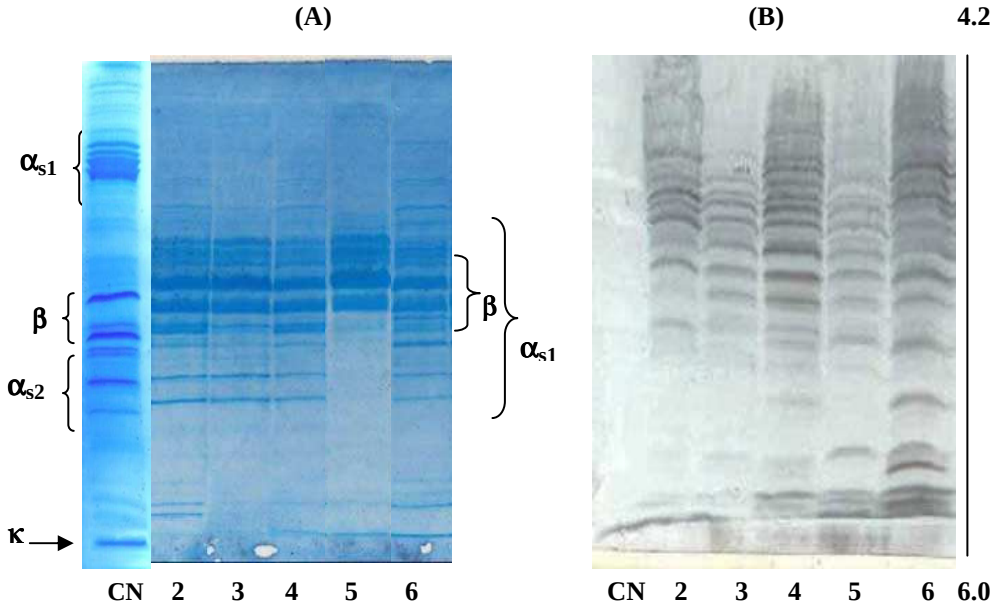

Fig 3.16: UTLIEF analysis of representative donkey caseins and bovine as reference (CN) after Coomassie Brilliant Blue staining (A) and immunospecific staining with polyclonal antibody against α_{s2}-CN (B).

3.3.16 Characterization of donkey α_{s2}-CN by two dimensional (2-DE) electrophoresis (IPG→PAGE-SDS)

The complexity of donkey α_{s2}-CN was also responsible of the overlapping phenomenon with other casein fractions. This phenomenon was partially resolved in 2-DE (IPG→PAGE-SDS) electrophoretic analysis of an individual donkey casein sample, either after Coomassie Brilliant Blue staining (Fig. 3.17A) and mainly after immunoblotting with specific polyclonal antibody against α_{s2}-CN (Fig. 3.17B). In fact, in this 2-DE map α_{s2}-CN components were arranged in two levels. A major level is associated to a greater apparent molecular mass and would correspond to the full-length protein with 221 aa, while a minor level is associated a very low apparent molecular mass and would correspond to its deleted component characterized by a splicing of many exons which encoded for this protein. At this regard, Cosenza et al. (2010) cloned, sequenced and analyzed the gene with 19 exons which encodes for the entire protein of 221 aa, but also a second gene (16 exons) characterized by the lack of the sequences that in the entire gene correspond to exons 6, 7, 8, 10,11,12, which encodes for a predicted peptide of 145 aa.

3.3.17 Characterization of donkey α_{s2}-CN by RP-HPLC/MS

As for α_{s1}-CN, to simplify the compositional heterogeneity of donkey α_{s2}-CN and subsequently to determine its mean expression level (paragraph 3.13.3), the individual donkey casein samples were analyzed by RP-HPLC. The identification of the eluted components, after AP action, was carried out using a mass detector coupled to an electrospray source (LC/ESI/MS) (table 3.4). After a screening of individual donkey caseins, a characteristic HPLC profiles was selected in (Fig. 3.18), and specifically in peak with elution time of 20 min the eluted protein corresponded to the full-length α_{s2}-CN component, with a molecular weight of 26027 Da, as reported by Chianese et al. (2010). The identification of α_{s2}-CN in this chromatographic peak will allow, in the chapter of the proteins quantitative analysis (paragraph 3.13.3), to determine the average amount of α_{s2}-CN in donkey milk by RP-HPLC.

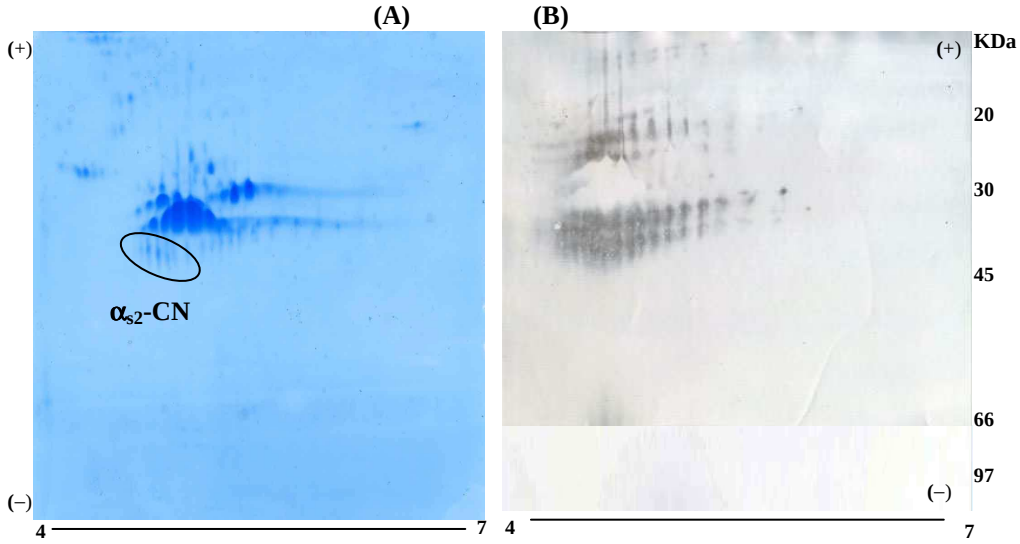

Fig 3.17: 2-DE (IPG→PAGE-SDS) analysis of representative donkey casein sample after Coomassie Brilliant Blue staining (A) and immunospecific staining with polyclonal antibody against α_{s2}-CN (B).

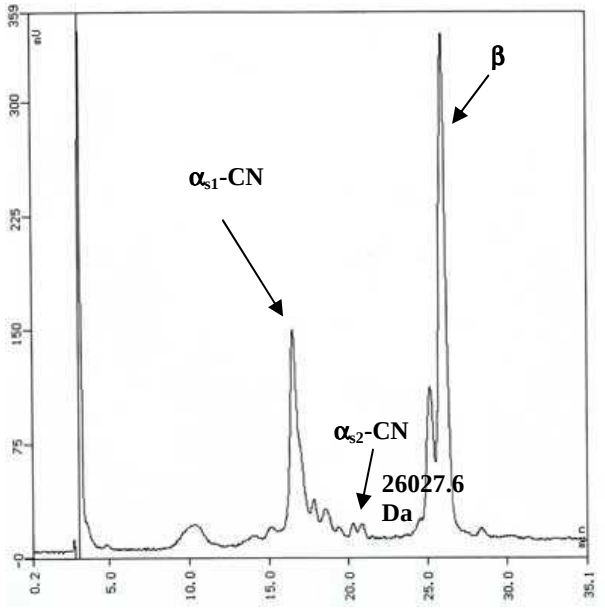

Fig 3.18: RP-HPLC of an individual donkey casein sample where α_{s2}-CN chromatographic peak was identified.

Table 3.4: Molecular masses (Da) of casein fractions identified in a individual donkey casein samples by LC-ESI-MS.

	Measured Mr (Da) Dephosphorylated	Theoretical Mr (Da) Dephosphorylated
αs1	24408.2	24406
αs2	26027.5	26029
β	25552.7	25558 (C variant)
	25537.6	25539 (B variant)

3.3.18 PAGE Analysis and Immunoblotting of donkey κ-CN

As far as donkey κ-CN, it was very difficult to detect its components only in CBB, likely depending either on the low amount of this fraction in the milk or on its lower reactivity to CBB staining (Fig. 3.19A). The results of this immuno-electrophoretic technique (Fig. 3.19B) showed the lowest mobility of κ-CN towards the anode compared to the other casein fractions and a different electrophoretic mobility of κ-CN in the different analyzed samples, probably due to the occurrence of genetic polymorphism also at this *locus*.

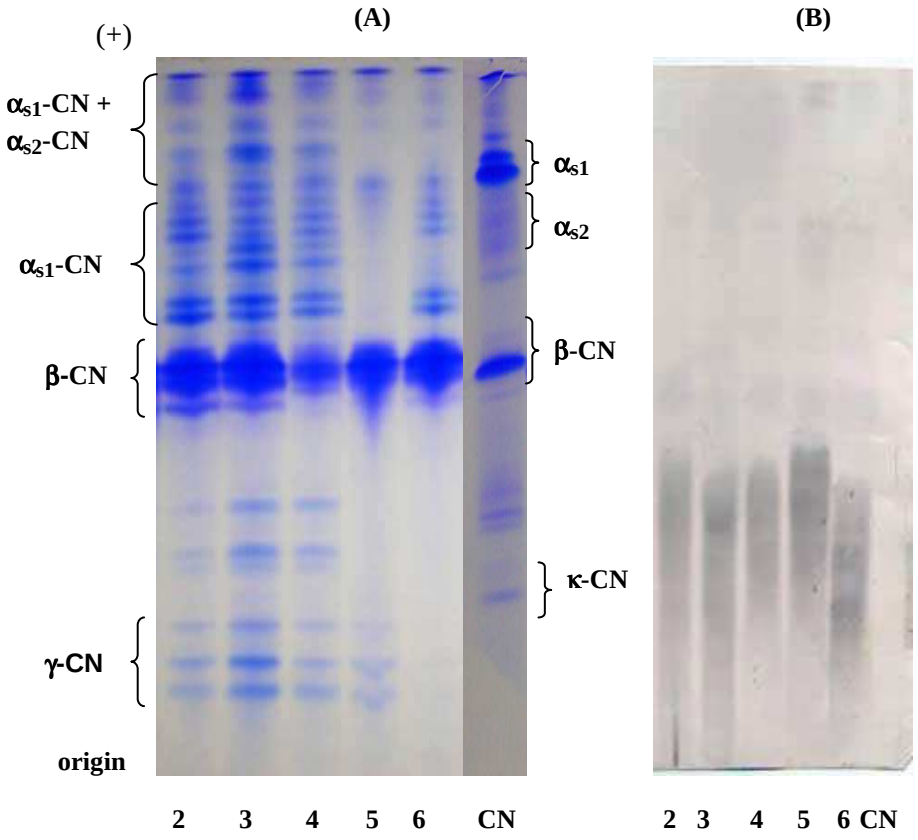

Fig 3.19: PAGE at pH 8.6 analysis of representative donkey casein samples and bovine as reference (CN) after Coomassie Brilliant Blue staining (A) and immunospecific staining with polyclonal antibody against κ-CN (B).

3.3.19 UTLIEF Analysis and Immunoblotting of donkey κ-CN

UTLIEF profiles of representative donkey caseins, either with Blue Coomassie (Fig. 3.20A) and mainly with polyclonal antibodies against κ-CN (Fig. 3.20B) showed the compositional heterogeneity of the asinine κ-CN.

In fact, all these casein fractions (α_{s1}-, β-, α_{s2}-CN) were overlapped by κ-CN components, which, as in ruminants, is the most heterogeneous casein fraction for the detection of components (10-12) focalizing throughout the pH gradient and probably this phenomenon is generated from a different glycosylation degree of the protein. In fact, considering the cDNA amino acid sequence of donkey κ-CN (without the first 20 aa of signal peptide), 12 Thr residues (Thr^{86}, Thr^{109}, Thr^{116}, Thr^{119}, Thr^{123}, Thr^{127}, Thr^{131}, Thr^{149}, Thr^{152}, Thr^{153}, Thr^{154}, Thr^{158}) may be glycosylated, as in mare counterpart, by using the program NetOGlyc 3.1, available at www.expasy.ch proteomic server in donkey κ-CN (without 20 aa of signal peptide) (Accession No. FOV6V5).

Many glycosides linked to the protein, giving it a more acidic nature. Therefore κ-CN forms which focused at cathode would correspond to the less glycosylated κ-CN components, which would also appear to be more abundant compared to the glycosylated κ-CN components, which focused at anode.

Even for κ-CN, a different intensity and migration of immunostained bands suggested not only quantitative, but also a qualitative polymorphism at its *locus*, and thus, the presence of more κ-CN variants.

No direct information is available on the phosphorylation state of donkey κ-CN. However, three putative phosphorylation sites, namely Thr^{119}, Thr^{127} and Thr^{149}, may be suggested based on the sequence similarity

studies with mare's κ-CN and on prediction analysis. The phosphorylation sites were determined using the program NetPhos 2.0, available at www.expasy.ch proteomic server in donkey κ-CN (without 20 aa of signal peptide) (Accession No. FOV6V5).

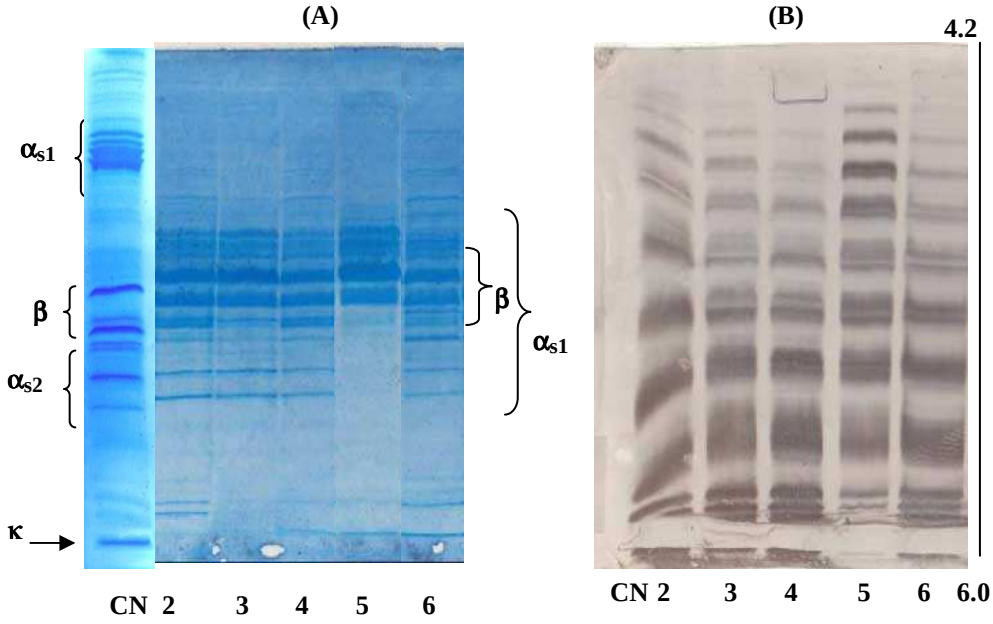

Fig 3.20: UTLIEF analysis of representative donkey casein samples and bovine as reference (CN) after Coomassie Brilliant Blue staining (A) and immunospecific staining with polyclonal antibody against κ-CN (B).

3.3.20 Characterization of donkey κ-CN by two dimensional (2-DE) electrophoresis (IPG→PAGE-SDS)

Concordantly with UTLIEF analysis (paragraph 3.3.19), all casein fractions (α_{s1}-, α_{s2}-, β-CN) were overlapped by κ-CN. In fact in the 2-DE map (PAGE→UTLIEF) analysis of an individual donkey casein, the most heterogeneous casein fraction was κ-CN, since at least 11 components were specifically immunostained in the 2-DE map, focalising throughout the entire working pH range (Fig. 3.21A-B).

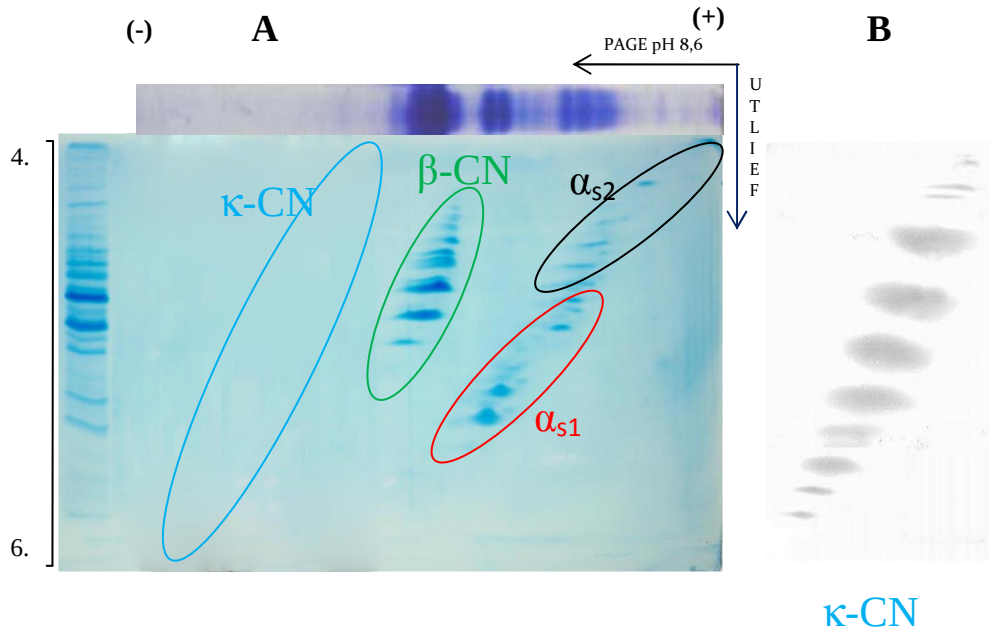

Fig 3.21: 2-DE (PAGE→UTLIEF) analysis of representative donkey casein sample after Coomassie Brilliant Blue staining (A) and immunospecific staining with polyclonal antibody against κ-CN (B).

3.3.21 Characterization of donkey caseins by PAGE-SDS and Immunoblotting analysis with fluorescent glycoprotein detection

An individual sample of donkey CN, before and after chymosin hydrolysis, was analyzed by PAGE-SDS and was shown in Fig. 3.22A after Blue Coomassie staining; with the aim to identify each donkey casein fraction it was immunostained using specific polyclonal antibodies against each donkey casein fraction (α_{s1}-, α_{s2}-, β-, and κ-CN) (Fig. 3.22B). The electrophoretic profile obtained for donkey casein fraction is shown to be very similar to that reported by Miranda et al. (2004) for mare and goat caseins. In fact, in the casein zone, the heaviest electrophoretic band was that corresponding to α_{s2}-CN, followed by α_{s1}-CN. For the latter, two main bands were separated, which probably could correspond to its two deleted forms resulting from the skipping of exon 7 and 5; instead β-CN has migrated into the intermediate zone between these two α_{s1}-CN components. The results of this immuno-electrophoretic technique also showed the fastest mobility of κ-CN towards the anode compared to the other casein fractions, suggesting, therefore, its lower molecular weight between asinine caseins. In addition, κ-CN migrated as a more or less extensive smear because of considerable variation in the level of post-translational glycosylation. This electrophoretic behaviour had previously been highlighted by Miranda et al. (2004) with respect to mare's milk.

The presence of κ-CN also in whey protein used as reference and corresponding to the casein zone, suggested that this smear would include highly glycosylated κ-CN forms, which could be either the heaviest and less abundant forms.

Moreover, this immuno-electrophoretic technique showed that β- and α_{s1}-CN are the most abundant casein fractions followed by α_{s2}- and κ-CN, and this feature makes donkey milk more similar to human milk, in which α_{s2}-CN has not yet been identified. Also peptides derived essentially from β- and α_{s1}-CN hydrolysis by endogenous enzymes of donkey milk were showed in the immunostained profiles.

The results obtained by this immuno-electrophoretic technique were also confirmed by Fluorescent Glycoprotein Detection (Fig 3.22C). In fact, the κ-CN band was more fluorescent than the other casein bands with a major molecular weight, thus suggesting its high glycosylation degree. It should be mentioned that the migration zone of donkey κ-CN spanned all the other casein bands.

Donkey κ-CN seems to be more glycosylated (about 12 glycosides) than bovine κ-CN which has six glycosylated Thr residues and considering the preferential peptide bond of donkey κ-CN susceptible to chymosin hydrolysis, it could probably be Phe^{97}-Ile^{98} and not Phe^{105}-Met^{106} bond (which would reflect differences in the mechanism of clotting of milk in ruminant and non ruminant mammals). In this way, donkey κ-CN, showing more similarities to equine and human κ-CN, compared to bovine κ-CN may belong to the κ-CN group II.

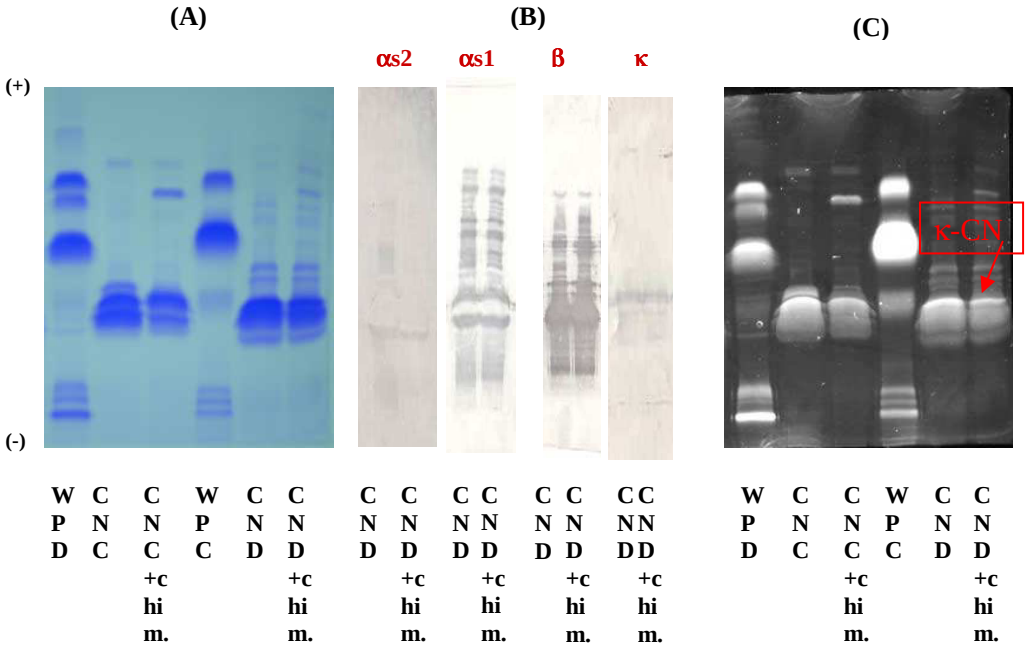

Fig 3.22: Characterization of donkey casein, before and after chymosin hydrolysis, by PAGE-SDS analysis (A) and immunoblotting with specific polyclonal antibodies against each casein fraction (B) with fluorescent glycoprotein detection (C).
WPD: whey protein donkey; **CNC:** casein cow; **CNC+chim.:** casein cow + chymosin; **CND+chim.:** casein donkey + chymosin.

Conclusions 1

The proteomic approach allowed the identification of the four casein fractions (α_{s1}-, α_{s2}-, β- e κ-CN) in donkey milk together with their related heterogeneity due to post-translational phenomena such as the different phosphorylation degree of the caseins (α_{s1}-, α_{s2}-, β-CN) and the high glycosylation level of κ-CN, incorrect splicing of primary transcript in mRNA (non allelic deleted forms of α_{s1}-, α_{s2}-, β-CN) and genetic polymorphism of α_{s1}- and β-CN (one or more variants in individual samples).
Specifically

- α_{s1}-CN was composed at least of three protein components, characterized by a different length of their amino acid sequence (210, 202, 197 aa) and also expressed at different intensity (202>197>210), where the minor component with 210 aa was found for the first time. This means, that unlike α_{s1}-CN mare, all exons are expressed in donkey α_{s1}-CN gene. The incorrect splicing of the primary transcript (210) determining the single and/or contemporary elimination of exons 7 and 5 gave rise to the two deleted components with 202 and 197 aa, respectively. Each of these three components (210, 202, 197) showed a microheterogeneity due both to the different phosphorylation degree (5, 6 e 7P) that the glutamine residue deletion. The screening carried out on the samples by RP-HPLC and MS analysis also revealed a qualitative polymorphism at α_{s1}-CN *locus* for the finding of a new α_{s1}-CN variant, in addition to the protein already known in literature (Cunsolo et al., 2009a), but also a quantitative polymorphism at this *locus* (paragraph 3.13.3), for the detection of samples characterized by the presence of α_{s1}-CN only in traces, as in goat milk.
- β-CN is always present in donkey milk as in human milk (paragraph 3.13.3). The immuno-electrophoretic and chromatographic analysis of analyzed samples showed three new genetic variants at this *locus*, each 226 aa long, which we named B, C, e D, while the mass spectrometry allowed to identify the characterizing amino acid substitutions. The structural study has indicated for each new variant the same phosphorylation degree of β-CN A reference (Cunsolo et al., 2009b), indicating that the involved mutations do not affect serine residues in the delegated code sequences. Each new variant, therefore, was heterogeneous for the presence of three phosphorylated components (5, 6, 7P) and for the contemporary presence of a non allelic deleted form lacking the peptide $E^{27}SITHINK^{34}$, encoded by exon 5, always with the same three phosphorylation degrees.
- α_{s2}-CN was identified and characterized in donkey milk by Chianese et al. (2010). Its heterogeneity is due to its high phosphorylation degree (10, 11, 12 P) and to the presence of deleted forms (Cosenza et al., 2010; Saletti et al., 2012). The electrophoretic and chromatographic analysis also suggested the existence of a possible quali-quantitative polymorphism at α_{s2}-CN *locus* (paragraph 3.13.3). The absence of α_{s2}-CN in some samples of donkey milk, as in the human milk, together with a lower expression level of α_{s1}-CN and β-Lg, confirm the compositional similarity of two milks and represent a scientific basis for donkey milk's use in nutrition of infants with CMP allergy.
- To date, for κ-CN only partial data on its cDNA sequence exist. We have experimental evidences of κ-CN presence in DM only after specific immunostaining, likely depending either on the low amount of this fraction in the milk or on its lower reactivity to Blue Coomassie staining. As in ruminants, κ-CN is the most heterogeneous casein fraction and probably this phenomenon is generated from its high glycosylation degree. In fact the fluorescent glycoproteins detection showed that κ-CN is not only the casein with the lowest molecular weight but also the most fluorescent, being a highly glycosylated protein. For this reason, it could have more similarity with human and equine κ-CN than other species. Finally, the immunoelectrophoretic profiles also showed the occurrence of a possible genetic polymorphism at this *locus* and the non-susceptibility of κ-CN to chymosin hydrolysis compared to α_{s1}- and α_{s2}-CN is due to the non para-κ-CN formation. This result is in agreement with cDNA κ-CN sequence which would lack of the specific site for chymosin action.

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3.6 Topic/theme 2: characterization of donkey milk whey proteins

At present, while the investigation of casein components is still at a relatively early stage of progress, more scientific data are available on donkey whey proteins consisting of α-La, β-Lg, serum albumin, lactoferrin and lysozyme as in ruminants and horse milk. The particularity of donkey milk is to contain two β-Lgs (β-Lg I and β-Lg II) because the donkey has two genes which encodes for this protein, absent in human milk and therefore, considered the principal milk allergen in neonates and children. This study carried out on donkey whey proteins of animals reared in Italy, is aimed to the characterization of their molecular composition (primary structure, disulphide bridges) and also to identification of possible new genetic variants, as for β-Lg II which, according to data reported in literature, showed a greater heterogeneity compared the other whey components. The heterogeneity of this protein, (due to its qualitative and quantitative polymorphism) could influence the milk composition and its allergenic properties, while the positive presence of minor whey proteins, lysozyme and lactoferrin, may influence nutritional, functional and biological properties of donkey milk.

3.7 Materials and methods 2

All chemicals were of the highest purity commercially available and were used without further purification. Tetra-methyl-ethylene-diamine (TEMED), 2-mercaptoethanol, ammonium persulphate, glycine, glycerol, TRIS (hidroxymethyl-amino methane), SDS (sodium dodecyl sulfate), acetic acid, methanol, urea, ammonium bicarbonate (AMBIC), HPLC grade H2O, formic acid (FA) and CH3CN were purchased from Carlo Erba (Milan, Italy). Trichloroacetic acid (TCA), Trifluoroacetic acid (TFA), sinapinic acid (SA), α-cyano-4-hydroxycinnamic acid (CHCA), iodoacetamide (IA), dithiothreitol (DTT) were obtained from Aldrich (St. Louis, MO, USA). Modified trypsin, sequencing grade was from Promega (Madison, WI, USA), alkaline phosphatase (AP) was from Roche (Mannheim, Germany). Dialysis tubing with molecular weight cut off (MWCO) 12–14000 Da was from Medicell International Ltd (London, UK). Amicon® Ultra centrifugal filters 3.000 MWCO and ZipTip™ C18 micro columns were obtained from Millipore (Bedford, MA, USA). Acrylamide, bis-acrylamide, Ampholine buffers were from GE Healthcare Amersham Biosciences (Buckinghamshire, UK). Coomaassie Brilliant Blue (CBB) R250 and G250 were purchased from Bio-Rad (Richmond, CA, USA).

3.7.1 Donkey milk sampling (sample whey protein preparation)

Individual donkey milk samples (77) were collected from two breeds reared in Italy (Sicilia and Abruzzo) and the whey proteins fraction was isolated by acidification at pH 4.6 of defatted milk (Aschaffenburg & Drewry, 1959). The supernatant, containing the whey proteins, was recovered by centrifugation (4000 rpm for 30 min) at room temperature and subsequently dialysed in tubing with molecular cut off 12000-14000 Da, individual whey protein samples were freeze-dried and stored at −20 °C before use.

3.7.2 Ultra-thin layer isoelectric focusing UTLIEF (on polyacrylamide gels) analysis

Sample Preparation: lyophilized whey protein samples (20 g/L) for electrophoretic analysis were dissolved in 9M urea solution, containing 2-mercaptoetanol (1 ml/L), respectively. 10 µl of these solutions were loaded on the gel.
UTLIEF analysis: Ultra-thin layer isoelectric focusing (UTLIEF) on polyacrylamide gels (124×258×0,25 mm) was carried out according to the procedure of Chianese et al. (2010). Briefly, the polyacrylamide gel in a 4.2-9.0 pH gradient was obtained mixing 1% (v/v) Ampholine buffers: 4.2–4.9 (350 µL), 4.5–5.4 (350 µL), 5.0–7.0 (150 µL), 7.0–9.0 (150 µL). UTLIEF analysis was performed with LKB Multiphore II instrument (Pharmacia LKB, Bromma, Sweeden) at 10°C and the electrophoretic analysis consists of three steps: pre-focusing (2000 V, 15 mA, 4 W, 30 min), focusing with the sample (2000 V, 15 mA, 4 W, 60 min), final focusing (3000 V, 5 mA, 20 W, 130 min). After the electrophoretic run, the fixation of the protein bands on the gel was carried out by gel's immersion in 20% (v/v) trichloroacetic acid solution. The gel was stained with CBB G-250 as described by Krause et al. (1988) and destained with two solutions of the same volume: aqueous solution containing 20% (v/v) acetic acid and aqueous solution containing 50% (v/v) methanol. This technique allows to separate whey proteins according to their isoelectric point values.

3.7.3 Immunoblotting analysis

This analysis is based on cross-reaction between specific polyclonal antibodies produced against the main whey proteins α-La and β-Lg and their respective antigens. Therefore, this technique allows to identify the

main whey proteins according to the specificity shown by specific polyclonal antibodies produced against these proteins after immunization of rabbits with the individual whey proteins α-La and β-Lg.
The reagents for immunoblotting were: PBS buffer (4.5% NaCl, 3.9% NaH_2PO_4, pH 7), wash buffer solution and saturating the membrane "blocking solution" (equine serum at 10% v/v in PBS), development buffer (0.5 mg/ml diaminobenzidine in 10 mM Tris-HCl 0.1 M pH 7.5, 0.6 mg/ml $NiCl_2$ 1%).
For immunoblotting analysis, the main whey proteins separated by UTLIEF analysis were transferred by capillary diffusion from the gel into a nitrocellulose membrane (0.45 μm, Trans-Blot, Bio-Rad, Richmond, CA, USA) and immunostained using polyclonal antibodies against bovine α-La and β-Lg as primary antibodies. These were prepared according to the following procedure: bovine α-La and β-Lg, fractionated by RP-HPLC, were used as antigen by Primm (Milan, Italy) to prepare rabbit polyclonal antisera against bovine α-La and β-Lg. Finally, the antisera were filtered using 0.45 mm filters (Millipore, Bedford, MA), divided into aliquots of 1 mL and stored at −20°C.
The immunoblotting analysis was conducted according to the procedure described in 3.2.5 paragraph for the recognition of the casein components in donkey milk.

3.7.4 Reversed-phase high performance liquid chromatography (RP-HPLC) analysis

The HPLC system was provided by Kontron Instrument (Milan, Italy) and is constituted by two pumps model 420, an injector of the sample (loop 50 μL) and a DATA SYSTEM 450 for the chromatography's management and for the integration of the peak areas of the chromatograms.
The whey protein samples were fractionated by RP-HPLC on a 214TP54 5μm Vydac C_4, 250 mm × 4.6 mm internal diameter column (Vydac, Hesperia, CA, USA) and the detection was at a wavelength of 220 nm with a UV detector Kontron (Mod. 430). The solvent used were: solvent A and solvent B. Solvent A was 0.1% (v/v) TFA in ultra pure water and solvent B 0.1% (v/v) TFA in acetonitrile. 50 μL of a solution containing 10 mg (whey protein sample)/ mL (solvent A) were loaded into a C4 column, equilibrated with solvent A. The elution program involved an isocratic step with 30% solvent B for 5 min and a linear gradient from 30 to 50% solvent B in 45 min, then from 50% to 100% solvent B in 2 min, with a flow rate of 1 $mLmin^{-1}$. Each eluted whey protein fraction was manually collected, freeze-dried and stored at −20°C.
This technique allows to separate proteins according to their hydrophobicity.

3.7.5 LC-ESI-MS analysis

The molecular mass determination of the main whey proteins (donkey α-La, β-Lg I and β-Lg II) and of the minor whey proteins with biological activity (donkey lysozyme, serum albumin and lactoferrin) was carried out using a single quadrupole instrument LC-ESI-MS. Also the screening on individual whey samples to discriminate β-Lg II A from B variant, otherwise comigrating in UTLIEF analysis, was carried out using LC-ESI-MS instrument.
Specifically, liquid chromatography/electrospray mass spectrometry (LC-ESI-MS) was performed using an HPLC modular system (HP1100-MSD, Agilent Technologies, Santa Clara, CA, USA) with UV detector, interfaced with a single quadrupole mass spectrometer equipped with a electrospray source (ESI) and monitored by HP Chem Station software for the acquisition, analysis and processing of the mass spectra.
The whey protein samples were fractionated by RP-HPLC on a 214TP54 5μm Vydac C_4, 250 mm × 2.1 mm internal diameter column (Vydac, Hesperia, CA, USA) and the UV detection was at a wavelength of 220 nm. Solvent A was 0.1% (v/v) TFA in ultra pure water and solvent B 0.1% (v/v) TFA in acetonitrile. 100 μL of a solution containing 5 mg (casein sample)/ mL (solvent A) were loaded into a C_4 column, equilibrated with solvent A. The elution program involved a gradient from 37 to 50% solvent B in 30 min, at a flow rate of 0.2 $mLmin^{-1}$.
The ESI mass spectra were scanned in the positive ion mode, from 800-2400 *m/z* at a scan cycle of 5 sec/scan. The source temperature was 120°C and the orifice voltage was 40. Mass values are reported as average masses. Signals recorded in the mass spectra were associated with the corresponding whey proteins on the basis of the molecular mass, taking into the account the reported amino acid sequences of mare and donkey whey proteins available on database Swiss-prot (ExPASy proteomics server).

3.7.6 β-Lg alkylation and trypsin enzymatic digestion

The digestion of the whey proteins from electrophoretic spots was carried out following the procedure reported by Mamone et al. (2003) in paragraph 3.2.9. As regards the tryptic digestion of the isolated β-Lg II, collected by RP-HPLC, it was dried using a Savant concentrator (Speed-Vac, Milan, Italy), lyophilized, denatured and alkylated before trypsin hydrolysis. Briefly, each isolated whey protein fraction (for example donkey β-Lg II) was denatured at room temperature, in a guanidine buffer (6M guanidine in 0.5 M Tris-HCl

with 1 mM EDTA) at pH 8. Subsequently, disulfide bridges were reduced by of 10 mM/L DTT for 60 min at 56°C. SH groups were subsequently alkylated with 55 mM/L IA in the above guanidine buffer, and maintained for 30 min in the dark. With the aim to remove the reagents, the alkylated whey protein was ultra-filtered on Amicon® Ultra filters 3000 MWCO, centrifugated at 4000 g for 25 min and washed with 50 mM NH_4HCO_3, pH 7.4. The retentate solution containing the purified alkylated whey protein (as β-Lg II), was added of trypsin at 1:50 (w/w) and incubated overnight (approximately 14 h) at 37°C. The digestion was stopped by frozen (–20°C).

3.7.7 Matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS) analysis

MALDI-TOF-MS experiments were carried out on a Voyager DE-PRO mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a N_2 laser (337 nm, 3 ns pulse width) operating in linear or in reflector positive ion mode, using the Delay Extraction technology. Mass spectra were acquired both in positive linear or in reflectron mode and using SA and CHCA dissolved in aqueous solution of 50% (v/v) CAN containing 0.1% (v/v) TFA, at a concentration of 10 mg/mL, as matrices for the analysis of proteins and peptides, respectively. MALDI-TOF-MS analysis of intact proteins were obtained in linear positive ion mode over the *m/z* range 10000-30000 and were averaged from about 150 laser shots.
As regards peptides analysis, the mixtures of tryptic peptides were subjected to desalting/concentration step with Zip-Tip C_{18} pre-packed micro-columns (Millipore, Bedford, MA, USA) previously equilibrated with aqueous 0.1% TFA (v/v) prior to analysis by MALDI-TOF. Spectra were obtained in reflectron positive ion mode over an *m/z* range 600-4000 and were averaged from about 150 laser shots. External calibration was performed by acquiring separate spectra of a mixture of standard peptides (PerSeptive Biosystems).
The identification of donkey whey proteins and their derived peptides was carried out using the known whey proteins available on Swiss-prot database (ExPASy proteomic server) of Swiss Institute of Bioinformatics (SIB) and with the online software FINDPEPT (website: http://www.expasy.org/tools/findpept.html).

3.7.8 Electrospray quadrupole-time of flight-mass spectrometry (ESI-Q-TOF-MS/MS) analysis

Tandem MS (MS/MS) data were obtained using a hybrid quadrupole-orthogonal acceleration time of flight Q-STAR instrument (Applied Biosystem, Foster City, CA) equipped with a nanospray source (Protana, Odense, Denmark), operating in positive ion mode. Dried samples were resuspended in 0.1% TFA, purified from residual salts by loading into ZipTip C_{18} Reversed Phase pre-packed micro-columns (Millipore Bedford MA, USA), and introduced in the source through borosilicate needles, gold coated (Protana Odense, Denmark). The capillary voltage used was 800 V. Double-charged ion isotopic cluster were selected by using the quadrupole mass filter and the induced to fragment by collision. The collision energy was 20 to 40 eV, depending on the size of the peptide. The collision-induced dissociation was processed by using Analyst 5 software (Appied Biosystems). The deconvoluted MS/MS spectrum was manually interpreted with the help of Analyst 5 software.

3.8 Results and discussion 2

3.8.1 Characterization of donkey whey proteins by UTLIEF and immunoblotting analysis

The most representative UTLIEF profiles of individual donkey whey proteins were shown in Fig. 3.23; after Comassie Brilliant Blue staining (Fig. 3.23A), the main whey proteins α-La and β-Lg, were identified according to known theoretical pI value and after immunoblotting with α-La and β-Lg specific polyclonal antibodies (B). With the exception of lysozyme which focused at alkaline zone of pH gradient, on the basis of the increasing pI value β-Lg II, β-Lg I and α-La focalized in A, B and C (acid) zone of UTLIEF pH gradient, respectively. The composition of each electrophoretic zone was heterogeneous for the presence of almost two components recognised by each specific polyclonal antibody. This was the case of B and C zones where β-Lg I and α-La were "excorted" by a and b satellite bands, respectively. While, both α-La and β-Lg I presented the same pI value in all analyzed samples, four different β-Lg II phenotypes were discriminated on the basis of their pI value. In particular, these latter were numbered in order of decreasing pI value (bands 1, 2, 3, 4) and occurred in homozygous (lanes 4, 5 and 6) and heterozygous form (lanes 1, 2, 3 and 8) (Fig. 3.23).
Taking into account the known theoretical pI values (Table 1.14), A and B genetic variants of β-Lg II focalized in band 2 (pI=4.70), β-Lg II C in band 1 (pI=4.72) and β-Lg II D (pI=4.64) in band 4. Since, to date, none genetic variant having a pI value intermediate between B and D β-Lg II was reported, the occurrence of a new variant E was supposed in band 3. Moreover, a defective coding allele for β-Lg II determined the absence of expressed protein in lane 7. A similar milk sample was found in Sicilian (Criscione et al., 2009) and Sardinian (Conti et al., 1989) donkey breeds. In conclusion, from a genetic point

of view, in examined donkey milk samples, α-La and β-Lg I were monomorphic and β-Lg II polymorphic, since known (B, C and D), new (E) variants and a defective quantitative allele occurred at this *locus*.

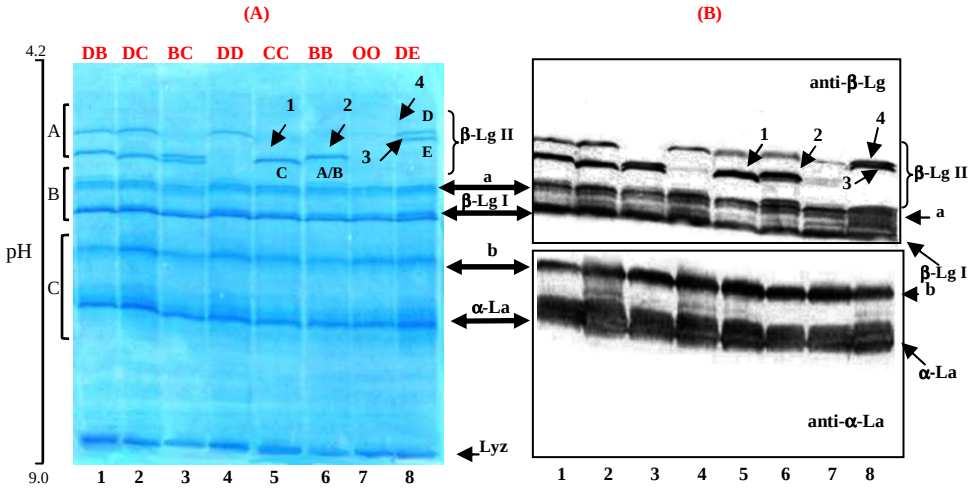

Fig 3.23: UTLIEF in pH gradient 4.2-9.0 of individual whey protein samples containing representative donkey phenotypes detected by double staining with Coomassie Brilliant Blue (A) and polyclonal antibodies against α-La and β-Lg (B).

3.8.2 Identification of donkey whey proteins, as β-Lg II genetic variants, by RP-HPLC coupled to LC/MS analysis

The screening, carried out by RP-HPLC analysis, on individual donkey whey protein samples, allowed to select the a and b representative chromatograms (Fig. 3.24). Each chromatogram consisted of five peaks. By LC/MS analysis, Lyz, SA/Lf, α-La, β-Lg II and β-Lg I, in increasing order of elution time, were identified in each of these five chromatographic peaks (Table 3.5).
In donkey, two variants of lysozyme, named A (Godovac-Zimmermann et al., 1988b) and B (Herrouin et al., 2000) were reported, whereas the sequences of minor components of whey fraction, as serum albumin and lactoferrin were only deduced from the corresponding cDNA sequences in donkey (Li et al., 2004) and mare (Sharma et al., 1999), respectively. No information is available for the primary structure of donkey lactoferrin. At this regard the identification of some antimicrobial proteins in donkey milk samples, such as lysozyme B and lactoferrin, could be responsible for the low microbial load found in this milk and may be useful to prevent intestine infections in infants.
However, comparing a and b chromatographic profiles (Fig. 3.24), a different quantitative expression of β-Lgs and α-La can be observed; in particular, β-Lg II was lacking in b, while a different quantitative ratio α-La/β-Lg I was assessed in each profile (a=1 and b=2, approximately).
The use of LC/MS allowed to identify the four β-Lg II variants (B, C, D and E), otherwise co-eluting in the same chromatographic peak 4 or co-focalising at the same pI value of UTLIEF pH gradient, as β-Lg II A and B (Table 3.5).
These results indicated β-Lg II B as the most common phenotype at donkey β-Lg II locus compared to β-Lg II A and confirmed the monomorphism of α-La and β-Lg I due to the occurrence of A and B variants, respectively. We found a more complex polymorphism of β-Lg II for the occurrence of known (B, C and D) new (E) variants and null allele at this *locus*.
Furthermore, the HPLC analysis of whey samples containing B, C and D β-Lg II variants (Fig. 3.25) showed a different peak area in the following decreasing order: (BB) < (CC) < (DD); therefore these genetic variants could be expressed by quantitative alleles (strong, intermediate, weak and null). It was well known that a similar quali-quantitative polymorphism was found at goat α_{s1}-CN *locus* and the related scientific studies reported in numerous research papers (Martin et al., 2002; Caroli et al., 2006; Marletta et al., 2007).

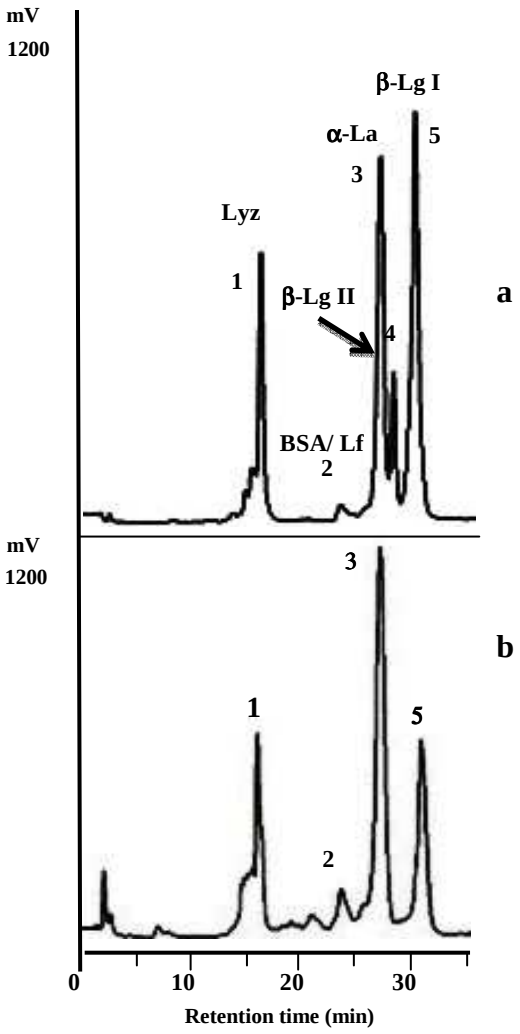

Fig 3.24: RP-HPLC profiles of representative donkey whey protein samples (profiles a and b) containing a different quantitative level of β-Lgs and α-La.

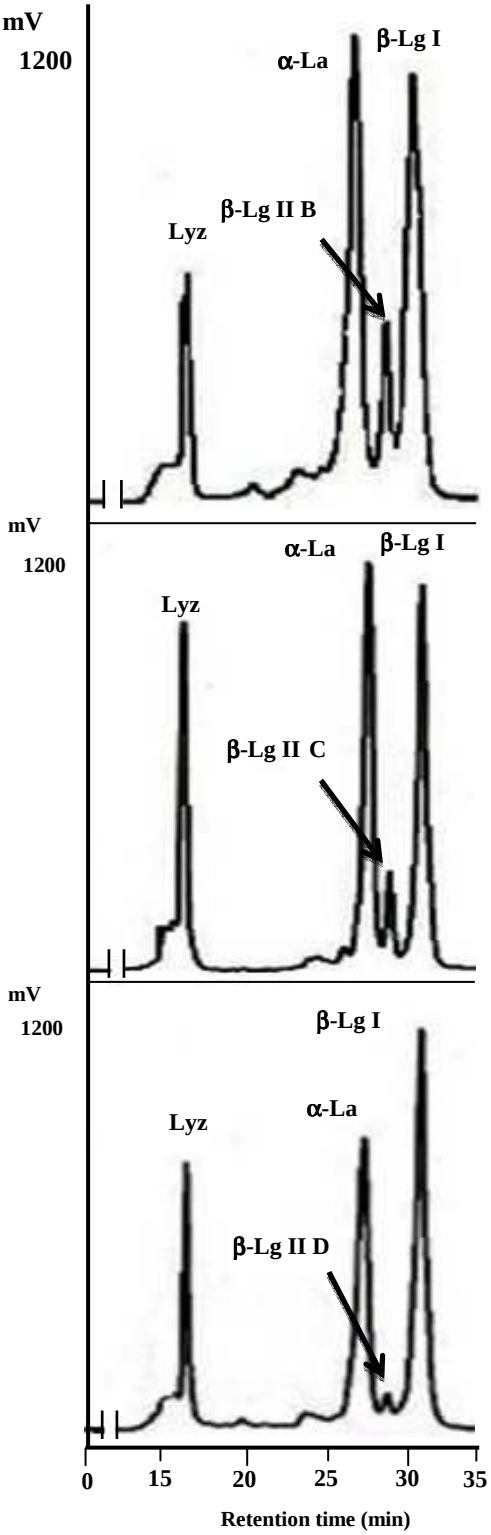

Fig: 3.25: RP-HPLC profiles of individual whey protein from donkey milk samples containing β-Lg II B, C, D variants.

Table 3.5: Identified donkey whey proteins by LC/MS analysis.

Retentetion time (min)	Peak	Molecular Mass (Da)		Protein identification
		Measured	Theoretical	
17,6	1	14.626	14.631	Lysozyme B
24,4	2	65.601	65.597	Serum Albumin
		75.536	75.420[a]	Lactoferrin
27,5	3	14.210	14.214	α-La A variant
28,6	4	18.221, 18.239, 18.314, 18.256[b]	18.227, 18.241, 18.311	β-Lg II B, C, D, E[b] variants
30,3	5	18.505	18510	β-Lg I B

[a] Horse Molecular Mass
[b] present research

3.8.3 Characterization of the new β-Lg II E variant by MALDI/TOF/MS analysis and ESI-Q-TOF-MS/MS analysis

To characterize the new β-Lg II E variant, the whey proteins of sample containing the variant, were separated by RP-HPLC (Fig. 3.26) and the eluted peak 4 manually collected, was submitted to LC/MS analysis.

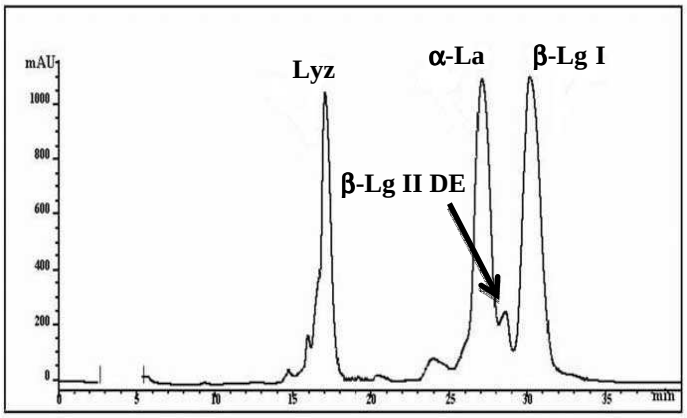

Fig 3.26: RP-HPLC profile of donkey whey sample containing D and E β-Lg II variants.

Two components at M_r 18314 and M_r 18256 Da, were determined in the peak sample. Of these, the first corresponded to expected β-Lg II D variant (Table 3.5) while the other one was assigned to the new genetic β-Lg II E variant. To identify the amino acid substitutions, β-Lg II D and E variants (co-eluted in peak 4) were digested by trypsin before MALDI TOF analysis. The obtained mass spectra (Fig. 3.27) gave rise to peptides with measured masses reported in Table 3.6; as expected, all signals were assigned to D variant except two new T_1 (E) and T_2 (E) peptides at M_r 2001.2 Da and 2356.8 Da, displayed in insets a and b. These peptides presented a molecular weight lower of 29 and 28 Da than T_1 (D) and T_2 (D) respectively. These achieved data were in close agreement with the 57 Da molecular mass difference measured between β-Lg II D and β-Lg II E.
The peptides T_1 (E) and T_2 (E) were submitted to ESI-Q-TOF-MS/MS analysis for sequencing and thus, determine the amino acid substitutions between them (Fig. 3.28): the peptide T_1 (E) corresponded to the sequence 1-18 with two amino acid substitutions $D^2 \rightarrow N^2$ and the C-terminal $R^{18} \rightarrow K^{18}$, while the peptide T_2 (E) corresponded to the sequence 19-40 and exchanged respected to peptide T_2 (D) for amino acid substitution $V^{25} \rightarrow A^{25}$. Overall, the β-Lg II E amino acid substitutions experimentally observed in this work were all confined in the N-terminal area of the protein sequence that was known to be conserved among the other β-Lg II genetic variants.

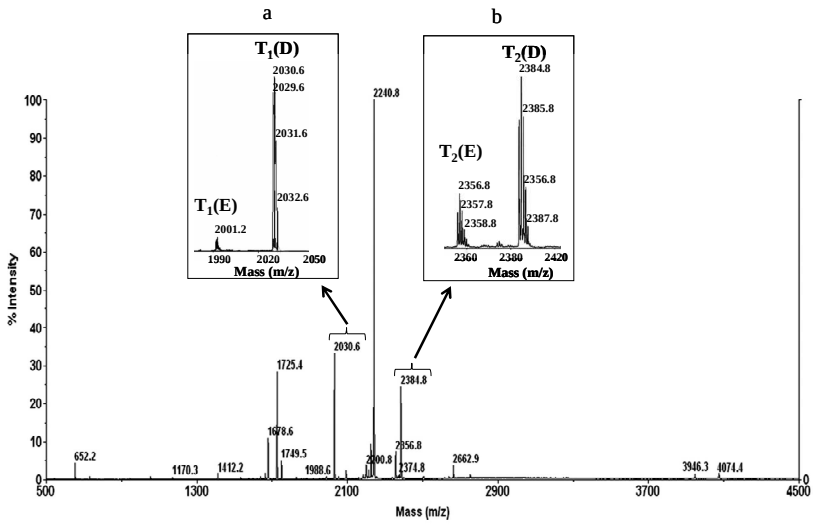

Fig 3.27: MALDI-TOF mass spectrum of tryptic digest of β-Lg II DE. Inset in panel a: peptide 1-18 T1(D) and T1 (E) in m/z range 1990-2050. Inset in panel b: peptides 19-40, T2(D) and T2(E) recorded in mass range 2360-2420.

Table 3.6: MALDI-TOF MS identification of RP-HPLC peak 4 eluted at 28.6 min (Fig 3 o 4) digested with trypsin.

Fragment	Position	Molecular Mass, Da		Peptide Sequences	β-Lg II variants
		Theoretical	Measured		
T1	1-18	2029.9	2030.6	T**D**IPQTMQLDDLQEVQG**R**	D
T1	1-18	2000.2	2001.2 (–29Da)	T**N**IPQTMQLDDLQEVQG**K**	E
T2	19-40	2384.2	2384.8	WHSVAM**V**ASDISLLDSESAPLR	D
T2	19-40	2355.8	2356.8 (–28Da)	WHSVAM**A**ASDISLLDSESAPLR	E
T3	41-59	2240.2	2240.8	VYVEELRPTPEGNLEIILR	D, E
T4	60-69	1112.5		EGANHVCVER	D, E
T5	70-75	671.4		NIVAQK	D, E
T6	76-90	1724.8	1725.4	TEDPAVFTVNYQGER	D, E
T8	92-125	3831.8	3945.7[a]	ISVLDTDYAHYMFFCVGPPLPSAEHGMV	D, E
T6+T8	91-125	3959.8	4091.6[a]	KISVLDTDYAHYMFFCVGPPLPSAEHGMV	D, E
T10	129-136	977.4		VDEEVMEK	D, E
T10+T11	129-139	1368.5		VDEEVMEKFSR	D, E
T12	140-160	2240.2		ALQPLPGHVQIIQDPSGGQER	D, E

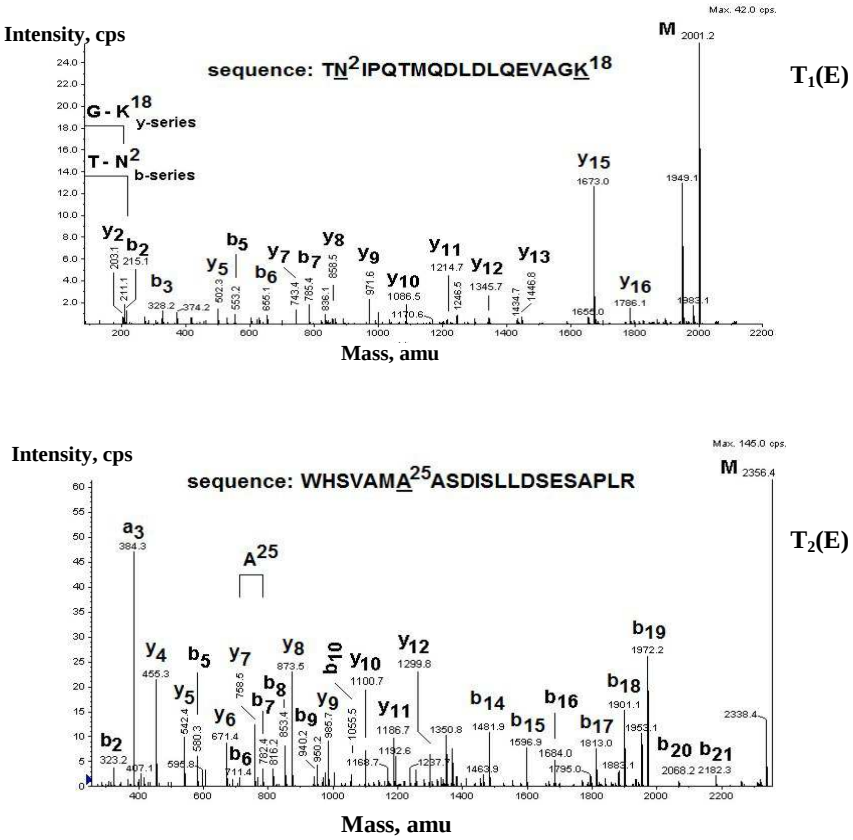

Fig 3.28: ESI-Q-TOF-MS/MS fragmentation of T_1 (1-18) and T_2 (19-40) tryptic peptides of β-Lg II E variant.

3.9 Conclusions 2

The proteomic characterization of the whey fraction in individual donkey milk samples allowed, together with the identification of already known whey proteins such as lysozyme B, α-La A and β-Lg I B, the detection of minor components (serum albumin and lactoferrin), whose sequences were only deduced from corresponding cDNA sequences in donkey and mare, respectively.

The application of proteomic methodology to the donkey whey proteins has also led to the identification of a novel β-Lg II genetic variant, which we named E, in addition to already known β-Lg II variants (A, B, C, D) in literature. The presence of these different molecular forms of β-Lg II explains its greater heterogeneity compared to other donkey whey proteins, as the monomorphic α-La A and β-Lg I B.

Three amino acid substitutions characterized β-Lg II E variant in comparison with D variant: D β-Lg II $Asp^{2}\rightarrow$ E β-Lg II Asn^{2}, D β-Lg II $Arg^{18}\rightarrow$ E β-Lg II Lys^{18} and D β-Lg II $Val^{25}\rightarrow$ E β-Lg II Ala^{25}. These results indicated a direct phylogenic origin of E from D variants. Moreover, since the percentage area of β-Lg II DE was very close to CD HPLC profile counterpart, it can be hypothesized that β-Lg II E is expressed by an intermediate alleles.

From a nutritional point of view the minor content of β-Lgs in donkey milk in addition to quantitative genetic polymorphism occurred at donkey β-Lg II locus can be related to the tolerance mechanism of donkey milk in the allergic patients to bovine milk.

Thus, the proteomic methodology is a powerful tool to underline the genetic biodiversity inter and/or intra species. In particular, the sensibility of based separative technique as UTLIEF was very high and able to discriminate between β-Lg II D and E variants and between β-Lg II C and B/A variants differing each other for 0.01 pH unit.

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3.11 Topic/theme 3: quantitative characterization of donkey milk proteins (caseins and whey proteins)

Donkey's milk may be considered a valid alternative, for infant nutrition, to powdered milks, soybean milk or other formulas, since its composition in lactose and proteins is very close to human milk. So, recent clinical studies confirm donkey milk feeding as a safe e valid treatment of most complicated cases of multiple food allergies compared other infant formulae. However, information on the donkey milk composition is more limited than that on mare's milk, which has also been studied as an infant food. The aim of the this part of the study is to characterize "donkey milk" product, especially with regard to the amount of protein fractions. In fact, although the mechanism of donkey milk's tolerance has not been fully clarified, it is reasonable to hypothesize that the hypoallergenic properties of donkey's milk can be related to structural and quantitative differences of its protein components with respect to bovine milk.

3.12 Materials and methods 3

All chemicals were of the highest purity commercially available and were used without further purification. Tetra-methyl-ethylene-diamine (TEMED), 2-mercaptoethanol, ammonium persulphate, glycine, glycerol, TRIS (hidroxymethyl-amino methane), SDS (sodium dodecyl sulfate), hydrochloric acid (HCl), sulphuric acid, sodium hydroxide (NaOH), boric acid, acetic acid, methanol, urea, potassium sulphate (K_2SO_4), ammonium bicarbonate (AMBIC), HPLC grade H2O, formic acid (FA) and CH3CN were purchased from Carlo Erba (Milan, Italy). Trichloroacetic acid (TCA), Trifluoroacetic acid (TFA), sinapinic acid (SA), α-cyano-4-hydroxycinnamic acid (CHCA), iodoacetamide (IA), dithiothreitol (DTT) were obtained from Aldrich (St. Louis, MO, USA). Modified trypsin, sequencing grade was from Promega (Madison, WI, USA), alkaline phosphatase (AP) was from Roche (Mannheim, Germany). Dialysis tubing with molecular weight cut off (MWCO) 12–14,000 Da was from Medicell International Ltd (London, UK). Amicon® Ultra centrifugal filters 3.000 MWCO and ZipTip™ C18 micro columns were obtained from Millipore (Bedford, MA, USA). Acrylamide, bis acrylamide, Ampholine buffers were from GE Healthcare Amersham Biosciences (Buckinghamshire, UK). Coomaassie Brilliant Blue R250 and G250 were purchased from Bio-Rad (Richmond, CA, USA).

3.12.1 Quantitative determination of the milk's nitrogen fractions (TN, SN, NPN) by Kjeldahl method

Eight significant donkey milk samples were selected in relation to the identification of quali-quantitative polymorphisms, and used for the determination of the nitrogen substance according to the FIL-IDF (1983) procedure with the Kjeldahl method.
The Kjeldahl method (FIL-IDF, 1993) is an analytical method for determining the nitrogen content of organic and inorganic substances. It includes three step:

- mineralization;
- distillation;
- titration.

The mineralization phase consists in the oxidation of the sample and in the processing of protein nitrogen in inorganic nitrogen with sulphuric acid. In order to accelerate the reaction, K_2SO_4 is added as catalyst which increases the boiling point of sulphuric acid and then the oxidation temperature. This phase lasts several hours and is completed when the solution is clear.
After mineralization, the cooled sample is added distilled water and 40% (w/v) NaOH to release NH_3. For direct injection of water vapor in the sample, the NH_3 is distilled in a vapor stream and transported to condenser, where it condenses together with water vapor. In a flask containing boric acid and a mixed methyl red-green bromocresol indicator (weakly reddish solution), the distillate is collected (blue solution). Finally, the distillate is titrated with 0.1N HCl. The titration is concluded at the turning of the indicator, that is when the indicator becomes colourless.
The % nitrogen is the result of the following ratio:

$$\frac{\text{mL HCl} \times \text{N HCl} \times 1.4007}{\text{sample weight}}$$

The protein content (%) is obtained by multiplying the nitrogen content (%) for a conversion factor, which, for milk proteins is equal to 6.38.

3.12.2 Preparation of the milk's nitrogen fractions (TN, SN, NPN)

The total nitrogen (TN) was determined directly in the milk; the nitrogen content of milk, soluble at pH 4.6 (SN) refers to the nitrogen content obtained after the precipitation of the casein fraction with acetic acid/acetate buffer. After the extraction of the precipitate by filtration, the filtrate contains pH 4.6 soluble nitrogen and was used for analysis according to the procedure described for total nitrogen.

It is possible to calculate the casein nitrogen content as difference between the total nitrogen and the pH 4.6 soluble nitrogen content.
The non protein nitrogen (NPN) refers to nitrogen content obtained after the precipitation of the proteins (caseins and whey proteins) in the sample by the addition of TCA solution to achieve a final concentration of TCA of 12% in the mixture. Extraction of the precipitate by filtration, with the filtrate containing NPN which was used for analysis according to the procedure described for total nitrogen. It is possible to calculate the true protein nitrogen as the difference between the total nitrogen and the non protein nitrogen content.

3.12.3 Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Sample Preparation: donkey milk samples (1 mL), after skimming by centrifugation at 4000 rpm for 30 min, were added of a denaturing solution (0.062 M Tris-HCl, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, pH 6.8) using about 90% of its water in donkey milk. The milk samples, after adding the denaturing solution, were added of 10 µl bromophenol blue, as tracer of electrophoretic run and then heated at 100°C for 5 min. Volumes of 10 µl of this final sample were loaded in the gels.
SDS-PAGE analysis: SDS-PAGE was carried out with a vertical electrophoretic apparatus (Protean II, Bio-Rad, Richmond, CA, USA) according to the procedure described by Laemmli, (1970), by using a 4% polyacrylamide stacking gel in 0.5 M Tris-HCl buffer (pH 6.8) and a 18% or 15% polyacrylamide resolving or running gel in 1.5 M Tris-HCl buffer (pH 8.6) in the presence of 10% SDS. The polyacrylamide gels were obtained by co-polymerizing acrylamide and bis-acrylamide with N,N,N',N'-tetrametiletilenediammina (TEMED) and ammonium persulphate (PER) added, respectively, as activator and catalyst of polimerization reaction. As molecular standard, a low molecular weight standard kit (LMW Calibration kit, Amersham, GE Healthcare, UK) was used. Electrophoresis was performed at constant voltage 200 V (for about 6 h) in the migration buffer containing 0.2M glycine, 0.025 M Tris and 0.1% SDS. The gel staining was performed with 0.1% (w/v) Coomassie Brilliant Blue CBB R-250 dissolved in a fixative solution (50% (v/v) methanol and 7% (v/v) acetic acid), followed by destaining in a aqueous solution containing 30% (v/v) methanol and 10% (v/v) glacial acetic acid.
This technique allows to separate proteins according their molecular weight.

3.12.4 Densitometry

The densitometric analysis of the electrophoretic SDS-PAGE profiles of the individual donkey milk samples were carried out with a scanning laser densitometer (LKB 2202 UltoScan XL Enhanced Laser) equipped with an integration program gel-Scan 2,0 and connected to an IBM computer PC/PS2. Gel image analysis (recognition of the bands, calculation of the molecular weight and amount of each band) were automatically performed by the software. The densitometry is based on the measurement of the transmitted light when a laser beam is made to pass through the gel allowing a quantitative evaluation of individual bands. The intensity of transmitted light when a laser beam is passed through the gel is inversely proportional to the amount of protein present in the band. The intensity of the bands is expressed in the form of peaks in "densitogramma", where the optical density detected by the instrument, for each single spot, is expressed as a percentage of the optical density of all spots corresponding to the different stained fractions to Coomassie Brilliant Blue. The densitometry should be used with caution, since the range of protein concentrations in which there is a linear relationship between transmitted light and concentration is very restricted and in addition, the proteins are often stained in a different way.

3.12.5 Quantitative analysis of donkey caseins and whey proteins by RP-HPLC

After the fractionation of donkey caseins and whey proteins by RP-HPLC, a quantitative analysis of components was performed by integration of the peak areas corresponding to each individual protein fraction.

3.13 Results and discussion 3

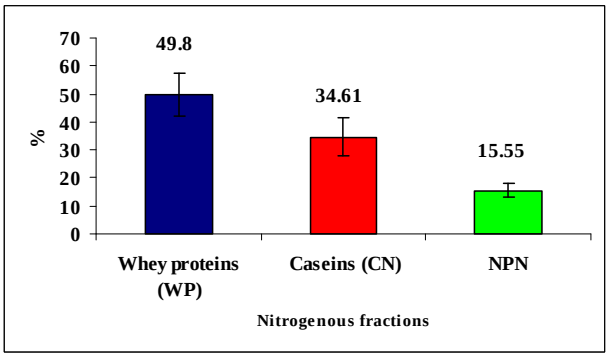
3.13.1 Quantitative determination of donkey milk proteins by Kjeldahl method

In eight individual donkey milks analyzed, the average protein content, consistent with data reported by Salimei et al. (2004) and Guo et al. (2007), was 1.48% ± 0.2, varying from a minimum (1.10%) to maximum value (1.81%) (Table 3.7-Fig. 3.29).
The reasons for this variability have aroused the interest in studying the quantitative distribution of the individual protein fractions in donkey milk.

The average percentages of casein, whey protein and NPN (contents) were 34.61%, 49.80% and 15.55% (Table 3.7-Fig. 3.29), respectively. These results showed remarkable differences compared with other mammalian milks and were within the range of variability reported in literature on donkey milk (Salimei et al., 2004; Guo et al., 2007), with the exception of measured CN content which was lower than that reported by Guo et al. (2007) for Chinese donkey milk.
The average NPN content is resulted very close to the values for human and mare's milk (Malacarne et al., 2002). The nutritional and biological significance of this milk fraction is still far from being completely understood, but seems to be related to the development of infant (Emmet & Rogers, 1997). In donkey milks analyzed, the whey protein content was more similar to human milk (defined typically as *albuminex*) than cow's milk (defined as a caséinoux milk) (Malacarne et al., 2002), while casein content was intermediate between human and ruminant milk casein (Travia, 1986).
The richness in whey protein content of donkey's milk, as mare's milk, make it more favourable to human nutrition than cow's milk, because of the relatively higher supply of essential amino acids (Hambraeus, 1994).

Table 3.7-Fig. 3.29: the average composition of proteins and nitrogenous fractions in analyzed donkey milk samples.

	Proteins (% NT × 6.38) (g ×100 mL^{-1})	% Whey proteins (WP)	% Caseins (CN)	% NPN
Mean	1.48	49.80	34.61	15.55
S.D.	0.21	7.49	6.89	2.32
Min.	1.10	40.38	21.33	11.71
Max.	1.81	63.33	41.03	18.82



3.13.2 Determination of casein: whey protein (CN:WP) ratio by SDS-PAGE analysis and Densitometry

The evaluation of the CN:WP ratio is important, since a milk with a reduced protein content, but especially with a ratio comparable to human milk (40:60), is less allergenic than cow's milk (80:20), and thus, could be a valid alternative non only for nutrition of infants with CMPA, but also a valid substitute if human milk is unavailable. Although it is not clear what are the major allergens in cow's milk, several studies demonstrated that most children with CMPA synthesize antibodies mainly against α-casein and β-Lg (Bevilacqua et al., 2001; Ametani et al., 2003). Even goat milk with a lower amount of α_{s1}-CN, may be responsible for the soft curd produced in the infant stomach and is less allergenic than cow's milk (Lara-Villoslada et al., 2004). In fact it has been previously demonstrated that goat's milk defective α-CN decreases sensitization to β-Lg, also ensuring its better digestion (Bevilacqua et al., 2001).
The mechanism by which a milk with a lower casein content can be less allergenic is unknown, but could be related to the biochemical interactions between casein and β-Lg. The digestion of β-Lg, a protein known to be resistant to gastric pepsin, might be facilitated by lower casein content, as it is known that caseins and β-Lg are tightly linked into the casein micelles. In this sense, the absence or reduction of α-CN content might inhibit the initiation of the allergenic course to other milk proteins, such as β-Lg (Lara-Villoslada et al., 2005). Moreover, using this scientific evidences, a milk with a minor content of caseins than whey proteins may be particularly suitable for human consumption, since the whey proteins are proteins with high biological and nutritional value and therefore, source of essential amino acids.
In order to enhance the donkey milk as a natural substitute of human milk, the CN:WP ratio was determined in the examined donkey population.

The most representative donkey milk samples, analyzed by SDS-PAGE, were shown in Fig. 3.30, after CBB staining. In the electrophoretic profiles, three areas were separated: A, in which proteins, with a high molecular weight (MW) are migrated, a central area B, in which proteins with an intermediate MW are migrated and a C zone, in which low-MW proteins are migrated.
By recognition of the protein marker bands and by comparing protein migration patterns with those previously published for mare (Miranda et al., 2004) and donkey (Salimei et al., 2004; Guo et al., 2007), it was possible to identify the following proteins Immunoglobulin IG (approximate relative molecular mass M_r 94 KDa), lactoferrin Lf (approximate M_r 75 KDa), serum albumin SA (approximate M_r 60 KDa), casein CN (approximate M_r of 21 to 35 KDa), β-Lg (approximate M_r 18 KDa), lysozyme Lyz (approximate M_r 15 KDa) and α-La (approximate M_r 14 KDa).
The casein fraction, with intermediate MW, has migrated in the central area and is well separated from whey proteins. After all, the whey proteins with high MW (Ig, Lf and SA) are quite distinct from those with low MW (β-Lg, α-La and Lyz), whereas the two β-Lg components (β-Lg I and β-Lg II) do not separate between them.

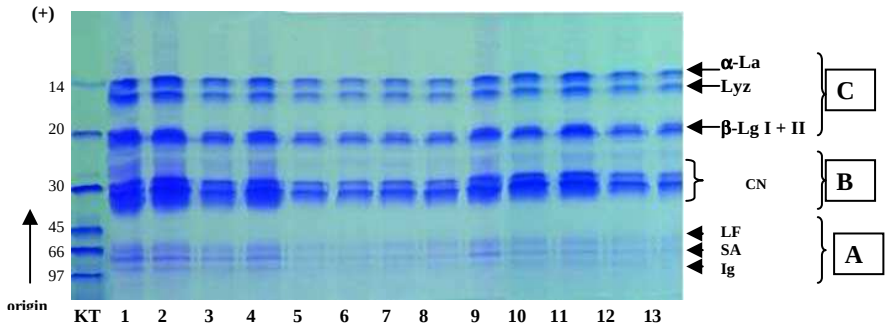
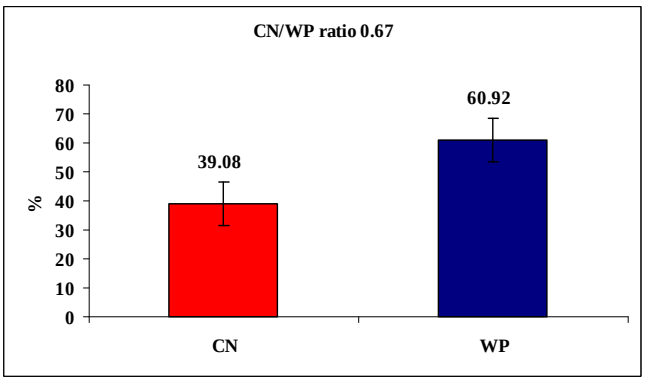


Fig. 3.30: determination of CN/WP ratio by SDS-PAGE analysis of representative donkey milk samples.

Since the casein fraction was separated from the whey proteins with SDS-PAGE analysis, the quantitative determination of these two protein fractions was carried out by densitometry in order to evaluate the CN:WP ratio in donkey milk samples. The data of densitometry (expressed as percentage of total protein) were shown in Table 3.8-Fig.3.31. It showed has shown that the average casein content was 39.08% ± 7.54, varying from a minimum of 23.11% to maximum value of 63.68% resulting, however, a lower value than that of cow's milk. The average whey proteins content was 60.91% ± 7.54 resulting a higher value than that of cow's milk and very close to human milk. It varied from a minimum of 36.32% to maximum value of 76.89%.
In this way, in analyzed donkey population the mean CN:WP ratio value was calculated to be 0.67 ± 0.24, resulting similar to that found in human milk, and specifically, it was intermediate between the lower value of human milk (0.60) and the higher value of cow's milk (4), consistent with the findings reported by Travia (1986). Moreover, this ratio value (0.67) was lower compared to that found in donkeys bred in China (1.4) indicating in the latter a major casein level as reported by Guo et. (2007).
In donkey milks analyzed, the values of CN:WP ratio indicated a high variability in the expression of individual protein fractions; in fact about 19% of analyzed donkey population exhibited a CN:WP ratio ≤ 0.5% and only 7% of the population exhibited a CN:WP ratio > 1. This result could be important for future genetic selection of donkeys producing "hypocaseinic" milk, because the casein and whey protein (as β-Lg) content influences milk's allergenicity.

Table 3.8-Fig. 3.31: determination of CN/WP ratio in examined donkey milk samples.

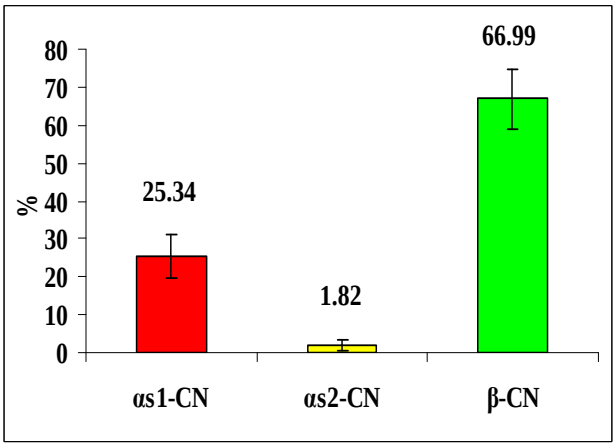


	% CN	% WP	CN/WP
Mean	39.08	60.91	0.67
S.D.	7.54	7.54	0.24
Min.	23.11	36.32	0.3
Max	63.68	76.89	1.75

3.13.3 Determination of expression level of donkey caseins by RP-HPLC

For the quantitative determination of donkey caseins, the individual casein samples were chromatographically separated by RP-HPLC (Fig. 3.32) and identified by mass spectrometry methods. Subsequently, the average expression level of donkey caseins (α_{s1}-, α_{s2}-, β-CN) was determined through the integration of chromatographic peaks. The data (expressed as percentage of total caseins) were shown in Table 3.9-Fig.3.33.

Table 3.9-Fig.3.33: determination of donkey casein content (% of the total caseins) in examined casein samples by RP-HPLC analysis.



	αs1-CN	αs2-CN	β-CN
Mean	25.34	1.82	66.99
S.D.	5.85	1.50	7.93
Min.	3.42	0.02	45.92
Max.	34.56	6.42	84.55

The results showed that the most abundant casein fraction in analyzed samples was β-CN (66.99 % ± 7.93), followed by α_{s1}-CN (25.34% ± 5.85) and α_{s2}-CN (1.82 ± 1.50), highlighting a high individual variability in the expression level of each casein fraction. Specifically, the mean content of β-CN in analyzed donkey caseins was about 66.99% (~ 3.87 mg/mL) of the total caseins, varying from a minimum 45.92 % to maximum value of 84.55%. This result obtained for donkey milk is in good agreement with the β-CN content in human (4.00 mg/mL) milk but is very low compared to mare (11 mg/mL) and cow (10 mg/ mL) β-CN content (Miranda et al., 2004).
The mean content of donkey α_{s1}-CN was about 25.34% (~ 1.5 mg/mL) of the total caseins varying, however, from a minimum of 3.42% to maximum of 34.56% and showing thus, a high variability in the expression level of the asinine protein. In fact, the HPLC analysis of individual caseins showed different peak areas for α_{s1}-CN (Fig. 3.32), in the following decreasing order (Table 3.10-Fig. 3.34): A (32.27%) (~ 2.00 mg/mL), B (24.05%) (~ 1.4 mg/mL) and C (3.42%) (~ 0,2 mg/mL); therefore the donkey α_{s1}-CN could be expressed by quantitative alleles (strong, intermediate, weak and/or null).

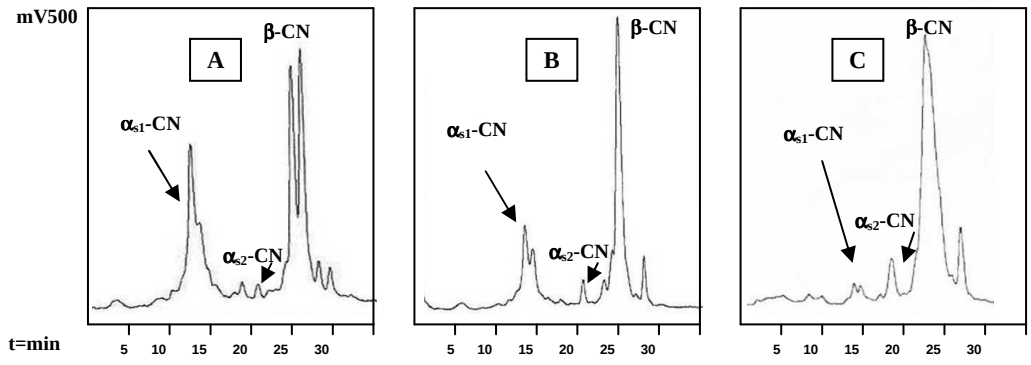
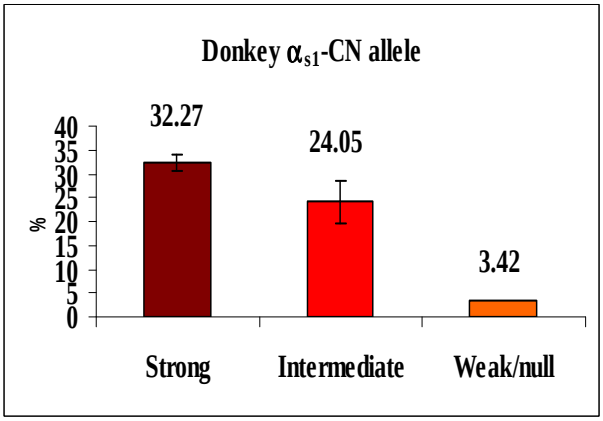


Fig. 3.32: expression level of donkey α_{s1}-CN by RP-HPLC analysis.

These results confirmed the quantitative polymorphism at donkey α_{s1}-CN *locus* and suggested that there are donkeys, as goats, which produce milk with a minor content of this allergenic protein. At this regard, the possible use of donkey milk for patients with CMPA could be encouraged by α_{s1}-CN expression level,

mainly for weak and intermediate phenotypes more similar to human (0.8 mg/mL) milk and much lower than cow α_{s1}-CN content (10.0 mg/mL) (Miranda et al., 2004).

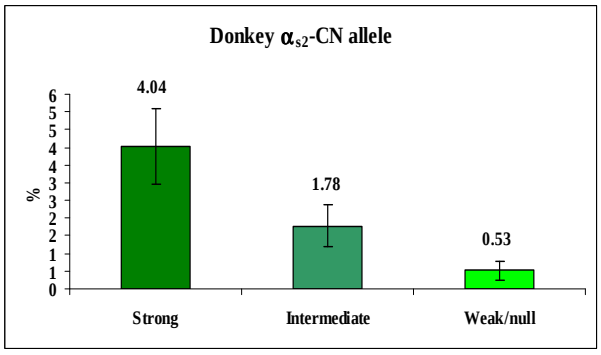
Table 3.10-Fig. 3.34: expression level (%) of donkey α_{s1}-CN by RP-HPLC analysis.



Donkey αs1-CN allele			
	Strong	Intermediate	Weak/null
Mean	32.27	24.05	3.42
S.D.	1.68	4.46	
Min.	30.08	11.71	
Max.	34.56	29.87	

In analyzed samples, the α_{s2}-CN was the less abundant casein fraction (1.82%) (~0.11 mg/mL) compared to α_{s1}-CN and β-CN, varying however from minimum of 0.00% to maximum value of 6.42% (Table 3.9-Fig. 3.33). In analyzed donkey milk samples, the mean value of α_{s2}-CN content was very close to that of mare's milk (0.2 mg/mL) and much lower compared to cow's milk (3.7 mg/mL), whereas the presence of α_{s2}-CN in human milk has not been demonstrated (Miranda et al., 2004).
As for donkey α_{s1}-CN, the HPLC analysis of individual caseins showed different peak areas for α_{s2}-CN (Fig. 3.32), in the following decreasing order (Table 3.11-Fig. 3.35): C (4.04%) (~ 0.2 mg/mL), B (1.78%) (~ 0.1 mg/mL) and A (0.53%) (~ 0.03 mg/mL); therefore the donkey α_{s2}-CN could also be expressed by quantitative alleles (strong, intermediate, weak and/or null).
Thus, a lower expression level or the absence of α_{s2}-CN in some samples of donkey milk, as in human milk, together with minor content of α_{s1}-CN and β-Lg confirm the compositional similarity of two milks and represent a scientific basis for donkey milk's use in nutrition of infants with CMPA.

Table 3.11-Fig. 3.35: expression level (%) of donkey α_{s1}-CN by RP-HPLC analysis.



Donkey αs2-CN allele			
	Strong	Intermediate	Weak/null
Mean	4.04	1.78	0.53
S.D.	1.06	0.58	0.20
Min.	3.04	1.03	0.00
Max.	6.42	2.95	1.00

The relatively low level of caseins, as α_{s1}-CN in donkey milk (1.5-2.00 mg/mL) compared with bovine milk (10 mg/mL) may be significant and, coupled with the low protein content, may be responsible for the soft curd produced in the infant stomach and foal. Goat milk lacking α_{s1}-CN has poor coagulation properties compared with milk containing α_{s1}-CN and also ensures a better β-Lg digestion.
These data, together with a greater homology of donkey caseins primary structure with human compared to cow's α_{s1}-CN represent a scientific basis to explain the higher digestibility and lower allergenicity of donkey milk than other species' milk (Table 3.12).

Table 3.12: Homology's percentage of donkey caseins in comparison with other species (expasy.org)

	Human	Donkey	Mare	Cow	Goat	Sheep
α_{s1}-CN	40%	100%	90%	36%	36%	36%
α_{s2}-CN		100%	89%	60%	60%	60%
β-CN	54%	100%	90%	49%	49%	49%

3.13.4 Determination of expression level of donkey whey proteins by RP-HPLC

For the quantitative determination of each donkey whey protein, the individual whey samples were chromatographically separated by RP-HPLC (Fig. 3.36) and identified by mass spectrometry methods (Table 3.5).

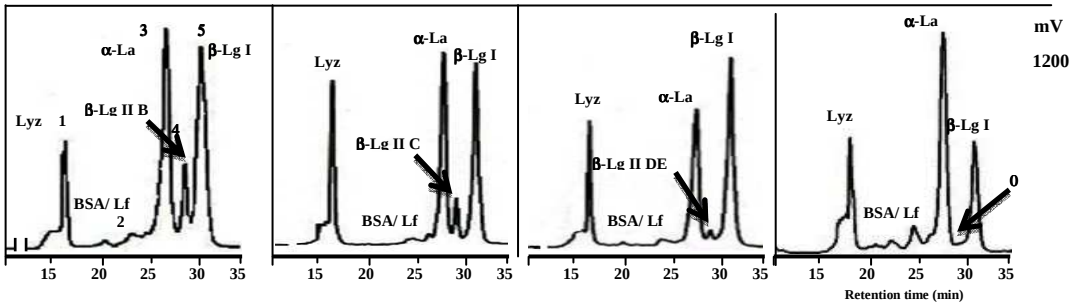
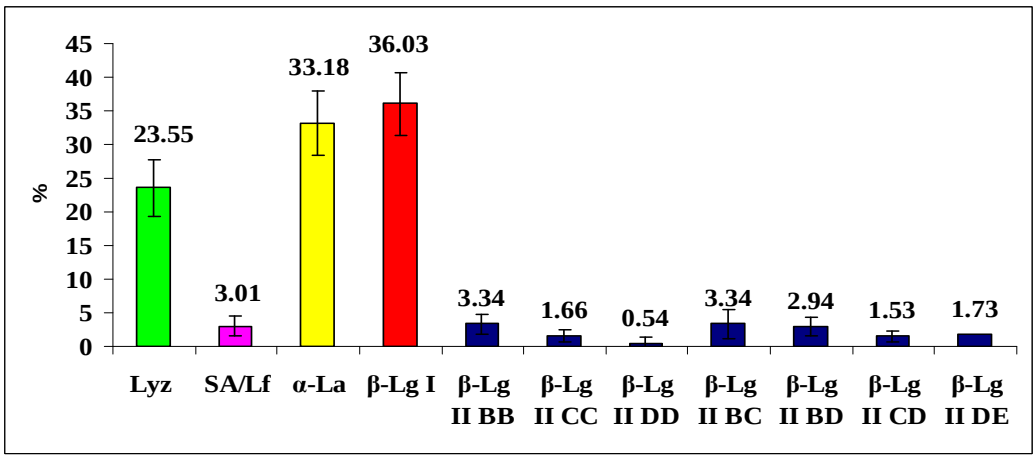


Fig 3.36: RP-HPLC of donkey whey proteins.

Subsequently, the average expression level of each donkey whey protein was determined through the integration of chromatographic peaks. The data (expressed as percentage of total whey proteins) were shown in Table 3.13-Fig.3.37.

Table 3.13-Fig. 3.37: determination of content of each donkey whey protein (% of the total whey protein) in examined whey samples by RP-HPLC analysis.

	Lyz	SA/Lf	α-La	β-Lg I	β-Lg II BB	β-LG II CC	β-Lg II DD	β-Lg II BC	β-Lg II BD	β-Lg II CD	β-Lg II DE
Mean	23.55	3.01	33.18	36.03	3.34	1.66	0.54	3.34	2.94	1.53	1.73
S.D.	4.16	1.44	4.68	4.70	1.47	0.87	0.72	2.09	1.31	0.83	
Min.	14.57	0.00	23.20	21.58	0.00	0.00	0.00	1.63	1.61	0.22	
Max.	33.91	15.23	46.80	47.05	8.10	5.05	2.38	7.62	4.23	3.03	



The mean content of Lyz in analyzed donkey whey proteins was about 23.54% (~ 2mg/mL), showing a considerable variability from a minimum (14.57%) to maximum value of 33.91%. The percentage of lysozyme in donkey whey proteins (23.54%) (~ 2mg/mL) was much higher than in human (0.50 mg/mL) and mare (1.10 mg/mL) milk, whereas only traces were found in cow's milk (Solaroli et al., 1993; Miranda et al., 2004). The large amount of Lyz in donkey milk was also confirmed by Salimei et al. (2004) and Guo et al. (2007) and may be responsible for its low bacterial count and could be useful to prevent intestinal diseases in infants. Many authors have shown interest in the large amount of Lyz in donkey milk, and according to

Coppola et al. (2002), donkey milk represents an optimal growth medium for certain strains of useful lactic acid bacteria. It must be noted that lysozyme can be also considered as an indirect "bifidogen factor".
The lactoferrin and serum albumin together accounted, on average, 2.88% (~ 0.25 mg/mL) of the total whey proteins, varying from a minimum 0.00% to maximum value of 15.23%. The determination of percentages of these two biological whey proteins represented our important result. In fact, the donkey lactoferrin, although representing together with SA a minor component than other whey proteins, not only in donkey, but also in other mammalian species, such as mare (0.82 mg/mL), human (2.3 mg/mL) and cow (0.47 mg/mL) (Malacarne et al., 2002), display many biological functions: regulation of iron homeostasis, cellular growth, anti-microbial and anti-viral functions, and protection against cancer development and metastasis (Vincenzetti et al., 2012).
The mean content of α-La in donkey whey proteins analyzed was about 33.18% (~ 3 mg/mL), varying from a minimum 23.20 % to maximum value of 46.80%. This result obtained for donkey milk is in good agreement with the α-La content in mare (3.30 mg/mL) milk but is very high compared to human (1.6 mg/mL) and cow (1.2 mg/ mL) α-La content (Miranda et al., 2004).
Regarding donkey β-Lg, in this study we were able to separate the two components of the protein (β-Lg I and II) by RP-HPLC, something that to date no one had been able to do. The mean content of β-Lg I in donkey whey samples analyzed was about 36.03% (~ 3.2 mg/mL), varying from minimum of 31.58% to maximum value of 47.05%. The mean content of donkey β-Lg I was very close to that of cow's milk (3.3 mg/mL) and mare's milk (3.0 mg/mL), whereas in human milk β-Lg is absent (Miranda et al., 2004).
The donkey β-Lg II was separated from β-Lg I by RP-HPLC analysis, which allowed to determine its mean content. Subsequently, the LC-MS analysis of individual whey proteins allowed to identify four β-Lg II variants (B, C, D and E) otherwise co-eluting in the same chromatographic peak or co-focalising at the same pI value of the UTLIEF pH gradient, as β-Lg II A and B.
We found a complex polymorphism of β-Lg II for the occurrence of different variants and null allele at this *locus*. Furthermore, the HPLC analysis of whey samples containing B, C, and D β-Lg II variants showed a different peak area in the following decreasing order: BB (3.34%), CC (1.66%) and DD (0.54%). The whey samples heterozygous for β-Lg II also showed a different peak area in the following order: BC (3.34%), BD (2.94%), DE (1.73%) and DC (1.53%). Donkey whey proteins with a lower or zero β-Lg II content (0.50-0.00%) were also identified in donkey population analyzed.
These results confirmed the quali-quantitative polymorphism at donkey β-Lg II *locus* and therefore the possibility that β-Lg II variants could be expressed by quantitative alleles (strong, intermediate, weak and null) as at goat α_{s1}-CN *locus*. Since the percentage area value of β-Lg II DE was very close to that of CD counterpart, it can be hypothesized that β-Lg II E (new variant) is expressed by an intermediate allele.
In donkey milk, the total β-Lg, absent in human milk, accounted for approximately 40% of the whey proteins, equal to the level in mare milk and lower than that in cow's milk, where β-Lg can account for up to 50% of the total whey proteins (Solaroli et al., 1993; Miranda et al., 2004). Other authors found a low β-Lg content in mare's milk compared with cow's and donkey milk (Malacarne et al., 2002).
However, from these analytical data, it appears that a high lysozyme content together with other antimicrobial proteins in donkey milk can be important from an immunological point of view and for the shelf life of the same milk. The two main whey proteins (α-La and β-Lg) are expressed at the same level, but with a prevalence of β-Lg and for this latter, β-Lg I is expressed at higher level than β-Lg II as known in literature (Godovac-Zimmermann et al., 1988; 1990). Moreover, the quali-quantitative polymorphism at donkey β-Lg II *locus* highlighted the possibility that β-Lg could be expressed at low level in donkey milk and this, may affect its composition and therefore, its allergenic properties.
These findings, together with the low casein content, are probably related to the hypoallergenic characteristics for both donkey and mare milk (Businco et al., 2000; Carroccio et al., 2000); β-Lg is, in fact, the probable major milk allergen not only in adults but also in children (Carroccio et al., 1999). This protein is absent in human milk and thus may react as an allergen. In fact, its particular folding makes β-Lg resistant to peptic digestion at low pH, even after denaturation (Dalgalarrondo et al., 1995). Conversely at pH 7.2, β-Lg is very susceptible to the pancreatic enzymes which completely hydrolyze it. The surviving peptides from β-Lg digestion can cross the gut mucosal barrier to sensitise the immune system and/or elicit an allergic response, acting as epitopes (Wall, 2004).
With respect to bovine β-Lg, seven IgE epitopes (AA 1-16, AA 31-48, AA 47-60, AA 67-78, AA 75-86 AA 127-144 and AA 144-151) were identified (Jarvinen et al., 2001). Among these, two the fragments 47-60 and 127-144 were identified as the main IgE binding immunoreactive epitopes. The knowledge about the allergenic epitopes of bovine β-Lg, together with the determination of the primary structure of different donkey β-Lgs provide the basis for some comparison of their sequences. According to Cocco (2007), the substitutions or deletions of one or more amino acids in a protein as donkey β-Lg could result in a partial reduction or elimination of IgE binding regions in majority of patients.

Comparison of the bovine β-Lg with donkey β-Lg I and II reveals that these proteins share a low sequence homology (56% and 51%) respectively. In particular, alignment reveals that the IgE-binding epitopes of cow's β-Lg and the corresponding domains present in donkey β-Lg have remarkable differences in their amino acid sequences. In fact, it is interesting to note that one of the strongest β-Lg epitope (125-135) is located in the 127-144 region, which is highly hydrophilic for the presence of five acidic residue and one Pro over total 11 residues, which also make it very resistant to proteolysis. An alignment of the 125-135 epitope of β-Lg from bovine with donkey showed 5 amino acid substitutions which lowering the acidic character of the donkey peptide (Table 3.14), drastically changes its susceptibility to proteolysis and therefore its allergenicity (Picariello et al., 2010).

Table 3.14: sequence alignment of the β-Lf f(125-135) from milk of cow and donkey species.

Cow	^{125}TPEVDDEALEK^{135}
Donkey β-Lg I	^{125}T**QM**VD**K**E**IM**EK^{135}
Donkey β-Lg II	^{125}T**QK**VD**K**E**VM**EK^{135}

3.14 Conclusions 3

The quantitative results indicated that the examined donkey milk samples were shown to be poor in protein, resulting more similar to mare and human milk than to other mammalian milks as cow's milk.
The analyzed donkey milk samples were also shown to be poor in caseins with allergenic properties, and rich in whey proteins with high biological and nutritional value, with a CN/WP ratio < 1. The richness and pattern of whey proteins and NPN of donkey's milk make it more favourable than cow's milk for human nutrition.
Specifically, regarding donkey caseins, the most abundant casein fraction was β-CN, followed by α_{s1}-CN and α_{s2}-CN; human milk is characterized by a prevalence of β-CN, as donkey milk, while cow casein is relatively rich in α_{s1}-CN, which is believed to be responsible for the onset of allergic forms in nursing infants.
The determination of α_{s1}-CN content in individual donkey milk samples also showed a quantitative polymorphism at this *locus*, for the presence, only in traces, of the protein.
For donkey whey proteins, the quantitative results showed an high level of lysozyme, which, together with other antimicrobial components of donkey milk, as lactoferrin, is responsible for the low bacterial count of this milk and could be useful to prevent intestine infections in infants. α-La and β-Lg content is similar with a β-Lg prevalence. Moreover, the detection of donkey milk samples characterized by a low β-Lg II content also suggested a quali-quantitative polymorphism at this *locus*.
The low casein (α_{s1}-CN) and β-Lg contents are probably related to the hypoallergenic characteristics reported in literature for donkey milk.
Finally, in analyzed donkey milk samples, an individual variability of either caseins and whey proteins amount suggests further studies in this direction aimed at a selection of animals producing milk with a composition "qualitatively and quantitatively" more similar to human milk, and "functionalized" for infant feeding.
The achieved results, confirming the biological and nutritional value of donkey's milk, similar to human milk, solicit detailed and analytic knowledge of donkey's milk, which could be used not only as a breast milk substitute but also as a new dietetic/bio-functional food. Moreover, the production and the wider use of donkey milk suggest the potential economic value of asinine breeding, providing an economic justification for breeding donkeys and preserving their natural environment, mostly represented by marginal and hilly areas, helping to protect certain donkey breeds from extinction in industrialised countries.

3.15 References

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3.16 Glossary

M_r	Molecular Mass
Aa	amino acid
UTR	untraslated region
Cys	cysteine
Ser	serine
Thr	threonine
SerP	serine phosphorylated
Glu	glutamic acid
NANA	N-acetyl neuraminic acid
GMP	glycomacropeptide
FA	fatty acids
SFAs	saturated fatty acids
MCFAs	medium chain fatty acids
LCFAs	long chain fatty acids
UFAs	unsaturated fatty acids
EPA	eicosapentanoic acid
DHA	docosahesaenoic acid
TAGs	triglycerides
MCT	medium chain triglycerides
Lyz	lysozyme
Lf	lactoferrin
SA	serum albumin
Ig	immunoglobulin
CMP	cow's milk protein
CMPA	cow's milk protein allergy
eHF	extensively hydrolyzed formulas
AAF	amino acid formulas
AD	atopic dermatitis
LAB	lactic acid bacteria
EFAs	essential fatty acids
PAGE	polyacrylamide gel electrophoresis
UTLIEF	Ultra-thin layer isoelectric focusing
PAGE-SDS	polyacrylamide gel electrophoresis-sodium dodecyl sulphate
MS	mass spectrometry
MALDI TOF	matrix assisted laser desorbtion/ionization time-of-flight
RP-HPLC	reversed phase- high performance liquid chromatography
LC-ESI-MS	liquid chromatography-electrospray ionization-mass spectrometry
ESI-q-TOF-MS/MS	electrospray quadrupole-time of flight-tandem-mass spectrometry
GRAVY	grand average of hydropathicity

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Appendix: Publications in three years of PhD

Articoli di riviste:

Chianese, L., C. De Simone, P. Ferranti, R. Mauriello, **A. Costanzo**, M. Quarto, G. Garro, G. Picariello, G. Mamone & L. Ramunno, in press. Occurrence of qualitative and quantitative polymorphism at donkey beta-Lactoglobulin II *locus.* Food Research International

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Articoli in atti di convegni:

Costanzo, A. & L. Chianese, 2012. Characterization of a new donkey β-Lactoglobulin II genetic variant by proteomic approach. Proc. 17th Workshop on the "Development in the Italian PhD Research on Food Science Technology and Biotechnology" (società editrice Il Ponte Vecchio), pp. 23-27, ALMA MASTER STUDIORUM, Cesena, ITALY.
ISBN: 978-88-481-6541-224-4

Costanzo, A. & L. Chianese, 2011. Characterization of Donkey alpha(s1)-CN by proteomic approach. Proc. 16th Workshop on the "Development in the Italian PhD Research on Food Science Technology and Biotechnology" (Tecniche Nuove ed.), pp. 213-214, Parco Tecnologico Padano, Lodi, ITALY.
ISBN: 978-88-481-2717-2

Costanzo, A., R. Mauriello, C. De Simone, P. Ferranti, M. Quarto & L. Chianese, 2010. Polimorfismo quali-quantitativo al locus della β-Lg II di asina. Proc. Giornate Scientifiche 2010 (CUES ed.), pp. 440-440, Università degli studi di Napoli – Federico II, Facoltà di Medicina e Chirurgia, Napoli, ITALY.
ISBN 978-88-95028-66-8 www.giornatescientifiche.unina.it/pdf/FO/440.pdf

Costanzo, A., & L. Chianese, 2010. Characterization of donkey's milk proteins by proteomic approach. Proc. Int. 15th Workshop on the "Development in the Italian PhD Research on Food Science Technology and Biotechnology" (CUES ed.) pp. 319-320, Univ. of Naples, "Federico II", Facoltà di Agraria, Portici, ITALY.
ISBN/ISSN: 978-88-95028-62-0

b) Abstracts per Poster e comunicazioni orali a Congressi

1) Abstract per comunicazione orale a Congresso:

Costanzo, A., C. De Simone, P. Ferranti, M. Quarto, G. Garro, MA. Nicolai, R. Mauriello & L.Chianese. La metodologia proteomica nella definizione della microeterogeneità compositiva dell'α_{s1}-CN asinina. In riassunti delle comunicazioni e dei poster 10° CISETA (Congresso Italiano di Scienza e Tecnologia degli Alimenti) (Chiriotti Editori), Fiera Milano Rho, Milano, 9-10 Maggio 2011, p.15-15.

2) Abstract per poster a Congresso:

Chianese, L., R. Mauriello, C. De Simone, P. Ferranti, **A. Costanzo**, M. Quarto, G. Garro, D. Marletta, L. Ramunno & A. Rando. Occurrence of qualitative and quantitative polymorphism at donkey β-Lactoglobulin II *locus*. In www.idfsheepgoatmilk2011.aua.gr. Athens, Greece, 16-18 May 2011, Brussels: Int. Dairy Fed., vol. Poster Abstract, session 2, p. 61-61.

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Occurrence of qualitative and quantitative polymorphism at donkey beta-Lactoglobulin II *locus*

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ABSTRACT

With the use of a proteomic approach a genetic screening of 70 individual donkey milk samples, from two autochthonous breeds reared in Italy, was carried out to determine the genetic polymorphism at donkey β-Lgs *loci*. The achieved results showed the occurrence of the new β-Lg II E genetic variant, differing from β-Lg II D reference for D Asp^{2}/E Asn^{2}, D Arg^{18}/E Lys^{18} and D Val^{25}/E Ala^{25} amino acid substitutions with a M_r 18 256 Da. Moreover a quantitative polymorphism seemed to affect the donkey β-Lg II *locus* since four phenotypes, with a relative quantitative percentage respect to total whey protein ranging from 0% to 3.65%, were detected by HPLC analysis.