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DONKEY MILK NUTRACEUTICAL CHARACTERISTICS: A BIOCHEMICAL EVALUATION OF NUTRITIOUS AND CLINICAL PROPERTIES

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In loving memory of Prof. Paola Maggiori Cantarini

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Introduction

“Milk has a special position among other food stuffs, since it has the most versatile composition. As abundant a use as possible is economically advantageous from the viewpoints of both an individual and of whole nations.”

Artturi Ilmari Virtanen, Nobel Prize in chemistry 1945

Better ride a donkey that carries me home than a horse that throws me.

English proverb

Milk has all nutritional requirements for every mammalian newborn of different species. Specificity depends by the needs of the neonate and must ensure survival and growth during the early months of life. Nutrition and life are tightly connected in the first period of life: colostrum (the breast secretion in the first days after birth) and milk contain antibacterial agents, anti-inflammatory factors and immunomodulators. The immaturity of the neonate’s immune system is overcome by the protection of several proteins such as secretive maternal immunoglobulins and enzymes like lactoferrin, lactoperoxidase, lysozyme. The secretive immunoglobulins are resistant to proteolytic activity of gastrointestinal tract and prevent the adherence of bacteria to mucosal surfaces neutralizing toxins from microorganisms. Lactoferrin has anti-inflammatory and antimicrobial activities at the mucosal sites competing with bacteria for ferric iron and preventing the growth of microorganisms. Lysozyme (EC 3.2.1.17, muramidase) exerts its antibacterial activity against a number of bacteria

catalyzing the hydrolysis of b1- 4 glycosidic bonds between N-acetylmuramic acid (Mur2Ac) and N-acetylglucosamine (GlcNAc) in the bacterial cell walls polysaccharides. Furthermore lysozyme may limit the migration of neutrophiles into a damaged tissue and therefore might function as an anti-inflammatory agent. Lysozyme may work synergistically with lactoferrin and immunoglobulins in antimicrobial functions. Lactoperoxidase is another antimicrobial factor that can exert their antibacterial effect by oxidative mechanism. This enzyme, in presence of hydrogen peroxide and thiocyanate ion (SCN^-) catalyzes the synthesis of hypothiocyanate ($OSCN^-$), compound responsible of the antimicrobial activity because inhibits by oxidation the bacterial metabolic enzymes (Lönnerdal, 2003). Because of its antimicrobial activity, lactoperoxidase has been used as preservative agent in reducing microflora in bovine milk. Besides the native proteins, active peptides liberated during the gastrointestinal digestion may exert a lot of physiological effects on the digestive, cardiovascular, immune and nervous systems; others bioactive molecules are hormones and growth factors. Specificity is equal to versatility: there can be a difference in composition at all the period of lactation, from one mother to another, the time of the day, and among the days. Moreover composition changes according to different ambient and climatic situation: for example, in cold regions milk is dense, rich of fats and highly energetic; in temperate districts it is more fluid and with a little content of fats. In human diet, milk keeps a considerable role in adults' diet for the presence of the important nutrients and constituents. Chemically milk is an emulsion of water and fat, a suspension of

proteins, a solution of sugar (lactose, a dimer of glucose and galactose, unobtainable elsewhere in nature), vitamins and minerals.

For human beings cow's milk represents the most common feeding during the infant weaning, but also the first allergen in life.. The European Academy of Allergy and Clinical Immunology distinguishes allergy from intolerance [1]. Allergy is an adverse reaction to food with an involvement of the immune system; intolerance is an adverse reaction to food that does not involve the immune system, does not reply to a precise and single fault and shows different symptoms. In many countries cow's milk is the most important food allergen in babies and children [2, 3]. Adverse reactions to cow's milk were found in 2% of babies during the first year of life: 30% of cases at the first month, 60% before the third and 96% within the twelfth [4, 5]. Symptoms can even appear during the breast-feeding because newborn reacts against a small amount of cow milk proteins present in maternal milk [6]. Children followed for the first 3 years of life, 56% of cases had recovered from cow's milk allergy at 1-year age, 77% at 2 years and 87% at 3 years age [7]. However allergy can persist for all life.

Lactose intolerance is a deficiency of lactase, an enzyme produced by brush border cells in the small intestine and results in gastrointestinal malabsorption. Lactose is a disaccharide; the products of hydrolysis are the monosaccharides glucose and galactose. When lactase is absent or deficient, intestinal bacteria must hydrolyse lactose; reduced capacity to ferment the undigested sugar and poor absorption are

responsible for intolerance symptoms [8]. Diarrhoea occurs because undigested sugar produces an osmotic effect pulling fluid into intestine; the bacterial fermentation produces excess gas. Infants with lactose intolerance may also present abdominal distension, vomiting dehydration, electrolyte abnormalities and even reduced growth [9, 10].

Lactase deficiency can be congenital, primary, and secondary. Congenital deficiency is a rare hereditary condition in which lactase activity is absent. Primary deficiency is the gradual decrease of lactase production during aging: this phenomenon is probably due to a normal reduction in need for milk after infancy and may explain the appearance of intolerance symptoms in adulthood [11]. Secondary deficiency occurs because of gastroenteritis, bowel surgery, immune disorders and antibiotic therapy.

The treatment of lactose intolerance is the removal of lactose from diet and the use of lactose free formulas. The American Academy of Paediatrics recommends the use of soy-based formulas in primary lactose deficiency. In secondary deficiency recovered infants should tolerate breast milk or cow's milk formula. However the routine utilization of soy-based formulas are not recommended to treat or prevent colic and are not nutritionally useful for the preterm infants less than 1800 grammas.

Allergy to cow's milk is more complex. Adverse reaction to cow's milk protein is the most common allergy in infancy. The reported incidence is between 0.3% and 7.5% [7, 12-13]. The variability in reporting is due to the differences in diagnostic criteria, plans of studies, areas and age of patients. Cow's milk proteins are processed by

gastric and pancreatic proteolytic enzymes. The macromolecules derived from the proteins digestion are absorbed through the intestinal mucosa and this results in a normal and physiological development of antibodies against cow's milk proteins. Genetic predisposition, changes in macromolecular absorption of intestinal mucosa may play a role in infant's allergic response toward cow's milk. Other factors are time, frequency and intensity of exposure. More than 30 proteins, the main of which are casein and whey proteins, represent cow's milk allergens. Caseins include α-, β- and ϰ-casein; whey proteins are classified into α-lactalbumin (α-la), β-lactoglobulin (β-lg), bovine serum albumin (BSA) and immunoglobulin. Several minor proteins are also present.

Cow's milk allergy (CMA) and cow's milk protein allergy (CMPA) are often used mutually in literature. The same is for **cow's milk intolerance (CMI)** and cow's milk protein intolerance (CMPI).

CMA is an abnormal immunological reaction to cow's milk caused by ingestion of an amount generally tolerated. The most common immune responses are immunoglobulin E (IgE) mediated, cell mediated (non-IgE) or the combination of the two.

CMI is not immune mediated and does not include the symptoms of lactose intolerance.

Children with CMA or CMI can present a multiplicity of symptoms like cutaneous (urticaria, atopic dermatitis, angioedema, rashes), gastrointestinal (nausea, vomiting, diarrhoea, enterocolitis, colitis, constipation) or respiratory (rhinoconjuctivitis,

asthma, laryngeal edema, otitis, anaphylaxis).

Breast-feeding may protect against CMA/CMI or retard the beginning of pathology. Secretive immunoglobulin A in breast milk can block the absorption of the macromolecules derived from the protein digestion. The incidence of CMA/CMI in infants exclusively breast-fed for more than one month is around 1% [14]. Reactions to cow's milk protein in human milk is in about 0.5% of infants. These infants may react to cow's milk protein ingested by their mothers. Small amounts of β-lactoglobulin have been found in maternal breast milk.

CMA/CMI is used to present within the first month of life and within the first week of exposure. Patients can have immediate reactions, even within minutes; some patients show symptoms, generally gastrointestinal, within 1-24 hours after exposure; others have manifestations from 24 hours up to 5 days after exposure. Symptoms tend to disappear at two or three years of age but sometimes they may persist up to four years. Children with one or both parents, brother, sister or relative with CMA or other allergic reactions are most at risk for CMA development. There is no single test to identify CMA: infants with normal IgE levels can still have CMA [15-17]. Specific skin or prick test can have high rates of false positives and false negatives [16, 18].

If CMA/CMI is suspected, the recommendation is the entirely removal of cow's milk from the diet for a period; at the cessation of symptoms, cow's milk can be carefully reintroduced but if symptoms reappear there is a highly probability of CMA. If the patient is exclusively breast-fed and there is a suspicion of CMA, the removal of cow's milk protein from the mother diet can reduce or eliminate symptoms [19].

Infants with a family history of CMA or other allergies should be exclusively breast-fed; if this is not possible, infants should be fed with hydrolysed formulas. The use of soy-based formulas is not opportune because of the potential allergenicity of soy proteins [20].

The American Academy of Paediatrics recommend breast milk for infant feeding during the first year of life and for infants with CMA or at risk of CMA. When maternal milk is not sufficient is advisable to use extensively hydrolysed formulas (proteins are pre-digested): the partially hydrolysed formulas are potentially dangerous because of the amount of intact cow's milk proteins. Extensively hydrolysed formulas may also induce allergic reactions but 90% of infants well tolerate these formulas [19]. Free amino acid formulas have further hydrolysed proteins and are also well tolerated. Soy formulas may be useful only in children with IgE symptoms (urticaria, atopic dermatitis, angioedema, cough) but not with non-IgE symptoms (enterocolitis, malabsorption, esophagitis).

Definitively, for the first six months to one year of life, breast-feeding is strongly recommended for all infants. Standard cow's milk formulas may well supplement breast milk in healthy children. Extensively hydrolysed or free amino acid formulas are the best and should be always the first choice for the needs of cow's milk allergy babies. Lactose free formulas must be used only for lactose intolerance. Soy-based formulas may be used with IgE mediated allergic reactions. Unfortunately the industrial formulas have a poor palatability and this may be a problem and often precludes their use in infant diet.

In conclusion, the best nutritional option for newborns is maternal milk. Frequently, some infants may not be exclusively breast-fed. So another substitute must be found. Nowadays, the most common alternatives are artificial formulas. However a considerable part of allergic children don't tolerate these derivates. The morbidity to infections is lower in breast-fed babies.

As always, man can find in nature all the answers to the hardest question: he must only be able to see. So an alternative substitute of human and cow milk may be milk from different animals such as goat, sheep, buffalo, camel, mare and donkey. Several studies have evaluated their nutritional properties. Evidences showed that milk of ruminant animals could be as allergic as cow's milk: their proteins have immunological cross-reaction with the counterparts in cow milk. The protein content is higher than in human milk, different in composition and structure; the proportion of whey proteins and caseins is inverted. Human milk is free of β-lactoglobulin, one of the major allergens; caseins differ in fraction number and composition; the concentration of soluble calcium is lower.

In Italy donkey's milk is probably the best natural substitute of human milk.

Better be the head of a donkey than the tail of a horse.

English proverb

The donkey (*Equus asinus*) is a member of the horse family and its progenitor was the small gray donkey of northern Africa (*Equus africanus*) domesticated around 4000 BC on the shores of the Mediterranean Sea. It worked together with humans for centuries; the most common role was for transport. It still remains an important work animal in the poorer regions.

Donkey is a complex creature, capable of many moods. It can be friendly, affectionate, patient, independent and especially intelligent. It has a keen sense of curiosity and an incredible memory. But it is also stubborn in an original way. It's a hardly animal and resistant to disease but it loves the hot and dry climates. Donkey has a slow gait and it is most active in the evening. The size varies considerably from 0.9 m to 1.6 m. The gestation is approximately of 12 months. Life is of 25-30 years. Now few species exist in the wild: some of them are the African, the Asiatic and the Hymalayan. In Italy it was used in the army till the beginning of the 20th century. The most important Italian domestic breeds are: Martina Franca donkey (original from Puglia), Sicilian or Ragusano donkey and Sardinian donkey.

In the Italian popular tradition of the last centuries **donkey's milk** was commonly used to feed infants when the maternal one was not sufficient and actually it seems to more attract the interest of consumers.

Collecting Ass' milk. Hospital St. Vincent de Paul. Paris, XIX century.

In 2006 the Italian Breeders Association introduced functional controls on its production. Compared with ruminant's milk, donkey milk has been less studied but in the last years it was investigated to clear up its legendary cosmetic and therapeutic properties.

The protein composition is marked different from cow's milk: the total content is lower (13-20 mg/ml) and quite similar to that of human and mare milk: this condition avoids an excessive renal load of solute [21, 22]. The main difference is the proportion of whey proteins: they are 35-50% of the nitrogen fraction while they represent only 20% in cow's milk [23].

The donkey's three major whey proteins are α-lactalbumin, β-lactoglobulin and lysozyme [24]. Donkey's milk α-lactalbumin has two isoforms with different isoeletric point [25]. The amount of α-lactalbumin in mare and cow milk is almost identical, about 3 mg/ml [26].

One of the main allergens in children is β-lactoglobulin that is the major whey protein in cow milk [27]. β-lactoglobulin is absent in human milk [26, 28, 29]. In donkey milk the content of β-lactoglobulin is approximately 40% of the whey proteins equal to the level in mare milk and lower than that in cow milk [26]. This condition may be related to the hypoallergenic characteristic of donkey and mare milk [30, 31, 32]. The mechanism for tolerance may be related to the specific levels of the major allergenic components in the milk. Donkey's milk has three genetic variants for β-lactoglobulin: one presents three amino acid substitutions while the others have two amino acid exchanges [23]. Donkey milk β-lactoglobulin is a monomer whereas this protein is a dimer in ruminant's milk.

The casein fraction, another main allergen, has been less investigated but is comparable to the content of woman milk.

The percentages of eight essential amino acids in the protein of donkey milk are higher than those of mare and cow milk; donkey milk also has higher levels of Ser, Glu, Arg, Val and a lower level of Cys.

Lysozyme is a natural antimicrobial agent because catalyses the hydrolysis of glycosidic bonds of mucopolysaccharides in bacterial cell walls. This enzyme with other factors including immunoglobulins, lactoferrin and lactoperoxidase reduce the

incidence of gastrointestinal infections in infants [30]. The high content may be responsible for the low bacterial concentration in donkey milk [21]. Donkey milk lysozyme presents two isoforms that differ in three amino acid substitutions at position 48, 52 and 61 [23]. The high content of lysozyme and lactose favours the growth of probiotic lactobacilli strains in the intestine [33].

The lactose content (7%) of donkey milk is similar to that of woman milk and is much higher than that of cow milk. The high content is responsible for the good palatability and facilitates the intestinal absorption of calcium that is essential for infant's bone mineralization [34].

Also the lipidic fraction is comparable to that of human milk and is characterized by high levels of linolenic acids [21]. Its addition in diet may be useful for the treatment of some atopic dermatitis. So donkey's milk could be used as a nutritional medicament for children suffering from this pathology [35].

The mineral composition is very close to that of human milk except for the highest level of calcium and phosphorus but the Ca-P ratio is similar [21]. The milk produced in the first month of lactation, when it is the only nutritional source for the foal, contained the highest levels of mineral elements that may be related to the fast growth stage of the foal. Afterwards, the mineral supply in milk decreases considerably [36].

In addition to use for infants, donkey milk may have multiple effects on the osteogenesis process, in arteriosclerosis therapy, in rehabilitation of patient coronary heart disease, premature senescence and in hypocholesterolemic diets [37].

Definitively, donkey milk has nutritional properties that make it more similar to woman milk than another mammalian one. Therefore it could be used not only as a breast milk substitute for allergic children but also as a new dietetic food for human consumption. Research interest increased in the last years. One of the most important problems in donkey milk supply is related to its seasonal availability during the year. Fertility of donkey female is strictly connected with photoperiod and the delivering period is normally limited in a range of a few months, basically from February to July. Furthermore donkey breeding is so dispersive and milk yield is very low but today the economic potential advantages are attracting capital investments.

This work would be a further contribution to increase the characterization of donkey milk. **The aim** of this thesis was the study the nutritional qualities of donkey milk by a proteomic approach. Through chromatographic techniques, SDS-PAGE, two-dimensional electrophoresis and N-terminal analysis it was possible to separate and identify components of the whey protein fraction and the casein fraction. The amount of the different components of donkey milk was also compared the relative amounts at different stages of lactation.

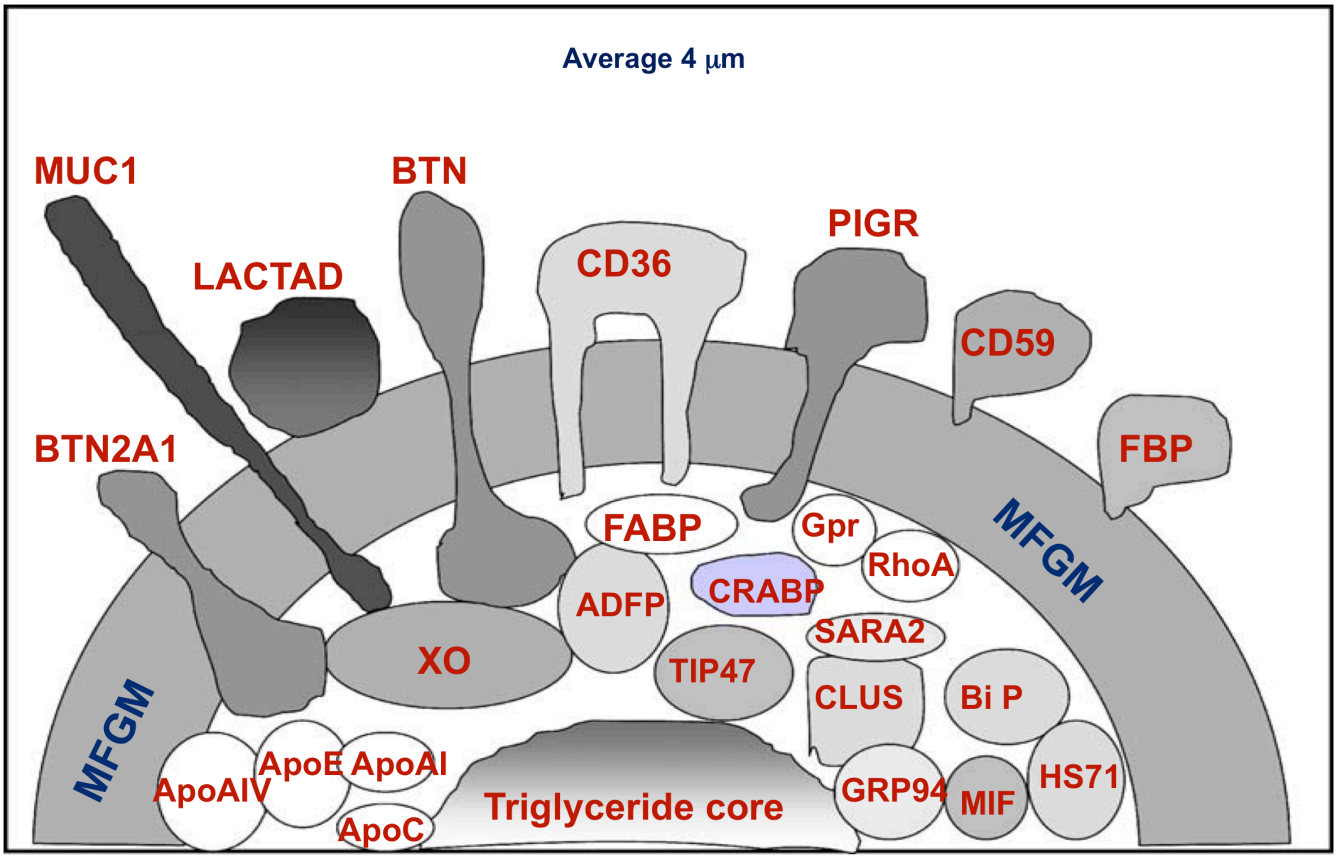
Furthermore, for the first time, lactoferrin from donkey milk was purified and quantified. In this work was also to evaluate the effects on the protein fractions of donkey milk of a particular storage temperature (-20°C), and of two specific technological process: spray-dryer in which milk is subjected to a strong thermal treatment to produce powdered milk and lyophilisation, in which the powderization process is obtained by low temperature and vacuum. It was investigated if these milk

processing may affect the activity of some antimicrobial factors such as lysozyme and lactoperoxidase. For this purpose the protein profile of donkey milk was investigated in the fresh milk, in the frozen milk and in the powdered milk (obtained by the spry-dry and by the lyophilisation processes). Furthermore the content and the enzymatic activity of lysozyme and lactoperoxidase was determined in order to verify if this important antimicrobial factor is preserved in different storage conditions or after a strong thermal treatment.

Vitamin C was quantified in fresh, frozen and powdered milk.

Lately, interest increased in characterization of the protein fraction in donkey milk fat globule membrane (MFGM), the membrane surrounding fat globules in milk, in attempt to identify heal-beneficial components.

In human milk the fat globule membrane (MFGM) is the most abundant component. The membrane of the globule is similar to other epithelial membranes being composed of cholesterol, phospholipids, proteins, and glycoproteins, which, in this case, surround a triglyceride core [38].



Cavalletto M et al. Clinica Chimica Acta (2004) 347: 41-48.

It has recently been suggested that the potent hydrolytic products of digested triglyceride core of the globule act to lyse enveloped bacteria, protozoa, or viruses, for example, herpes simplex or human immunodeficiency virus if these are present in the stomach of newborn infants.

The glycoproteins of the MFGM are also thought to act as specific viral and bacterial ligands that may contribute to the prevention of the attachment of pathogenic organisms to the intestinal mucosa of the infant (Hamosh, M.1999). The glycoproteins of this membrane are able to maintain their original structure and function even in the acidic environment of the infant's stomach, a characteristic that may be due to the extreme diversity in the glycans found on these proteins.

Materials and Methods

Materials

Bis-tris, tris (hydroximethyl) amminomethane (trizma base), dithiotreitol (DTT), ammonium acetate, sodium dodecyl sulphate (SDS), lysozyme (from egg white), α-lactalbumin, β-lactoglobulin (from bovine milk), lyophilized *Micrococcus lysodeikticus* 2',2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and other reagents were from Sigma Chemical Co. (St. Louis, MO - USA).

Superdex 75 HR 10/30, Mono Q HR 5/5, Mono S HR 5/5, Mono P HR 5/5, HiTrap Desalting, Polybuffer 96 and 74, Blue Dextran 2000, Low molecular weight gel filtration calibration kit and FPLC system ÄKTA Purifer were produced by Amersham Biosciences (Uppsala, Sweden).

Reversed-phase column C4 prosphere (150x4.6 mm, 300 Å pore size, 5 μm particle size) was from Alltech (Wankegan Red Deerfield, IL - USA).

Mini Protean II, Protean II, Trans-Blot cell apparatus, polyvinylidene difluoride (PVDF) membrane and SDS-PAGE molecular weight standard low range, IPGphor Isoelectric Focusing System, Immobiline DryStrip 2-D Clean-Up kit were produced from Bio-Rad Laboratories (Hercules, CA - USA).

Beckman DU 640 spectrophotometer, Beckman Microguge were from Hewlett Packard (Palo Alto, CA – USA).

Ultrafiltration cell and PM10 membranes were produced from Amicom Inc. (Beverly, MA – USA).

Sterile filter 0.2 μm pore size was from Schleicher & Schnell (Dassel – Germany).

Enzyplus Eza 941+ L-ascorbic acid test kit was from Raiso Diagnostics (Rome - Italy).

Human lactoferrin was from Gen Way Biotech Inc. (San Diego, CA – USA).

Samplig

Samples were obtained from Ragusana breed pluriparous females manually or machine milked. Milk was immediately refrigerated at 4° C until analysis or frozen later and finally stored at -20° C or -80° C until analysis. To evaluate the effects of a specific technological treatment, sometimes the samples were dry-powdered or lyophilized and stored at 4° C until reconstitution (9% w/v in distilled water) and analysis.

Skimmed milk was obtained by centrifugation at 3.000 g for 30 min at 16° C. The casein fraction was obtained from skimmed milk by adjusting the pH to 4.6 with 10% (v/v) acetic acid and centrifuged at 27.000 g for 20 min at 4° C. Supernatant represented the whey protein fraction while the isoelectrically precipitated caseins were found as pellet. The casein fraction was resuspended in clarifying buffer (CL Buffer): 100 mM bis-tris pH 8.0, 8 M urea, 1.3% trisodium acetate, 0.3% DTT. The protein concentration was determined according to the method of Bradford [40].

The fresh whole milk was powdered using the technique of two-stage drying system: the milk was firstly treated at 90°C for 25 sec, than dried at 170°C, the volume of milk in the tank was around 8 L/hour. Before to be analyzed, 0.9 g of powdered milk was completely dissolved in 10 ml of distilled water, the reconstituted milk was skimmed as described before.

Lysozyme turbidimetric assay

Donkey milk lysozyme activity was performed by turbidimetric assay according to the method of Jenzano [41]. The spectrophotometer used in this assay was a Beckman DU 640 Hewlett Packard. Lysozyme catalyzes the hydrolysis of glycoside bonds of muramic acid of bacterial cell walls: bacterial cells lysed gave rise to the clarification of culture medium. *Micrococcus lysodeikticus* was used as substrate and was prepared under sterile conditions by mixing gently 3 mg of lyophilized bacteria with 10 ml of sterile 100 mM sodium phosphate pH 7.0 and incubated for 1 hour at 25° C. The reaction mixture contained 20 μl of skimmed donkey milk and substrate up to 1 ml. The time-depended decrease in optical density at 490 nm was monitored for 10 min and at least 5 determinations were made for each sample. The control solution contained only 1 ml of substrate. One unit was defined as the amount of lysozyme that cause the decrease of 0.001 unit of absorbance at 490 nm per minute at 25° C. The ΔA_{490nm}/min obtained from each single reaction was multiplied for the dilution factor resulting in U/ml. Standard lysozyme solutions were prepared as follows: a stock solution of egg white lysozyme 10 mg/ml was prepared in 100 mM sodium phosphate pH buffer filtered through a sterile filter 0.2 μm pore size and stored at 4° C. The real lysozyme stock solution concentration was determined by Bradford's method [40]. The standard lysozyme solutions were diluted from stock solution: 2, 4, 6, 8 mg/ml. The reaction mixture contained 10 μl of each standard solution and 990 μl of substrate in sterile phosphate buffer. The standard solutions of lysozyme were used to test the linearity of turbidimetric assay.

Optimum temperature and stability of donkey's milk lysozyme toward temperature

The effect of temperature on lysozyme activity was investigated in the range +4°C to +70°C. The enzymatic reaction was started by adding the sample to the assay medium adjusted to the specific temperature. The effect of temperature on lysozyme stability was checked by incubating the enzyme at different temperatures (from +4 to +70°C) for 6 min, rapidly cooled in ice, centrifuged by a microfuge at high speed and finally assayed at + 25°C. For each temperature tested were made at least five determinations.

Lactoperoxidase activity

Enzymatic assay of donkey milk lactoperoxidase was performed by a continuous spectrophotometric method using ABTS as substrate [42, 43]. The method is based on the ABTS oxidation in presence of H_2O_2. The increase of absorbance, due to the oxidized ABTS, can be read at 436 nm as a function of time at 25° C. At least 5 determinations were made for each sample. The final reaction mixture contained: 27 mM ABTS, 34 mM sodium acetate buffer pH 4.6, 50-100 μl of skimmed milk in a final volume of 1 ml. A standard solution of lactoperoxidase at different concentration (0.1; 0.2; 0.3; 0.5; 0.7; 1.0 μg/ml) was prepared in 50 mM sodium phosphate buffer pH 6.0 and was used to test the linearity of the enzymatic assay. The reaction mixture, prepared as describe above, contained 10-50 μl of each standard solution in a final volume of 1 ml.

Isolation of lactoferrin from donkey milk

In addition to nutritional constituents milk contains proteins crucial against infections of both the digestive and respiratory tract such as lactoferrin, a member of the system of innate immunity.

Lactoferrin is a protein of the transferring family and is particularly resistant to the proteolytic degradation in contrast to other milk protein [49]. It controls the proper composition of the intestinal microflora suppressing the growth of pathogenic bacteria and promoting the multiplication of non-pathogenic *Lactobacillus* and *Bifidobacterium*.

Skimmed donkey milk was prepared as described in the previous session (see sampling).

Whey protein fraction was subjected to cationic-exchange chromatography on FPLC. The column (Mono S HR 5/5, 1 ml bed volume) was equilibrated in buffer A (50 mM ammonium acetate pH 5.5) and eluted by a linear gradient between buffer A and buffer B (1 M ammonium acetate pH 5.5, 1 M NaCl). 10 mg of sample were loaded onto the column. The gradient was: %B=0, Column Volume=10; %B=100, CV=100; %B=100, CV=10. UV 900 monitor of FPLC system monitored elution at 280 nm. Fractions corresponding to chromatographic peaks eluted from the column were analyzed by 15% or 7.5% SDS-PAGE. Coomassie Blue stained bands.

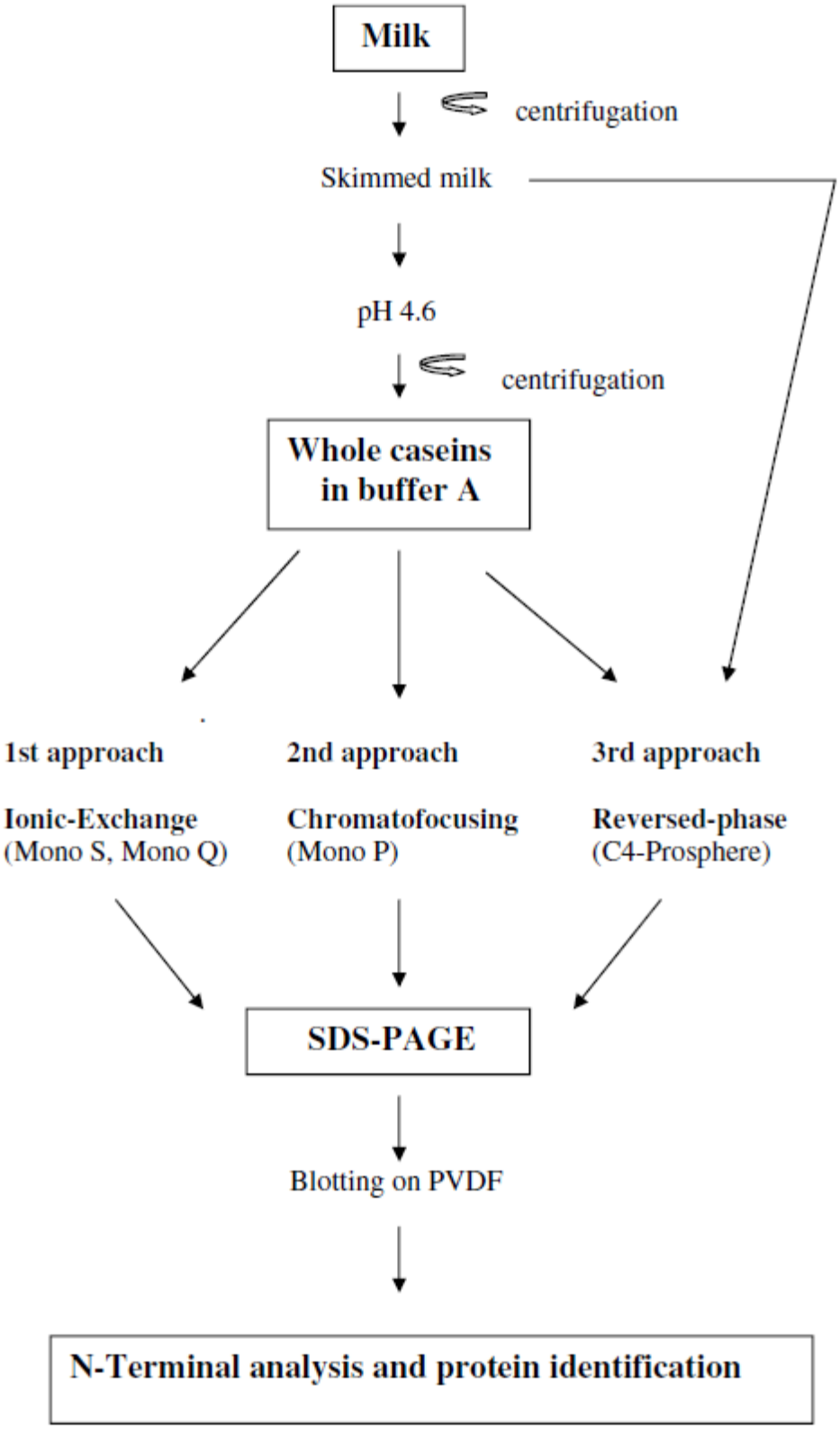
Whey proteins eluted from Mono S and separated by 7.5% - SDS-PAGE in acrylamide were subjected to western blotting (constant voltage of 50V for 2 hours at 4° C). The nitrocellulose membrane was blocked with TBST (Tris Buffer Saline,

0.1% Tween 20), 1% BSA and then was incubated with rabbit anti-human lactoferrin antibody diluted 1:2000 in TBST. After a series of washing in TBST the membrane was incubated for 30 min with the horseradish peroxidase-conjugated anti-rabbit secondary antibody diluted 1:4000 in TBST. The complex antibody-protein was visualized by the chromogenic-substrate 3'-1' diamminobenzidine (HRP conjugate substrate kit, Bio-Rad).

Quantitative determination of donkey milk lactoferrin was performed by a cationic-exchange chromatography on FPLC (MonoS HR 5/5 column, 1.0 ml bed volume). Column was equilibrated and eluted as described above (see first approach: ion-exchange chromatography). Stock solutions (20, 30, 40, 60, 80 μg/ml) were prepared from a standard solution of human lactoferrin 3 mg/ml from Gen Way Biotech and 1 ml of each stock solution was injected. Calibration curve was designed by relating the peak area of each solution eluted from Mono S as a function of μg loaded.

Chromatography

Three different chromatographic approaches were used to characterize the protein fraction by the ÄKTA Purifer FPLC system: ion-exchange chromatography (first approach), chromatofocusing (second approach) and reversed-phase (third approach). Each peak obtained after chromatography was subjected to dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The purified caseins were identified by Nterminal sequencing. The procedure is resumed in the following scheme.


Milk
centrifugation
Skimmed milk
pH 4.6
centrifugation
Whole caseins in buffer A
1st approach
Ionic-Exchange
(Mono S, Mono Q)
2nd approach
Chromatofocusing
(Mono P)
3rd approach
Reversed-phase
(C4-Prosphere)
SDS-PAGE
Blotting on PVDF
N-Terminal analysis and protein identification

Ion-exchange chromatography (first approach)

After isoelectric precipitation at pH 4.6, one aliquot of whole caseins, resuspeneded in CL buffer was subjected to a cation-exchange chromatography: 1 ml was injected onto FPLC Mono S HR 5/5 column, 1 ml bed volume, equilibrated in buffer A (50 mM ammonium acetate, 8 M urea, pH 5.5 or pH 7.0) and eluted by a linear gradient between buffer A and buffer B (1 M ammonium acetate, 8 M urea, pH 5.5 or pH 7.0). The gradient was: %B=0, Column Volume=10; %B=100, CV=100; %B=100, CV=10. After the chromatographic course, the fractions obtained from Mono S elution were pooled and were desalted by gel filtration chromatography on FPLC HiTrap Desalting column (Sephadex G-25 Superfine), equilibrated with 50 mM NH_4HCO_3 pH 7.9.

Then, the caseins were subjected to anionic-exchange chromatography on FPLC by a Mono Q HR 5/5 column, 1 ml bed volume, equilibrated with 50 mM NH_4HCO_3 pH 7.9 and eluted by a linear gradient between 50 mM NH_4HCO_3 and 1 M NH_4HCO_3 pH 7.9. The gradient was: %B=0, Column Volume=20; %B=100, CV=140; %B=100, CV=10. In all cases elution was monitored at 280 nm by UV 900 Monitor included in FPLC system.

Chromatofocusing (second approach)

Another aliquot of casein from isoelectric precipitation at pH 4.6 was subjected to gel filtration chromatography on FPLC using a Superdex 75 HR 10/30 column

equilibrated and eluted in buffer A pH 5.5 (50 mM ammonium acetate pH 5.5, 8 M urea). The void volume of the column (V_0) was determined using Blue Dextran 2000 (2000 kDa) and the column was calibrated by the Low Molecular Weight Gel Filtration Calibration kit (ribonuclease A 13.7 kDa, chymotrypsinogen A 25.0 kDa, ovalbumin 43.0 kDa, bovine serum albumin 67.0 kDa). The effluent was monitored at 280 nm and collected in 0.5 ml fractions. The calibration curve was obtained relating the logarithm of the molecular weight of each standard protein to the K_{av} ($K_{av}=V_e-V_0/V_t-V_0$) of relative standard.

Pool 1 and 2 from gel filtration were subjected to a chromatofocusing analysis on FPLC performed in two pH ranges, 6.0-4.0 and 7.0-5.0, using a Mono P HR 5/5 column, 1.0 ml bed volume. In the pH range 6.0-4.0 the chromatofocusing column was equilibrated with starting buffer at pH 6.3 (bis-tris 25 mM pH 6.3, 8 M urea) and eluted by a linear gradient with the eluent buffer at pH 4.0 (100 ml containing 10 ml Polybuffer, 8 M urea, adjusted to pH 4.0 with 1 M HCl). The gradient was: %B=0, Column Volume=0; %B=100, CV=50. In the case of the pH range 7.0-5.0 the starting buffer was at pH 7.0 and the eluent buffer at pH 5.0.

Reversed-phase chromatography (third approach)

Whole casein fraction obtained after isoelectric precipitation at pH 4.6 was suspended in CL buffer. Before injection, samples were diluted 1:3 in CL buffer and 100 µl were loaded onto RP column (C4 prosphere Alltech, 2 ml of bed volume) [26].

The samples for reversed-phase HPLC (RP-HPLC) from skimmed donkey's milk were clarified by the addition of two volumes of CL buffer. One hundred microliters of clarified samples were loaded into the RP-HPLC column.

The reversed-phase column was a C4 Prosphere Alltech, 2 ml of bed volume. The column was equilibrated in trifluoroacetic acid TFA/H_2O 1:1000 v/v (buffer A) and elution was achieved by the following step gradient with TFA/H_2O/acetonitrile 1:100:900 v/v (buffer B): %B=0, Column Volume=5; %B=20, CV=5; %B=40, CV=5; %B=60, CV=20. The proteins eluted were monitored at 280 nm.

This reversed-phase method was also used to study the chromatographic profile of skimmed milk. Sample was diluted in clarifying buffer 1:3 and then loaded onto the column. Before injection, all the samples were centrifuged at 13.000 rpm for 5 min by Beckman Microfuge. The proteins eluted from RP-HPLC columns were monitored at 280 nm by a UV 900 Monitor included in the HPLC system.

Each chromatographic peak eluted from each chromatographic course was collected and subsequently analyzed by SDS-PAGE.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done as described by Laemmli [42] under reducing conditions using a 15% or 13% acrylamide-bis acrylamide solution and Mini Protean II apparatus Bio-Rad. The markers were Bio-Rad molecular weight standards low range (phosphorylase b 97.4

kDa, bovine serum albumin 66.2 kDa, ovalbumin 45.0 kDa, carbonic anhydrase 31.0 kDa, soybean trypsin inhibitor 21.5 kDa, lysozyme 14.4 kDa). Five to ten μg of each sample were incubated with the denaturing loading solution (12% glycerol, 1.2% SDS, 5.4% β-mercaptoethanol, satured bromophenol blue) at 100° C for 5 min and then loaded onto the gel. Electrophoresis was performed with a constant voltage of 200 V. Coomassie Blue staining (0.1% Coomassie Brilliant Blue R 250 in 50% methanol and 10% acetic acid) visualized the proteins on the gel.

The chromatographic fractions of whey proteins were analyzed by 15% SDS-PAGE whereas the chromatographic fractions of caseins were analyzed by 13% SDS-PAGE.

Proteome analysis: PROTEin complement expressed by a genOME

This technique separates proteins in two steps: IEF (isoelectric focusing) and SDS-PAGE [50, 51].

IEF, the first-dimension step, separates proteins according to their isoelectric points. Ampholytes, a mixture of a low molecular weight organic acids and bases, establish a pH gradient and distribute themselves in an electric field generated across the strip-gel. When a protein mixture is applied each protein migrates until it reaches the pH value identical to its pI. Proteins with different isoelectric points are distributed differently throughout the strip-gel. SDS-PAGE, the second-dimension step, separates proteins according to their molecular weights. The strip-gel is then laid horizontally on a second gel and the proteins are separated by SDS-PAGE.

In this two-dimensional gel, horizontal separation reflects differences in pI; vertical separation reflects differences in molecular weight.

The proteomic analysis of donkey milk, based on two-dimensional electrophoresis (2-DE) followed by N-terminal analysis, may be a powerful tool to identify more proteins from the complex mixture of milk simultaneously [52, 53]. Milk proteins are characterized by genetic variants and post-translational modifications such as phosphorylation and glycosilation. Donkey milk contains a large number of proteins present in low concentrations such as lactoferrin, immunoglobulins, hormones that may be identified through the proteomic approach.

Donkey milk was initially skimmed by centrifugation at 3.000 g for 30 min at 16° C. The whey protein fraction was separated from caseins by isoelectric precipitation at pH 4.6 with 10% v/v acetic acid followed by centrifugation at 27.000 g for 20 min at 4° C. Supernatant represented whey fraction. Precipitated caseins were resuspended in clarifying buffer (100 mM bis-Tris pH 8.0, 8 M urea, 1.3% trisodium citrate, 0.3% DTT). Before the 2-DE analysis 200 μg of skimmed milk or whey fraction or casein fraction were processed with the 2D-Clean-Up kit to increase the electrophoresis resolution. The proteins precipitated after this treatment were resuspended in rehydration buffer (8 M urea, 2% CHAPS, 40 mM DTT, 0.002% bromophenol blue and 0.5% IPG buffer). The range of pH was 4-7 for casein, 3-11 for whey fraction and skimmed milk.

First dimension: isoelectric focusing (IEF) on immobilized pH gradient (Immobiline DryStrip gel, 18 cm) range 3-11 for analysis of skimmed milk and whey fraction; pH

range 4-7 for caseins. Terms of run: 50 μA/strip, rehydration of Immobiline DryStrip gel for 12 h, then 1 h at 500 V, 1 h at 1000 V and finally 4 h at 8000 V.

Second dimension: SDS-PAGE 12.5% for separation of casein fraction and skimmed milk while 15% for separation of whey fraction. Electrophoresis was performed by Bio-Rad Protean II at constant amperage of 30 mA. Spots were stained by Coomassie Blue or were transferred onto PVDF membrane by Western blotting (at constant amperage of 25 mA at 4° C) for N-terminal analysis.

N-terminal analysis and identification of proteins

Following electrophoresis the polyacrylamide gel was equilibrated for 10 min in 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) pH 11, 10% (v/v) methanol and blotted onto a PVDF membrane (Sequi-blot, Bio-Rad) equilibrate in the same buffer for at least 30 min. Before CAPS equilibration, PVDF membrane was soaked in 100% methanol for few seconds and in water for 4 min.

N-terminal amino acid sequence was determined by Edman degradation using an automatic protein sequencer (Protein Sequencer G 1000 A, Hewlett Packard) on passively transferred electrophoretic bonds on PVDF using a Trans-blot cell Bio-Rad at constant amperage of 250 mA at 4° C. Coomassie Blue staining (0.1% Coomassie Brilliant Blue R 250 in 50% methanol) visualized the bands on the gel.

The identification of proteins and peptides was performed by consulting the Swiss-Prot and TrEMBL protein databases available on the ExPASy (Expert Protein Analysis System) proteomic server (http://us.expasy.org/) at the Swiss Institute of

Bioinformatics. The α_{s1}- and β-caseins from donkey milk were identified by comparing the sequence obtained after N-terminal analysis with those of α_{s1}- and β-caseins from mare's milk. The accession numbers were: Q9GKK3 (β-casein precursor, *Equus caballus*); Q8SPR1 (α_{s1}-casein, *Equus caballus*). The accession numbers for donkey's milk lysozyme, α-lactalbumin and β-lactoglobulin, consulted to compare the obtained N-terminal sequences, were P11375, P28546, P13613 respectively. In all cases the sequence homology for the first 10 aminoacids was 100%.

Lysozyme and whey proteins content determination

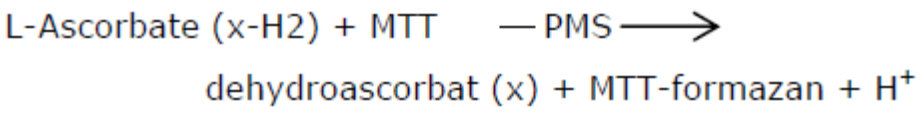
For the quantitative determination of lysozyme, α-lactalbumin and β-lactoglobulin milk samples were taken at different stage of lactation: 60, 90, 120, 160, 190 days after parturition.

Donkey's milk lysozyme and whey protein content was determined by an FPLC method using a reversed-phase column. Sample milk preparation, column equilibration and elution were performed as describe above. Each standard solution of egg white lysozyme (2.0, 1.0, 0.5, 0.25, 0.15 mg/ml), bovine milk β-lactoglobulin (0.17, 0.3, 0.75, 1.0, 1.5 mg/ml), bovine milk α-lactalbumin (0.25, 0.35, 0.5, 0.75, 1.0, 1.5 mg/ml) was prepared in clarifying buffer and 100 µl of each standard were separately loaded onto reversed-phase column. The area of each standard peak was measured using the valley-to-valley integration mode and quantification was achieved by a calibration curve obtained relating the concentrations in µg of each

standard loaded to the peak area corresponding to each concentration. The quantity of lysozyme, α-lactalbumin and β-lactoglobulin was determined by the calibration curve.

Vitamin C determination

The content of donkey milk L-ascorbate was determined by using a colorimetric method (Raiso Diagnostics kit) [45, 46]. The kit is based on the following principle: L-ascorbate and some more reducing substances reduces the tetrazolium salt MTT [3-(4,5dimethylthiazolyl-2)-2,5diphenyltetrazolium bromide] to a MTT-formazan in the presence of the electron carrier PMS (5-methylphenazinium methosulphate). In the assay with the sample the sum of the reducing substances is measured



The MTT-formazan was the measuring parameter and was determined by means of its light absorbance in the visible range at 578 nm. The absorbance difference between the sample and the sample blank (where ascorbate oxidase oxidized L-ascorbate to dehydroascorbate) was equivalent to the quantity of L-ascorbate in the sample. The dehydroascorbate formed did not react with MTT/PMS.

The absorbance was monitored by Beckman DU 640 spectrophotometer. The pH value of skimmed milk was adjusted to 3.5-4.0 by addition of citric acid to precipitate the proteins.

Pipette into cuvette:

Pipette into cuvette	Blank Sample	SAMPLE
Distilled Water	1.480 mL	1.500 mL
Vial 1 (buffer solution)	1.000 mL	1.000 mL
Vial 3 (AAox rec.)	0.020 mL	-
Sample solution	0.100 mL	0.100 mL

Mix and incubate for 6 min at 37°C.

Read the absorbance of the sample blank and sample (A1).

Start the reaction by the addition of:

Vial 2 (PMS) [2]	0.100 mL	0.100 mL

Mix (1) and allow the solutions to stand for 10 min at 37°C.

Read the absorbance of sample blank and sample immediately one after another (A2).

Calculation:

Determine the absorbance difference (A2-A1) for both sample blank and sample. Subtract the absorbance difference of the sample blank from the absorbance difference of the sample, thereby obtaining ΔAD-Ascorbic acid.

$$\Delta A_{\text{L-Ascorbic Acid}} = (A_2\text{-}A_1)_{\text{sample}} - (A_2\text{-}A_1)_{\text{sample blank}}$$

The value of ΔAL-Ascorbic acid should be as a rule at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of L-Ascorbic Acid can be calculated as follows:

$$C = \frac{VxMW}{\varepsilon x d x v x 1000} x \Delta A (g / L)$$

Where

V= Final Volume (mL)

v= sample Volume (mL)

MW= Molecular weight of L-Ascorbic Acid[176.13 g/mol]

ε= extinction coefficient of NADPH at 578 nm= 16.9 [$l \times mmol^{-1} \times cm^{-1}$]

d= light path (cm)

If sample has been diluted during preparation, the result must be multiplied by the dilution factor, F.

Optimum temperature and stability of donkey milk lysozyme toward temperature

The effect of temperature on lysozyme activity was investigated in the range 4°C-70°C. Adding the sample to assay medium at relative temperature started the enzymatic reaction.

The effect of temperature on lysozyme stability was monitored by incubating the enzyme at different temperatures (from 4°C to 70°C) for 6 min, rapidly cooled on ice, centrifuged and finally assayed at 25° C. At least 5 determinations were made for each point of temperature.

Powdered donkey milk

The lyophilized donkey milk was obtained in this manner: milk was sprayed into a reaction chamber, and immediately cooled under vacuum: the tiny ice crystals formed in this way from milk, instantly sublimated (changed directly from solid to water

vapour) and then were removed from the gas stream, while the milk powder was collected at the bottom.

Donkey milk was pulverized using the technique of spray-dryer with two steps: first the milk was treated for 25 sec at 90 ° C, then pulverized to 170 ° C under a flow of about 8 L / hour. The milk powder was reconstituted by dissolving 1.0 g of powder in 10 ml of distilled water.

Results and Discussion

Chromatographic proteome analysis

Ion-exchange chromatography followed by SDS-PAGE (first approach)

Total protein content determined on the whole casein by the method of Bradford [40] was 6.6 mg/ml. Casein fraction was separated into 5 peaks named A, B, C, D, E and in another 5 named F, G, H, I, J by cationic-exchange chromatography (Mono S) performed at pH 5.5 and pH 7.0 respectively (fig. 2a and 2b). Each peak was subjected to 13% SDS-PAGE (fig 2c and 2d respectively) to choose which one was suitable to N-terminal sequence: this analysis revealed mainly β-caseins (sequence: RKEELNVSS) and α_{s1}-caseins (sequence: RPKLPHRQPE) that represented considerable homology with α_{s1}- and β-caseins from mare milk [54, 55].

In particular peak A and peak B were identified as β-casein having a molecular weight of about 34.5 and 33.3 kDa respectively; peak D and peak E showed the same electrophoretic pattern with 2 bands of 33.0 and 30.7 kDa both identified as α_{s1}-caseins, as well as peak I that proved to be a mixture of two α_{s1}-caseins with a molecular weight of 31.3 and 29.4 kDa. Peak J, with a molecular weight of 29.4 kDa, was identified as α_{s1}-casein. The amount of peaks B and C was not sufficient for N-terminal analysis; however peak C could be a mixture of 2 α_{s1}-caseins since it showed the same electrophoretic pattern of peaks D and E. After SDS-PAGE peak G and H resulted not homogeneous: both of them showed one diffuse band of 36.1 kDa and 3 neighbouring bands with a molecular weight of 35.4 kDa (probable β-casein), 31.3 and 29.4 kDa (probable α_{s1}-caseins).

The anionic-exchange chromatography (Mono Q) separated donkey milk casein fraction into peaks consisting mainly of β-caseins.

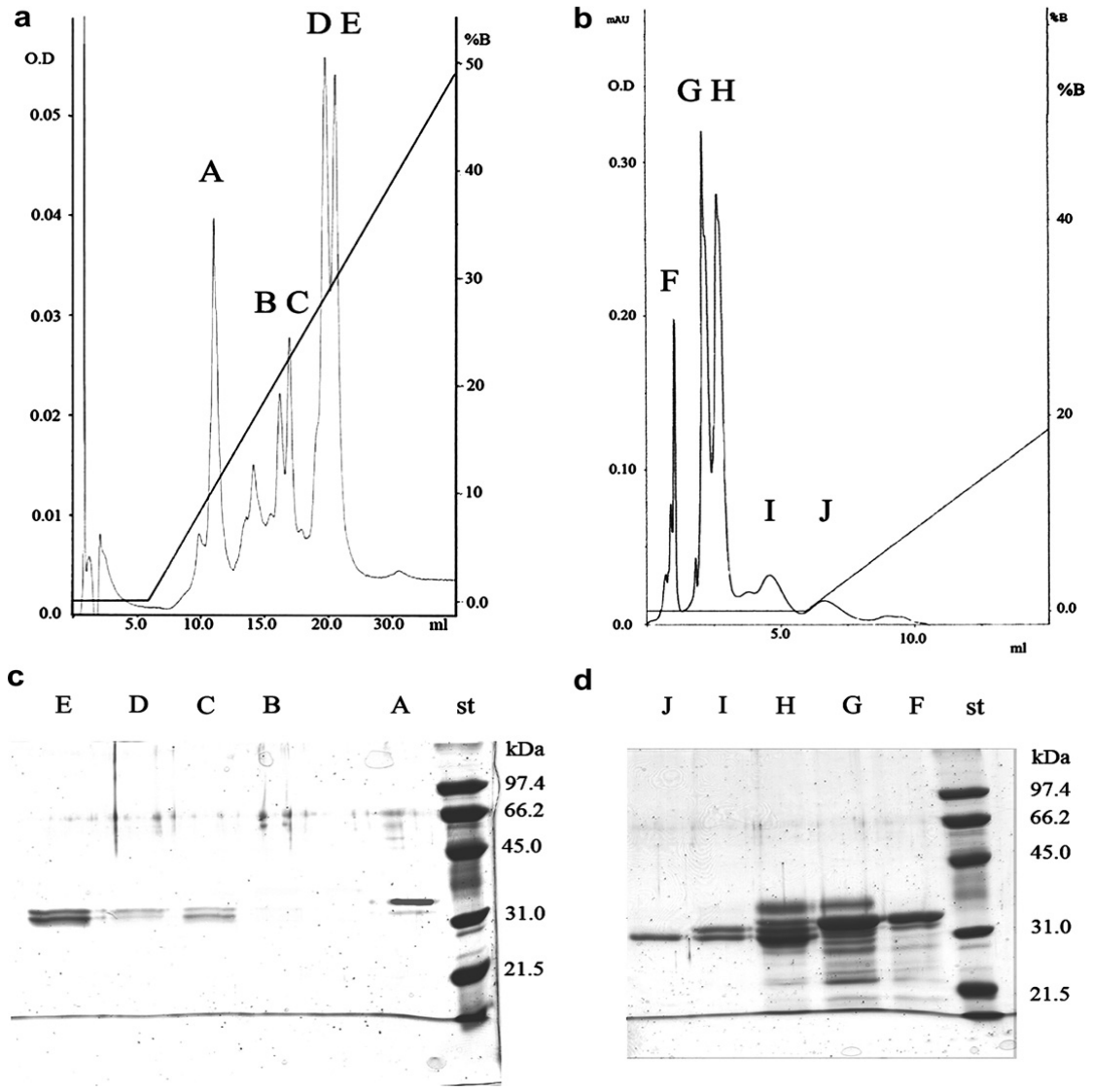


Fig. 2 Cationic-exchange chromatography on HPLC (Mono S HR 5/5) analysis on whole casein performed at pH 5.5 (a) and at pH 7.0 (b). 13% SDS- PAGE of the peaks separated by Mono S analysis at pH 5.5 (c) and pH 7.0 (d). (st) Bio-Rad low molecular weight standard (97.4 kDa, phosphorylase b; 66.2 kDa, bovine serum albumin; 45.0 kDa ovalbumin; 31.0 kDa carbonic anhydrase; 21.5 kDa, soybean trypsin inhibitor; 14.4 lysozyme).

Chromatofocusing followed by SDS-PAGE (second approach)

Another protocol based on the different isoelectric points and the different molecular weight of caseins was set up. As shown in fig. 3, three peak proteins were obtained from FPLC gel filtration. The molecular weight of peak protein was estimated using a calibration curve (see fig. 3, inset). Peaks 1, 2 and 3 revealed a molecular weight of 48.9, 32.3 and 7.0 kDa respectively. The retention time of the void volume was 15.1 min corresponding to a volume of 7.55 ml. High molecular weight of peak 1 might derive from the anomalous behaviour of caseins in this chromatography.

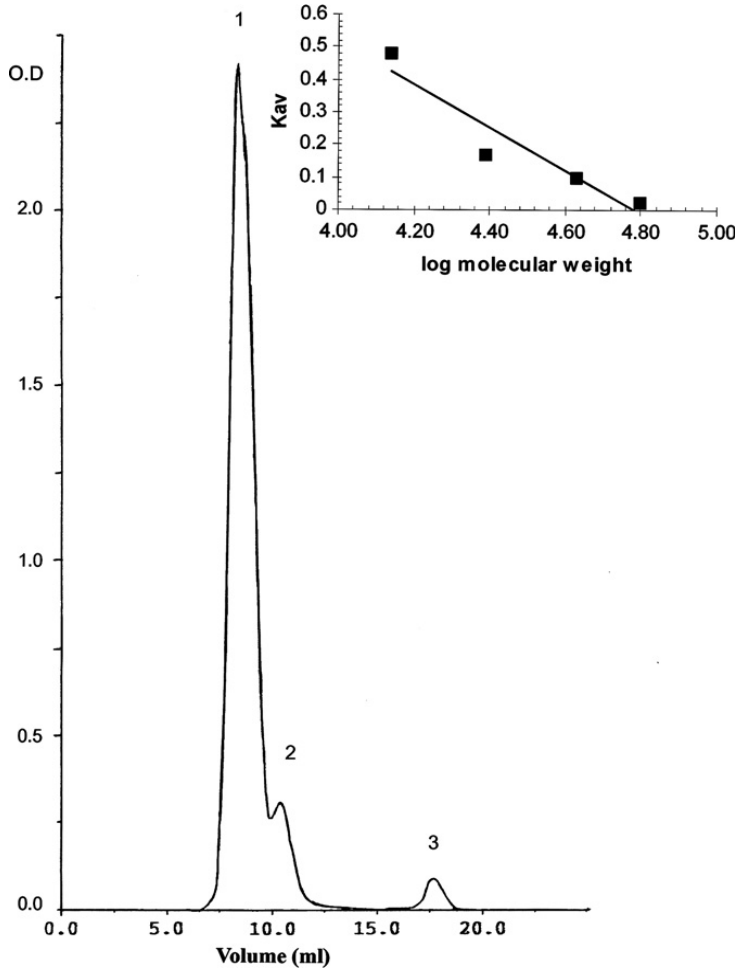


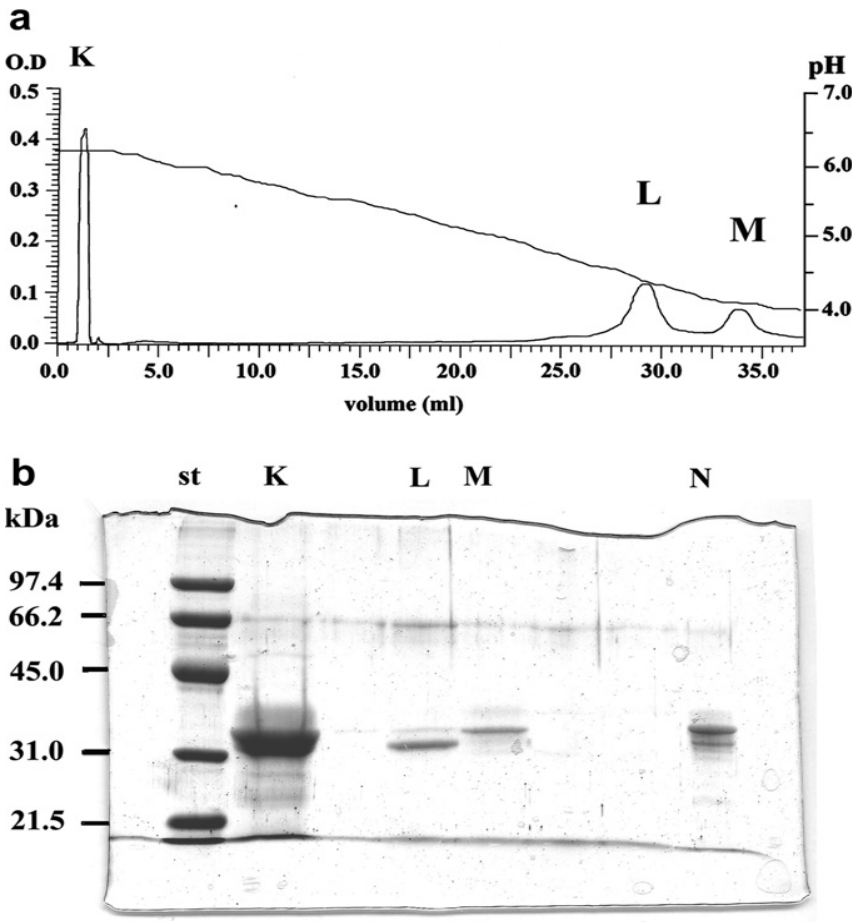
Fig. 3 Gel filtration analysis of whole caseins. Inset: calibration curve of the Superdex 75 obtained by LMW Gel Filtration Calibration Kit (ribonuclease A, 13.7 kDa; chymo- trypsinogen A, 25.0 kDa; ovalbumin, 43.0 kDa; bovine serum albumin, 67.0 kDa).

Peak 1 and peak 2 from gel filtration were subjected to chromatofocusing analysis whereas protein content of peak three was not enough to run another chromatography.

Chromatofocusing of peak 1, in the pH range 6.0-4.0, resulted in other three peaks named K, L and M (fig. 4a) while chromatofocusing of the same peak, in the pH range 7.0-5.0, showed only one peak named N (data not shown).

After a 13% SDS-PAGE analysis (fig. 4b) peak K was not homogeneous with a diffuse band having a molecular weight ranging from 34.5 to 28.7 kDa; peak L showed one band of 29.9 kDa not identified by N-terminal sequencing and peak M proved one band of 35.4 kDa. Peak N showed one intense band with a molecular weight of 35.4 kDa that resulted to be a β-casein by N-terminal analysis (sequence: REKEELNVSS) and another unidentified weak band of 31.2 kDa.

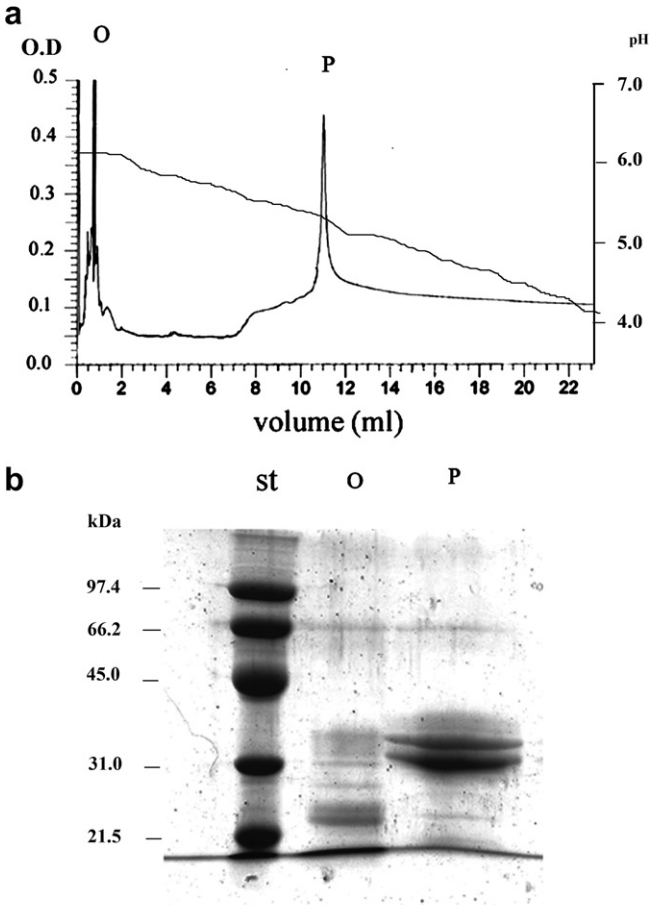
Fig. 4 Chromatofocusing analysis (Mono P H/R 5/5) in the pH range 6.0–4.0 of peak 1 eluted from the gel filtration. (b) 13% SDS-PAGE of peaks eluted from chromatofocusing. Peaks K, L, M, were obtained from chromatofocusing analysis in the pH range of 6.0–4.0. Peak N was from chromatofocusing analysis in the pH range of 7.0–5.0. (st) Bio-Rad low molecular weight standard (97.4 kDa, phosphorylase b; 66.2 kDa, bovine serum albumin; 45.0kDa, ovalbumin; 31.0kDa, carbonic anhydrase; 21.5 kDa, soybean trypsin inhibitor; 14.4 lysozyme).



Chromatofocusing of gel filtration peak 2 (pH range 6.0-4.0) resulted in two peaks named O and P (fig. 5a). After a 13% SDS-PAGE the protein amount in peak O was too low for sequencing while peak P showed one band of 35.4 kDa that resulted to be a β-casein and another band of 31.2 kDa (fig. 5b). Since peak 1 and peak 2 from gel filtration were not completely resolved, it may be possible that peak M, N and P (β-caseins having the same molecular weight) may come from the same gel filtration peak.

Fig. 5. (a) Chromatofocusing analysis (Mono P H/R 5/5) in the pH range 6.0–4.0 of peak 2 eluted from gel filtration. (b) 13% SDS-PAGE of peaks O and P eluted from chromatofocusing analysis in

the pH range of 6.0– 4.0. (st) Bio-Rad low molecular weight standard (97.4 kDa, phosphorylase b; 66.2kDa, bovine serum albumin; 45.0kDa, ovalbumin; 31.0kDa, carbonic anhydrase; 21.5 kDa, soybean trypsin inhibitor; 14.4 lysozyme).



Chromatofocusing did not result in a good separation of casein fraction; therefore a reversed-phase chromatography was undertaken.

Reversed-phase chromatography followed by SDS-PAGE (third approach)

Reversed-phase chromatography on FPLC (RP-FPLC) followed by 15% SDS-PAGE was performed on skimmed donkey milk and on casein fraction respectively.

Three main peaks were recovered from RP-FPLC of skimmed donkey milk (fig. 6a) named Q, R, S and identified after 15% SDS-PAGE and N-terminal sequence.

Peak Q with a molecular weight of 14.0 kDa resulted to be lysozyme (sequence KVFSKXELA); peak R was identified as α-lactalbumin 14.1 kDa (sequence KQFTKXELSQVLXSM); peak S with a molecular weight of 22.4 kDa was β-lactoglobulin (sequence TNIPQTMQ).

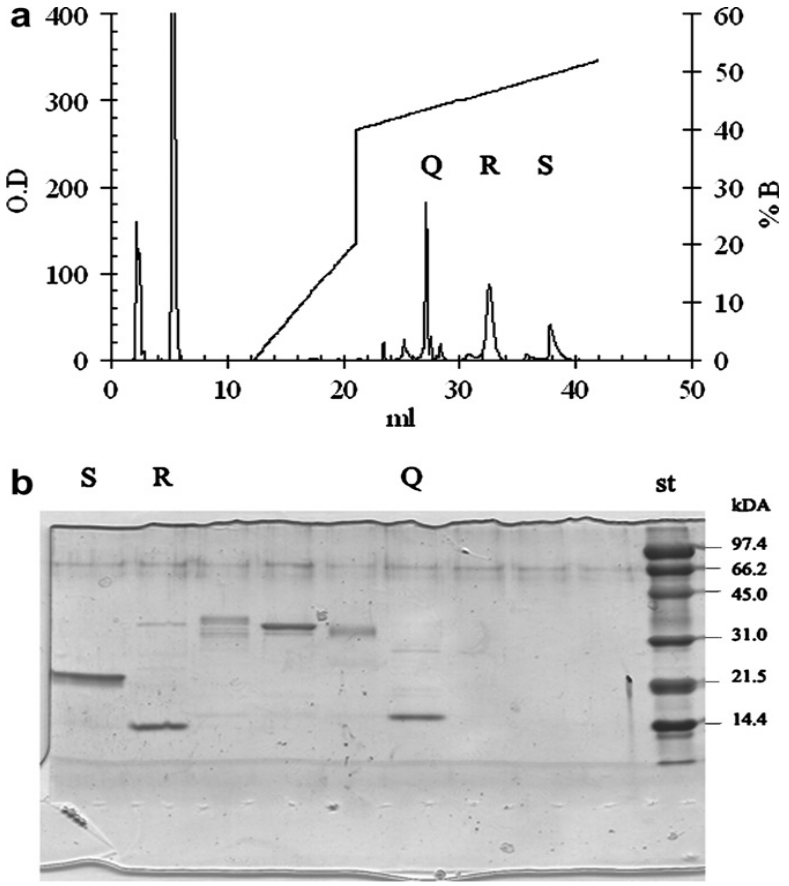


Fig. 6. (a) Reversed-phase HPLC of skimmed donkey's milk. (b) 13% SDS-PAGE of peaks Q, R and S eluted from RP-HPLC. (st) Bio-Rad low molecular weight standard (97.4 kDa, phosphorylase b; 66.2 kDa, bovine serum albumin; 45.0 kDa, ovalbumin; 31.0 kDa, carbonic anhydrase; 21.5 kDa, soybean trypsin inhibitor; 14.4 lysozyme).

RP-FPLC was also performed on donkey milk precipitated caseins. Five peaks were recovered (named from T to X fig. 7a) each of them run onto 13% SDS-PAGE (fig. 7b). Protein amount of peak T (a widespread band of 35.8 kDa) and peak U (the main band of 30.1 kDa) was not sufficient for N-terminal sequence. Peak V showed a more intense band with a molecular weight of 33.3 kDa identified as α_{s1}-casein (RPKLPHRQPE) and an unidentified weaker band of 35.1 kDa. Both peak W and peak X, with a molecular weight of 37.5 kDa, were identified as β-caseins. Furthermore, β-casein sequence of peak X (REKEALNV) showed an E→A substitution in the fifth amino acid.

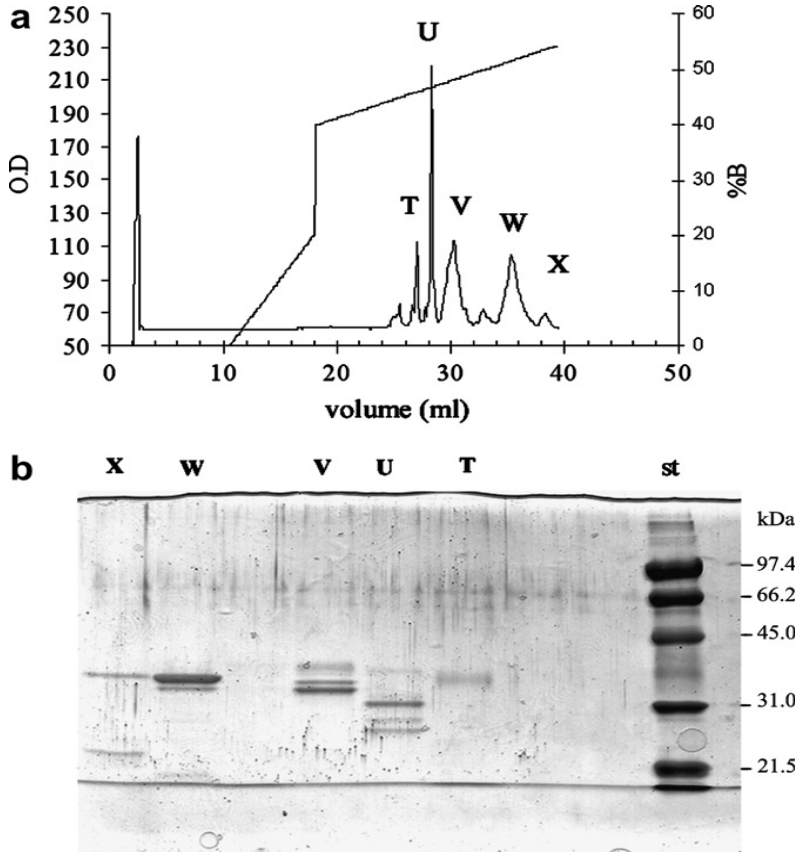


Fig. 7. (a) Reversed-phase HPLC of whole caseins precipitated at pH 4.6. (b) 13% SDS-PAGE of peaks T, U, V, W and X eluted from RP-HPLC. (st) Bio-Rad low molecular weight standard (97.4

kDa, phosphorylase b; 66.2 kDa, bovine serum albumin; 45.0 kDa, ovalbumin; 31.0 kDa, car- bonic anhydrase; 21.5 kDa, soybean trypsin inhibitor; 14.4 lysozyme).

Lysozyme and whey protein content determination

The concentration of total protein was 7.5 mg/ml [40].

Lysozyme, α-lactalbumin and β-lactoglobulin concentrations calculated quantitatively by RP-FPLC method at different stages of lactation (60, 90, 120, 160 and 190 days after parturition) are shown in table.

Quantitative determination of lysozyme, β-lactoglobulin, α-lactalbumin in different stages of lactation

Days after parturition	Lysozyme (mg/ml)	β-lactoglobulin (mg/ml)	α-lactalbumin (mg/ml)
60	1.34	Not determined	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

Lysozyme content was variable during different stages of lactation with a marked decrease at 160 days after parturition. The mean content was about 1.0 mg/ml. In human milk a quantitative determination of lysozyme showed a considerable variability during the entire period of lactation.

α-lactalbumin content 60 days after parturition was 0.81 mg/ml and increased two-fold at 90 days remaining relatively stable with a mean of about 1.81 mg/ml till the last stage of lactation [56, 57].

β-lactoglobulin content was stable during lactation with a mean value of about 3.75 mg/ml.

Through three different chromatographic methods followed by electrophoresis under reducing condition it was possible to separate and identify α_{s1}-caseins having N-terminal sequence RPKLPHRXPE and different molecular weights: 30.3, 31.3, 33.0 kDa and β-caseins having N-terminal sequence REKEELNVS and molecular weights varying from 33.0 to 37.5 kDa.

All donkey milk proteins identified are summarized in table 3.

It was not possible to determine the presence of other types of caseins such as α_{s2}-, γ- and κ- that were found in small amounts in mare milk. Probably in donkey milk these caseins are present in minute and therefore in undetectable quantities making their characterization difficult. The presence of α_{s2}-casein has not been demonstrated in human milk [26].

Lysozyme content varied considerably during different stages of lactation with a mean value of 1.0 mg/ml. This value is higher than in cow (traces), human (012 mg/ml) and goat milk (traces) but is close to mare milk (0.79 mg/ml) [58].

The mean content of β-lactoglobulin was 3.75 mg/ml and remained unchanged during the period of monitoring. This value is close to that of cow milk (3.3 mg/ml)

and pony mare milk (3.0 mg/ml) whereas in human milk β-lactoglobulin is absent [26, 28, 29].

α-lactalbumin content showed a considerable increase (up to 1.8 mg/ml) at three months after parturition and remained almost stable during lactation. This result is in good agreement with α-lactalbumin in human milk (1.6 mg/ml) but is lower compared to pony mare milk (3.3 mg/ml).

Table 3 shows a comparison of relative amounts of whey proteins and caseins in donkey, human and mare milk.

Donkey's milk proteins identified by three proteomic approaches

Chromatographic peak	Protein	Molecular weight (kDa)	N-terminal sequence
A (cationic exchange, pH 5.5)	β-Casein	35.4	REKEELNVS
B (cationic exchange, pH 5.5)	–	–	–
C (cationic exchange, pH 5.5)	Probable α_{S1}-casein	33.0	–
	Probable α_{S1}-Casein	30.7	–
D (cationic exchange, pH 5.5)	α_{S1}-Casein	33.0	RPKLPHRQPE
	α_{S1}-Casein	30.7	RPKLPHRQPE
E (cationic exchange, pH 5.5)	α_{S1}-Casein	33.0	RPKLPHRQPE
	α_{S1}-Casein	30.7	RPKLPHRQPE
F (cationic exchange, pH 7.0)	β-Casein	33.3	REKEELNVS
G–H (cationic exchange, pH 7.0)	–	36.1	–
	Probable β-Casein	35.4	–
	Probable α_{S1}-Casein	31.3	–
	Probable α_{S1}-Casein	29.4	–
I (cationic exchange, pH 7.0)	α_{S1}-Casein	31.3	RPKLPHRQPE
	α_{S1}-Casein	29.4	RPKLPHRQPE
J (cationic exchange, pH 7.0)	α_{S1}-Casein	29.4	RPKLPHQPE
K (chromatofocusing, pH 6–4)	–	34.5 ÷ 28.7	–
L (chromatofocusing, pH 6–4)	–	29.9	–
M (chromatofocusing, pH 6–4)	Probable β-Casein	35.4	–
N (chromatofocusing pH 7–5)	β-Casein	35.4	REKEELNVS
	–	31.2	–
O (chromatofocusing, pH 6–4)	–	–	–
P (chromatofocusing, pH 6–4)	β-Casein	35.4	REKEELNVS
	–	31.2	–
Q (reversed phase)	lysozyme	14.6	KVFSKXELA
R (reversed phase)	α-Lactalbumin	14.1	KQFTKXELSQVLXSM
S (reversed phase)	β-Lactoglobulin	22.4	TNIPQTMQ
T (reversed phase)	–	35.8	–
U (reversed phase)	–	30.1	–
V (reversed phase)	α_{S1}-Casein	33.3	RPKLPHQPE
	–	35.1	–
W (reversed phase)	β-Casein	37.5	REKEELNVS
X (reversed phase)	β-Casein	37.5	REKEALNVS

Different storage conditions

Some whey proteins may be used as quality indicators of milk. Different kinds of storage may induce changes in these components regarding fresh milk characteristics.

Fresh, frozen and powdered donkey milk whey protein content determination

The total whey protein content, determined by the method of Bradford [40], resulted to be similar in fresh, frozen and powdered milk: about 7.50 mg/ml.

The concentrations of α-lactalbumin, β-lactoglobulin and lysozyme were quite similar for fresh and frozen milk and these results seem to indicate that the freezing and thawing out process does not affect milk quality under this point of view.

Powdered formula, instead, showed a considerable reduction (about 50%) both of lysozyme and β-lactoglobulin whereas α-lactalbumin content resulted to be almost similar to the others two.

	lysozyme	**α-lactalbumin**	**β-lactoglobulin**
	(mg/ml)	(mg/ml)	(mg/ml)
Fresh milk	1.75±0.41	2.10±0.38	5.79±0.55
Frozen milk	1.52±0.30	2.21±0.42	4.47±0.81
Powdered milk	0.84±0.28	1.56±0.37	3.00±1.30

Table 4. Determination of lysozyme, α-lactalbumin and β-lactoglobulin content in fresh, frozen and powdered donkey milk. Reported values are the mean of five determinations.

Fresh, frozen and powdered donkey milk analysis by Reversed Phase-FPLC and SDS-PAGE

The RP-FPLC protein profile resulted to be similar in fresh and frozen milk while it was rather different in powdered formula concerning position, shape and size of peaks. Each chromatographic peak was run on a 15% SDS polyacrylamide gel. The electrophoretic analysis showed the main whey proteins in the three kind of milk: lysozyme (peak B) with a molecular weight of 14.60 kDa, α-lactalbumin (peak E) with a molecular weight of 14.12 kDa, β-lactoglobulin (peak F) with a molecular weight of 22.40 kDa. Peak D and E corresponded also to casein fraction with a molecular weight ranging from 44.0 to 29.0 kDa.

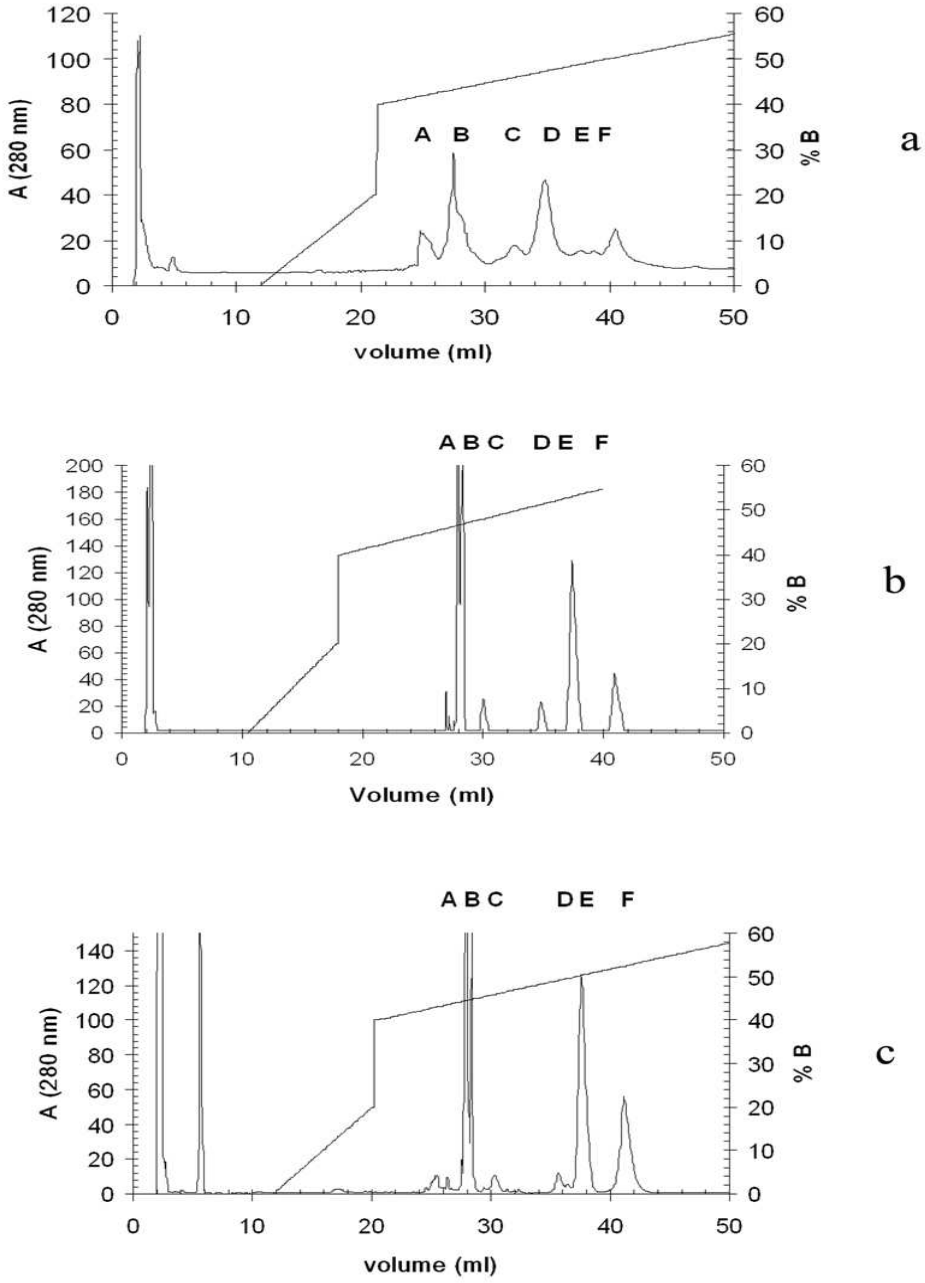


Fig 8. Reversed-Phase FPLC of skimmed powdered milk (a), frozen milk (b) and fresh milk (c)

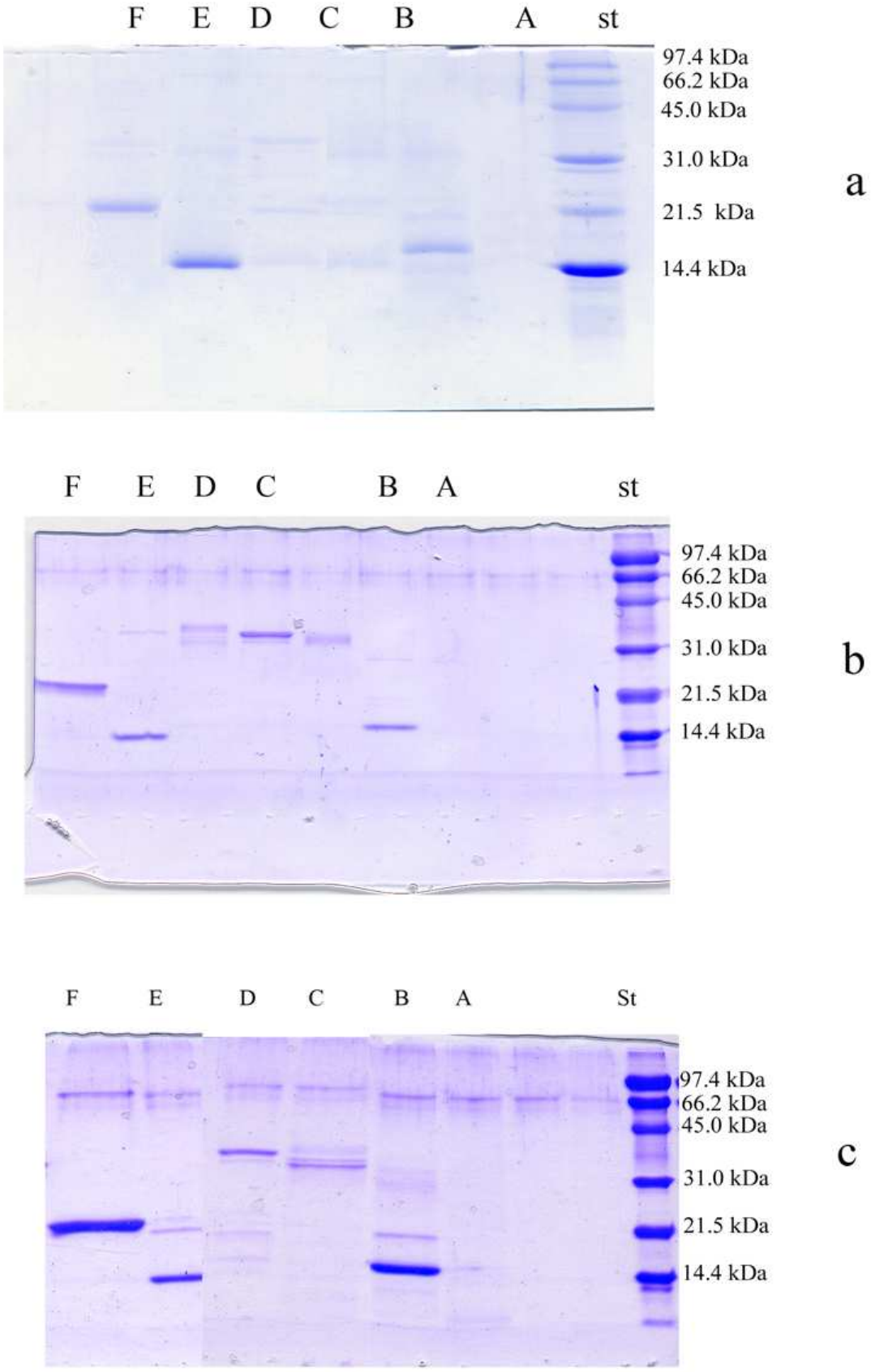


Fig 9. 15% SDS-PAGE of the peaks A, B, C, D, E, F eluted from the RP-FPLC. a) powdered milk, b) frozen milk, c) fresh milk. st: Bio-Rad low molecular weight standard (97.4 kDa, phosphorylase

b; 66.2 kDa, bovine serum albumin; 45.0 kDa, ovalbumin; 31.0 kDa, carbonic anhydrase; 21.5 kDa, soybean trypsin inhibitor; 14.4 kDa, lysozyme).

Lysozyme and lactoperoxidase activity in fresh, frozen and powdered donkey milk

Lysozyme activity in fresh and frozen milk resulted to be very similar: 0.66±0.0084 U/ml in the first kind, 0.61±0.1 U/ml in the second. Probably freezing and thawing out process does not affect the activity of donkey milk lysozyme.

In powdered formula lysozyme showed a 50% lower activity (0.32±0.01) than that in fresh sample. The high temperature use in spry-drying process for milk powderization strongly affects the activity of the enzyme.

	Lysozyme activity (U/ml)	**Lactoperoxidase activity (mU/ml)**
Fresh milk	0.66±0.0084	4.83±0.35
Frozen milk	0.61±0.1	1.39±0.23
Powdered milk	0.32±0.01	2.88±0.51

Table 5. Lysozyme and lactoperoxidase activity determination on fresh, frozen and powdered donkey milk. The reported values are the mean of five determinations

The very low peroxidase activity found in donkey milk (4.83±0.35 mU/ml in fresh milk) may be indicative of low peroxidase concentration in this kind of milk. Also in human milk the amount of lactoperoxidase is very low about 770 μg/ml [59] whereas in cow milk is about 0.03↔0.1 mg/ mg/ml [60].

Frozen milk showed the lowest value of 1.39±0.23 mU/ml whereas in powdered milk the result was 2.88±0.51 mU/ml and this higher value may be due to the thermostability of the enzyme [61].

Optimum temperature and stability of donkey milk lysozyme toward temperature

As shown in figure 10 donkey milk lysozyme started to be active at temperature of about 15° C showing an optimum of temperature of 35-40° C. At 50° C it retained 80% of activity and finally it showed a 50% of residual activity when temperature increased until 70° C.

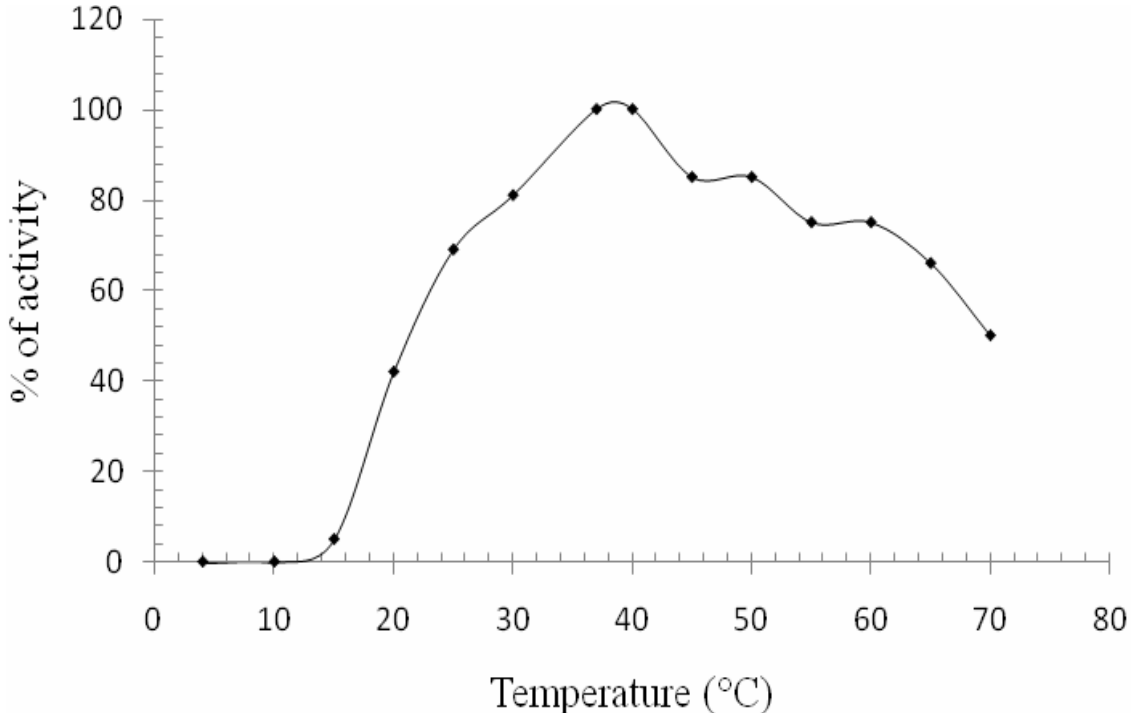


Figure 10. Effect of temperature on lysozyme activity. The enzymatic activity was measured for 6 min at different temperatures (from 4 to 70°C). Each point is the mean of five determinations.

Lysozyme thermostability was also monitored in the range from 4° C to 70° C (fugure 11). The enzyme was thermostable (about 100% of residual activity) from 4°

C to 65° C; after this temperature the activity decreased indicating that an irreversible denaturing process of the enzyme started from this temperature.

These observations may be important in the choice of a valid method of storage for donkey milk, in fact the presence of an active lysozyme may be useful to prevent or to reduce intestinal infections in infants.

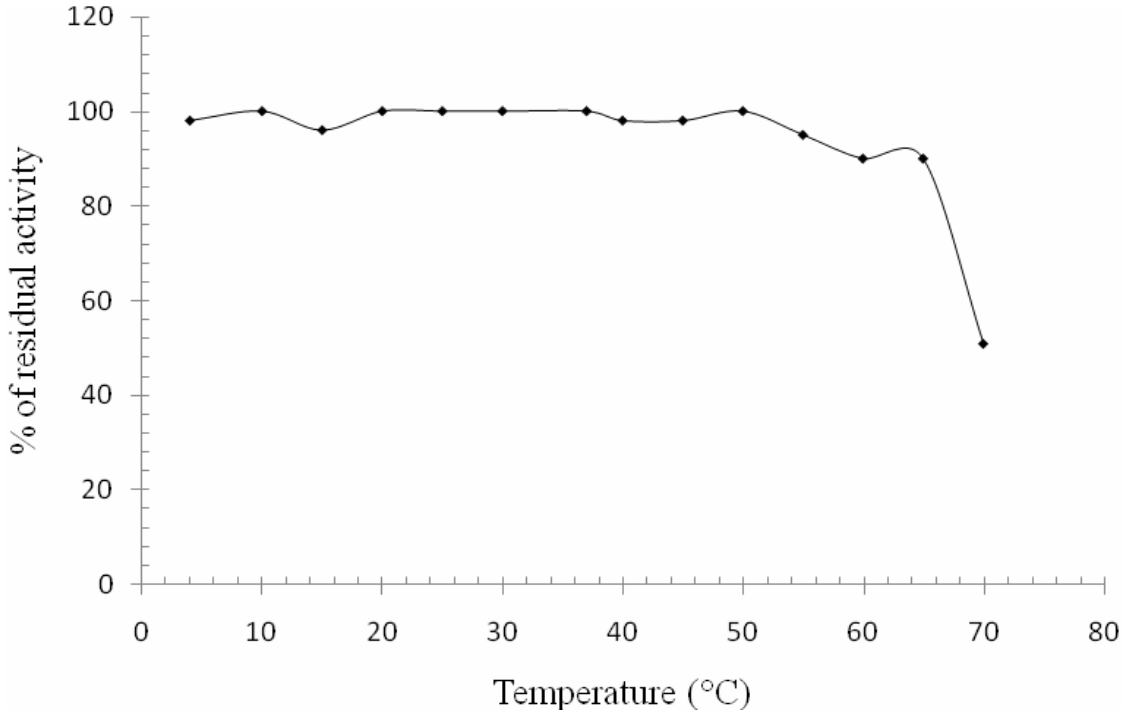


Figure 11. Effect of temperature on lysozyme thermostability checked by incubating the enzyme at different temperatures (from 4 to 70°C) for 6 min and then assayed at 25°C. Each point is the mean of five determinations.

These results seem to indicate that frozen donkey milk has a nutritious value not so different compared to fresh donkey milk whereas the thermal process of spry-drying in production of powdered donkey milk causes the loss of some nutritional characteristics.

2D Proteome analysis

By 2D analysis was performed to characterize total skimmed donkey milk, whey fraction and casein fraction.

Some spots separated from whey fraction (fig. 12) were analyzed by N-terminal sequence and it was possible to determine the following proteins: spot A (molecular weight of about 68.0 kDa) serum albumin and immunoglobulins; spot B (molecular weight of about 22.0 kDa, sequence TNIPQTMQDLDLQEV) β-lactoglobulin; spot CI and spot CII (molecular weight of about 12 kDa, sequence KQFTKHELSQVLKSM) two isoforms of α-lactalbumin; spot D (molecular weight of about 14.0 kDa, sequence KVFSKHELAHKL) lysozyme C.

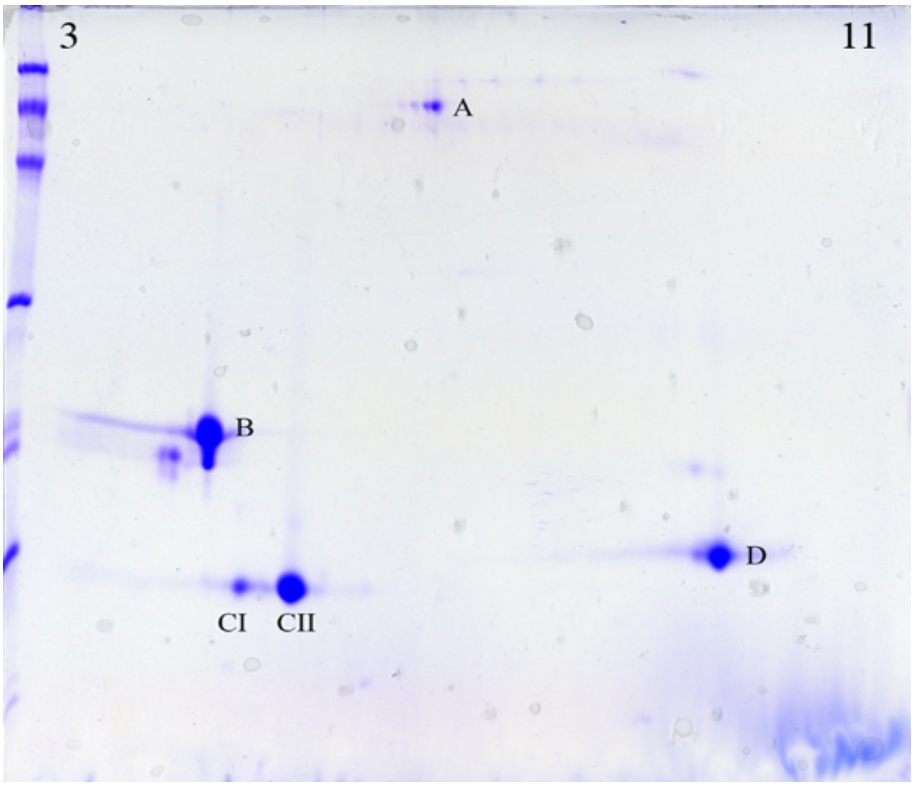


Fig. 12. IEF on immobilized pH gradient (range 3-11) for analysis of skimmed milk and whey fraction. Second dimension: SDS-PAGE 15%. Spots were transferred onto PVDF membrane by Western blotting.

Casein fraction (fig. 13) showed a considerable number of spots with molecular weights ranging from 25.7 to 38.0 kDa.

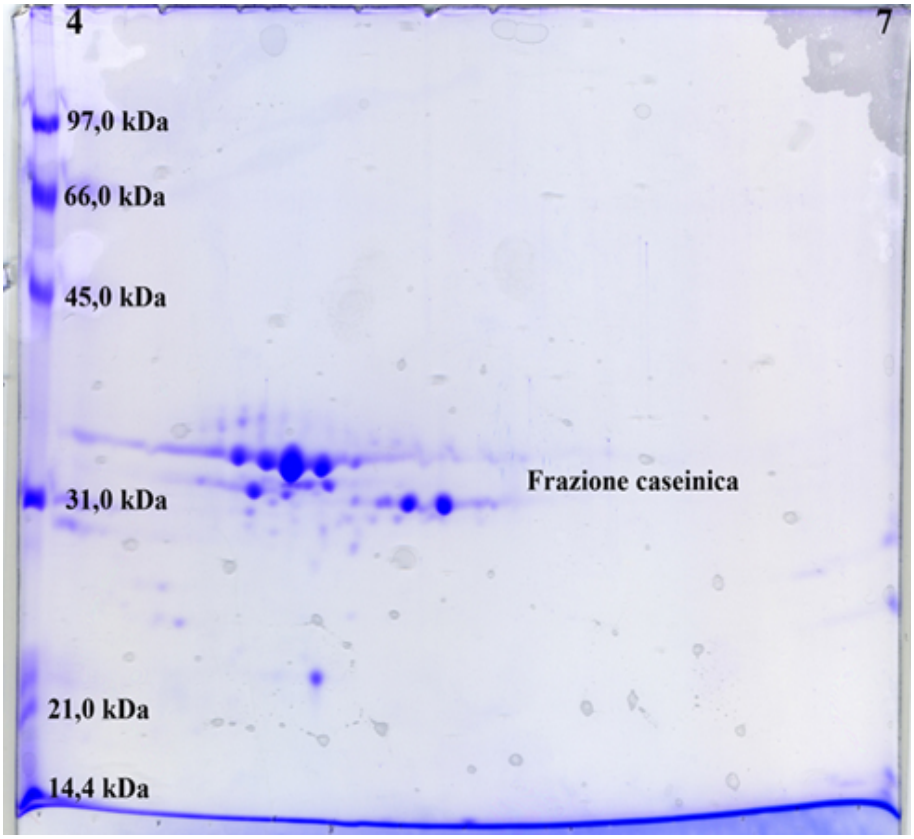


Fig. 13. IEF on immobilized pH gradient (range 4-7) for analysis of casein fraction. Second dimension: SDS-PAGE 12.5%. Spots were transferred onto PVDF membrane by Western blotting.

Isolation of lactoferrin from donkey milk

In addition to nutritional constituents milk contains proteins crucial against infections of both the digestive and respiratory tract such as lactoferrin, a member of the system of innate immunity.

Lactoferrin is a protein of the transferring family and is particularly resistant to the proteolytic degradation in contrast to other milk protein [45-47]. It controls the

proper composition of the intestinal microflora suppressing the growth of pathogenic bacteria and promoting the multiplication of non-pathogenic *Lactobacillus* and *Bifidobacterium*.

Skimmed donkey milk was prepared as described in the previous session (see sampling). Whey protein fraction was subjected to cationic-exchange chromatography on FPLC. The column (Mono S HR 5/5, 1 ml bed volume) was equilibrated in buffer A (50 mM ammonium acetate pH 5.5) and eluted by a linear gradient between buffer A and buffer B (1 M ammonium acetate pH 5.5, 1 M NaCl). 10 mg of sample were loaded onto the column. The gradient was: %B=0, Column Volume=10; %B=100, CV=100; %B=100, CV=10. UV 900 monitor of FPLC system monitored elution at 280 nm. Fractions corresponding to chromatographic peaks eluted from the column were analyzed by 15% or 7.5% SDS-PAGE. Coomassie Blue stained bands.

Whey proteins eluted from Mono S and separated by 7.5% - SDS-PAGE in acrylamide were subjected to western blotting (constant voltage of 50V for 2 hours at 4° C). The nitrocellulose membrane was blocked with TBST (Tris Buffer Saline, 0.1% Tween 20), 1% BSA and then was incubated with rabbit anti-human lactoferrin antibody diluted 1:2000 in TBST. After a series of washing in TBST the membrane was incubated for 30 min with the horseradish peroxidase-conjugated anti-rabbit secondary antibody diluted 1:4000 in TBST. The complex antibody-protein was visualized by the chromogenic-substrate 3'-1' diamminobenzidine (HRP conjugate substrate kit, Bio-Rad).

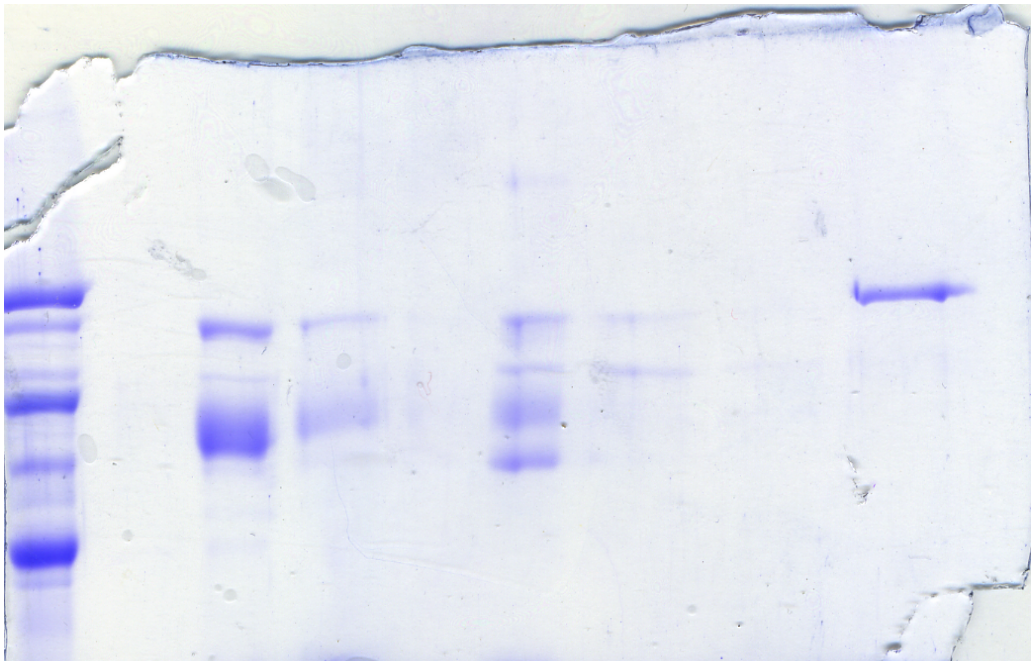


Fig. 14. Fig 9. 7.5% SDS-PAGE of peaks eluted from Mono S. Donkey milk lactoferrin showed a molecular weight of about 80.0 kDa. Bio-Rad low molecular weight standard (97.4 kDa, phosphorylase b; 66.2 kDa, bovine serum albumin; 45.0 kDa, ovalbumin; 31.0 kDa, carbonic anhydrase; 21.5 kDa, soybean trypsin inhibitor; 14.4 kDa, lysozyme).

Vitamin C

Vitamin C is a powerful reducing agent and antioxidant and is essential in many enzymatic reactions. Recent studies have shown that intake of vitamin C from milk may be associated with a reduced risk of atopic dermatitis.

The total vitamin C content determined in donkey milk was 35µg/ml. This value remained identical only in lyophilized donkey milk (data not shown).

Conclusions

It was possible to identify α_{s1}-caseins having N-terminal sequence RPKLPHRXPE and variable molecular weight (from 30.0 to 33.0 kDa). It was also possible to identify β-caseins having N-terminal sequence REKEELNVS and molecular weights varying from 33.0 to 37.5 kDa. Molecular mass of each class of equids caseins is in the range of 19.0-25.0 kDa [26] but in the present study they showed a molecular mass ranging from 30.0 to 37.5 kDa. However an abnormal behaviour of monomeric caseins in Laemmli separation was observed in earlier reports [63]. Instead it was not possible to detect presence of α_{s2}-, γ- and κ-caseins. Perhaps these caseins are present in undetectable quantities and could be separated by different procedures of those described here [54, 55].

It was studied some whey proteins in fresh, frozen and powdered donkey milk in order to better characterize the behaviour of this kind of milk at different storage conditions. These whey proteins may be used as quality indicators related to modifications in milk protein structure induced by a specific technological treatment [64, 65, 66]. These results seem to indicate that frozen donkey milk has a nutritious value not so different compared to fresh one while thermal process utilized in production of powdered formula causes the loss of some nutritional characteristics. Further investigations may be useful considering the need of donkey milk availability.

The high lysozyme and α-lactalbumin content found in donkey milk may be responsible for the low bacterial count reported in literature. Lysozyme,

lactoperoxidase and lactoferrin have been recognized as antimicrobial and antibacteriostatic agents and could be useful to prevent intestine infections in infants. Their action may extend the conservation of fresh donkey milk and the relative potential commercial distribution.

On the basis of results obtained donkey milk may be considered suitable for feeding young children affected by severe cow's milk allergy. In past it has been widely used to replace human milk because chemical composition and particularly protein content are close to that of human.

Food is called functional if it contains one or more components that can provide a benefit to human health, beyond their traditional nutritional role. Donkey milk may be configured as functional food in early childhood and not only.

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