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Chapter 3

CASEINS CHARACTERISTICS IN EQUID AND HUMAN MILK

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ABSTRACT

Equid milk is similar in composition to human milk, even if equids and humans are phylogenetically distantly related. The protein content of milk varies considerably between species and reflects the growth rate of the young. For humans, one of the slowest growing and maturing species, it takes 120-180 days to double birth weight and only 7% of calories come from protein. Equid species take between 30 and 60 days to double their birth weight and, like humans, have an exceptionally low level of protein in their milk.

Caseins are the predominant class of proteins in bovine milk, about 80% of total milk protein, while equid and human milk contains less caseins and more whey proteins. The biological function of the caseins lies in their ability to form macromolecular structures, casein micelles, which transfer large amounts of calcium to the neonate with a minimal risk of pathological calcification of the mammary gland. Casein micelles

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are hydrated spherical structures with dimensions in sub-micron range. Equine micelles are larger than bovine or human micelles, while those of donkey milk are similar in size to bovine micelles. Caseins are precipitated by gastric acid and enzymes, forming a clot in the stomach that entraps fat. The hardness of this clot depends on the casein content of the milk: high casein-containing milks will produce firm clots. Generally, species that nurse their young at frequent intervals, for example equids and humans, tend to produce dilute milk in which less than 60% of total proteins is casein and which form a soft clot, whereas animals that nurse infrequently, such as bovine and ovine, produce milk that is high in fat and caseins and has much longer gastric retention. The contents of casein and whey proteins in human milk change deeply early in lactation: the concentration of whey proteins is very high, whereas casein is virtually undetectable during the first days of lactation. As a consequence, there is not a fixed ratio of whey to casein in human milk, it varies throughout lactation. The major constituent of the family of human caseins is β-casein, a highly phosphorylated protein. During digestion, phosphopeptides are formed and have been shown to keep Ca^{2+} soluble, thus facilitating its absorption. It is therefore likely that phosphopeptides formed from β-casein contribute to the high bioavailability of calcium from human milk. K-casein, a minor casein subunit in human milk, is a glycosylated molecule that has been shown to inhibit the adhesion of *Helicobacter pylori* to human gastric mucosa, by acting as a receptor analogue. The presence of α-casein in human milk has not been clearly demonstrated.

Protein profile of equid milk is quite similar to human milk; therefore, equid milk may be more suitable in human nutrition than bovine milk. In this work the attention was focused especially on the molecular characteristics of caseins from equid and from human milk compared also to those of bovine milk. Furthermore, the different allergenic properties of casein from equid and bovine has been taken into account.

Keywords: caseins, human milk, equid milk

INTRODUCTION

Milk is thought to be the main source of biologically active compounds for infants, providing antibacterial and antiviral activities, facilitating nutrient absorption, promoting bone growth, enhancing immunological protection and supporting the development of host immune competence (Sun and Jenssen,

2012). After childhood, milk has become a common component of the diet of many people probably because it is a palatable product, easily available, well tested, and finally because is a food which reminds us of pour first life experience. Milk proteins appear to be an interesting link between nutrition, dietetics and therapy (Fiat et al., 1993). Milk proteins can be classified into 3 groups: mucins, caseins and whey proteins. Mucins, also known as milk fat globule membrane proteins, surround the lipid globules in milk and contribute only a small percentage of the total human content of human milk (Lönnerdal, 2003). Because the fat content of human milk does not vary during the course of lactation, the milk mucin concentration is most likely constant. The contents of casein and whey proteins change significantly early in lactation; the concentration of whey proteins is very high, whereas casein is virtually undetectable during the first days of lactation (Kunz & Lönnerdal, 1992). Subsequently, casein synthesis in the mammary gland and milk casein concentrations increase, whereas the concentration of total whey proteins decreases, partially because of an increased volume of milk being produced. As a consequence, there is no "fixed" ratio of whey to casein in human milk, it varies throughout lactation. The frequently cited ratio of 60:40 is an approximation of the ratio during the normal course of lactation, but it does vary from 80:20 in early lactation to 50:50 in late lactation. The main role attributed to caseins is mineral binding and their capacity as carriers, mainly for calcium and phosphorus, forming a coagulum and improving tehir digestibility in the stomach (Holt et al., 2013).

Bioactive peptides have been defined as specific protein fragments that have a positive impeact on body functions and conditions and may ultimately influnece health (Kitts & Weiler, 2003). Milk proteins are the main source of many bioactive peptides, with different important physiological functions. The bioactive peptides that result from the hydrolysis of caseins and whey proteins, or that are generated during digestion, can act in several ways, including promoting immunomodulation, stimulating bone formation, and improving an array of other body functions as a result of their opioid, antimicrobial and antiviral properties ((Pacheco et al., 2008).

Caseins, representing about 80% of total cows milk proteins, consist of four families, α_{s1}, α_{s2}-, β- and k- families, in the approximate ratio 38:11:38:13 (Pinto et al., 2012).

CASEINS MICELLES STRUCTURE

Casein Proteins

Milk is composed of numerous specific proteins, the major milk proteins, including caseins, β-lactoglobulin and α-lactalbumin, are synthesized in the mammary epithelial cells and are only produced by the mammary gland in response to lactogenic hormones. Once synthesized, caseins are secreted as large colloidal aggregates termed micelles. Caseins have an appropriate amino acid composition that is important for growth and development of the newborn. This high quality protein in cow milk is one of the key reasons why milk is such an important human food. Caseins have been and still are the most studied food proteins precisely to the fact that form micelles that are responsible for the physical properties of milk. The four types of caseins named α_{s1}-, α_{s2}, β-, and κ-caseins present a heterogeneity due to post-translational processing, alternative splicing of the gene product or genetic polymorphisms. The caseins have been studied by several authors especially in bovine milk, where there are numerous articles on the topic. Casein proteins can be divided into two groups: the calcium-sensitive and the non-calcium-sensitive. As regards to bovine milk, κ–casein is calcium-insensitive whereas α_{s1}-, α_{s2}, β-caseins are the three calcium-sensitive members and, differently to the k-casein, are highly phosphorylated (Horne, 2006).

Bovine α_{s1}-caseins represent the major protein fraction, are present in two forms, a larger and a smaller one, and each has different degrees of phosphorylation (Eigel et al., 1984). α_{s1}-caseins aminoacidic sequence contains multiple phosphorylation that occurs at serine or threonine level and is part of the motif: Ser-Thr-X–Y, where X indicates the position of a generic aminoacid and Y the position of an acidic residues. Bovine α_{s1}-casein exists in two phosphorylated forms containing 8 and 9 phosphates/mol of protein (Mercier, 1981). Furthermore have been described naturally occurring variants of the α_{s1}-caseins in the milk of several dairying species: for example in bovine milk there are two variant (named B and C) that differ for the presence of a glutamate and glycine residue respectively (Ng-Kwai Hang & Grosclaude, 1992). There is also a rare A variant with a 13 residue deletion between Glu14 and Ala26 (in the mature peptide).

The α_{s2}-like caseins represent a more disparate group than the α_{s1}-caseins, also this class of casein has a high degree of phosphorylation, for example

bovine milk α_{s2}- caseins contains four differentially phosphorylated isoforms with 10-13 phosphate groups/mol of protein.

In bovine milk the β-caseins are particularly rich in glutamines and present a single major phosphorylation site containing 5 phosphate/mol of protein, located near the N-terminus, therefore the phosphorylation level is less than α_{s1}- and α_{s2}-caseins. However, in the milk of other species (e.g., human and goat) β-casein has multiple phosphorylation state (Greenberg et al., 1984). Furthermore, in bovine milk has been identified a number of fragment of peptides named γ1, γ2 and γ3-caseins and the proteose peptone components which derived from the partial proteolysis of β-casein by plasmin. For bovine milk β-casein three variants have been reported: A1, A2 and B that differ among them in single aminoacidic substitutions (Ng-Kwai Hang & Grosclaude, 1992; FitzGerald, 1997).

κ-casein is characterized by the lower phosphate content than the other caseins and by the fact that is the only casein soluble in the presence of calcium ions. In the κ-casein there is a single phosphorylation site located in the C-terminal region of the protein. Furthermore it has been shown that κ-casein this is the only class of casein to contain carbohydrate moieties (Fiat et al., 1980; Brignon et al., 1985; Gorodetskii & Kaledin, 1987). Carbohydrate groups are linked to serine and threonine residues (by a O-glycosidic bond) located at the C-terminal aminoacidic part of κ-casein. Glycosylation occurs by a post-translationally modification within the Golgi apparatus of mammary epithelial cells, is increased during the colostral period, and in response to infection but decreases with successive periods of lactation (Dziuba & Minkiewicz, 1996). In bovine k-casein the carbohydrate portion contains galactose, N-acetyl galactosamine and N-acetyl neuraminic acid. κ-casein may be subjected to a proteolytic cleavage by chymosin (rennin), an aspartate protease that recognizes a specific Phe-Met bond (in cow, sheep, goat) or a Phe-Ile or Phe-Leu bond in (rat, mouse, pig, human) located in the C-terminal portion of the protein giving as products the glycosylated C-terminal fragment (named glycomacropeptide) and the hydrophobic N-terminal peptide (named *para-k*-casein) (Jolle's et al., 1968). In bovine milk has been described the presence of two variants of κ-casein, A and B that differ at residues 136 and 148 (A: Thr and Asp; B, Ile and Ala respectively) (Grosclaude et al., 1972). As regard casein concentration, in bovine milk the total amount is about 25-26 g/L, distributed as follows: α_{s1}- and β-casein (9 and 11 g/L respectively), α_{s2}- and κ-casein (3g/L each). In table 1 is reported the total concentration (g/L) of the single fraction of caseins from bovine, human and donkey milk.

Table 1. Composition of the casein fraction of bovine, human, equine and donkey milk. Furthermore the casein micelles size of each species is reported in the last row

	Bovine (g/L)[a]	Human (g/L)[a]	Equine (g/L)[b]	Asinine (g/L)[c]
Total caseins	27.2	5.8	14.0	6.60
α_{s1}-caseins	10.0	0.8	2.5	n.d.
α_{s2}-caseins	3.7	--	0.2	unknown
β-caseins	10.0	4.0	11.0	n.d.
κ-caseins	3.5	1.0	0.25	unknown
Micelle size (nm)	182	64	255	100-200

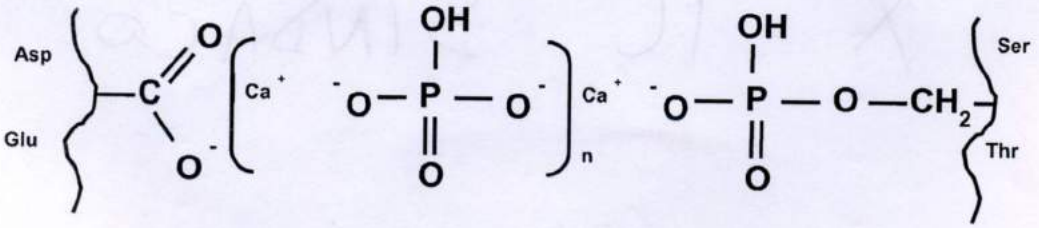
[a] Martin and Grosclaude, 1993; [b] Miranda et al., 2004; [c] Vincenzetti et al., 2008.
[d] Uniacke-Lowe and Fox, 2012.

Casein Micelles

Caseins are present in whole milk as macromolecular aggregates of proteins and minerals forming colloidal particles named micelles with a mean size of 120 nm (Fox & Brodkorb, 2008). This particular supramolecular aggregate is responsible of the opalescence characteristic of skim milk. The function of the micelles is to fluidize casein molecules and solubilize the calcium and the phosphate.

A micelle contains about 94% protein and 6% colloidal calcium phosphate (CCP, which include calcium, magnesium, phosphate, and citrate). The function of CCP is to cement casein micelle, in fact its removal by chelating agent causes micelle dissolution at low temperature (Horne, 2006). One micelle contains 10^4 casein molecules that are linked to each other by hydrophobic and electrostatic interactions and by their interactions with calcium phosphate, the latter fundamental for the maintenance of the micelle structure (Farrell &Thompson, 1988; Holt, 1992). Calcium can bind directly to the phosphorylated residue of the caseins or can be associated to the micelle but not directly bound to casein. Also the carboxylic group of aspartate or glutamate at phosphate clusters can bind calcium (see the scheme below).

Although the proportions of the various caseins vary widely among species, and there are variations in casein components, all species can form colloidal casein micelles for the transport of calcium and phosphate and at the ultra-structural level, the casein micelles of most species seems to be similar.



Several model of bovine milk casein micelle structure has been proposed, the most accepted structure models are the coated sphere model, the sub-micellar model, the Holt model and the Horne model. These models will be briefly explained below.

The coated sphere model describes the initial formation of monomer complexes with a charged phosphate loop between α_{s1}- and κ-caseins in absence of calcium, then the monomers start to aggregate to a limiting size while calcium ions are added forming the caseinate core. When low weight α_{s1}- κ-casein complexes is formed aggregation of the caseinate stops. In this coat complex κ-casein monomers are located on the surface of the micelle determining its size. The coated sphere model is consistent with the fact that the micellar hydrodynamic diameter decrease during renneting and that decrease after the action of chymosin which remove the external portion of the κ-casein molecule. Another model that fit in this category described a micelle core formed by α_{s1}-caseins and CCP and the β-caseins which are bound by hydrophobic interactions. This core is surrounded by another complex of α_{s1}-, α_{s2}-, caseins and κ-casein (Rollema, 1992).

The sub-micelle model has been proposed firstly by Morr (1967), in this case uniform sub-micelle are formed by α_{s1}-, β-, and κ-casein monomers bounded by hydrophobic interactions and calcium caseinate bridges. The sub-micelles are held together by CCP in order to form a micelle structure which is covered by α_{s1}-, and κ-casein. In another sub-micelle model proposed later (Slattery and Evard, 1973; Brunner, 1977; Wong, 1988), The casein composition in the sub-micelle is variable and depends on the interaction of the single casein. In the surface of casein micelle are present mainly κ-caseins rich subunits whereas those lacking κ-caseins are buried internally stabilized by hydrophobic interactions. In this way the micelle is covered by the hydrophilic κ-casein regions.

Finally Walstra (1999), proposed a new sub-micelle model in which spherical subunits of sub-micelles of 12-15 nm in diameter, containing about 20-25 casein are hold together by hydrophobic interactions between proteins, and by calcium phosphate linkages to form a casein micelle. A typical micelle is composed by two types of sub-micelles: the first one consists mainly of αs-

and β-caseins with the hydrophobic region inside the sub-micelle and are located in the centre of the micelle, the other one is more hydrophilic since consists of αs- and κ-caseins, the latter is located in the surface of the micelle molecule with the sugar residues of the C-terminal end that protrude from the micelle molecule to form a "hairy" layer which ensure the integrity of casein micelles avoiding further aggregation of sub-micelles by steric and electrostatic repulsion (Figure 1).

In this way casein micelle do not tend to flocculation (Walstra, 1999; Walstra et al., 1999). In the Holt model (Holt, 1992; Holt and Horne, 1996) the casein micelle is considered a cross-linked protein gel formed by a cross-linking between the casein proteins and CCP nanoclusters in order to give rise to an intricate network. The growth of the CCP nanoclusters begins the process of micelle formation: more protein can coat this new surface, more calcium phosphate is bound until the formation of a size limited micelle (Farrell et al., 2006). The C-terminal region of κ-casein form the "hairy" layer protruding to the micelle surface which stabilize the structure. Subsequently Horne refined this model (Horne, 1998), suggesting that the protein binding in casein micelles is a balance between the attractive hydrophobic interactions which is driving force for the formation of casein micelles and the electrostatic repulsion which define the degree of polymerization. The particular conformation of α_{s1}- and β-caseins favor their polymerization by hydrophobic interactions, CCP binds to the negatively charged phosphoserine residues of α_{s1}- and β-caseins reducing the electrostatic repulsions, as a consequence the hydrophobic interactions between caseins are predominant and more association of proteins occurs. The reaction of κ-casein which contains only one phosphoserine residue, limits micellar growth and acts as a propagation terminator (see Figure 2), (Farrell et al., 2006).

More recently, it has been proposed that the micelle consists of tubules, presumably of caseins, of about 20 nm in diameter (consistent with the dimension of CCP/casein nanocluster proposed by Holt) the ends of which protrude from the micelle structure thus protecting the micelle itself from the approaching of other large particles (such as other micelles) but leaving sufficient space to permit to individual protein or small aggregates of proteins to reach the micelle (Dalgleish et al., 2004).

In all casein micelle models, κ-casein is located around the micellar surface with the aim to stabilize the structure. Furthermore the location of k-casein allows a specific hydrolysis of chymosin (rennin) in the neonate stomach which specifically cleaves one bond in κ-casein to initiate aggregation

of the micelles and allows the complex formation with whey protein after heating treatment.

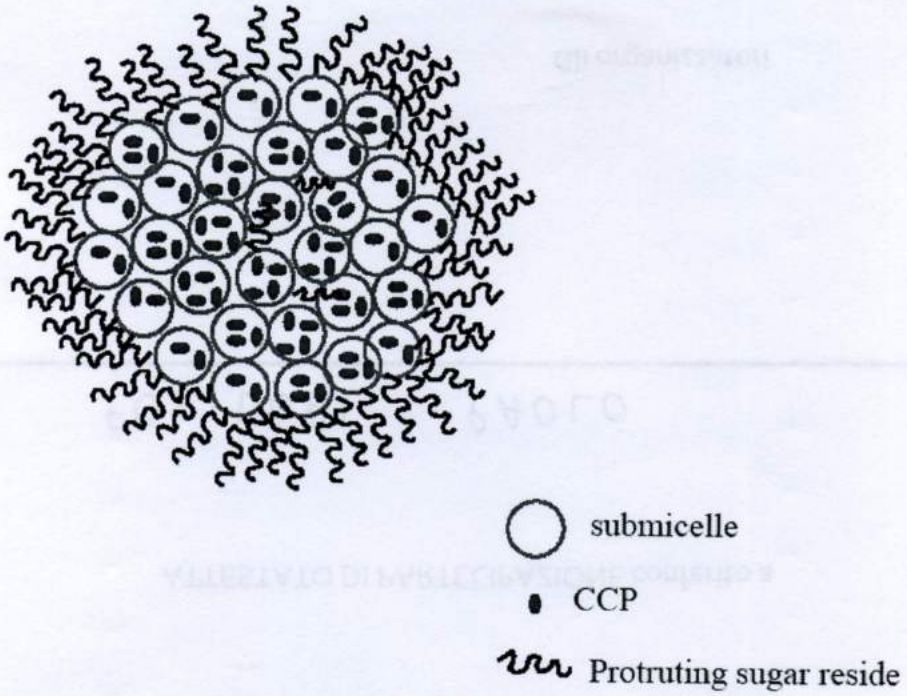


Figure 1. Schematic structure of a bovine casein micelle (Source: adapted from Walstra, 1999).

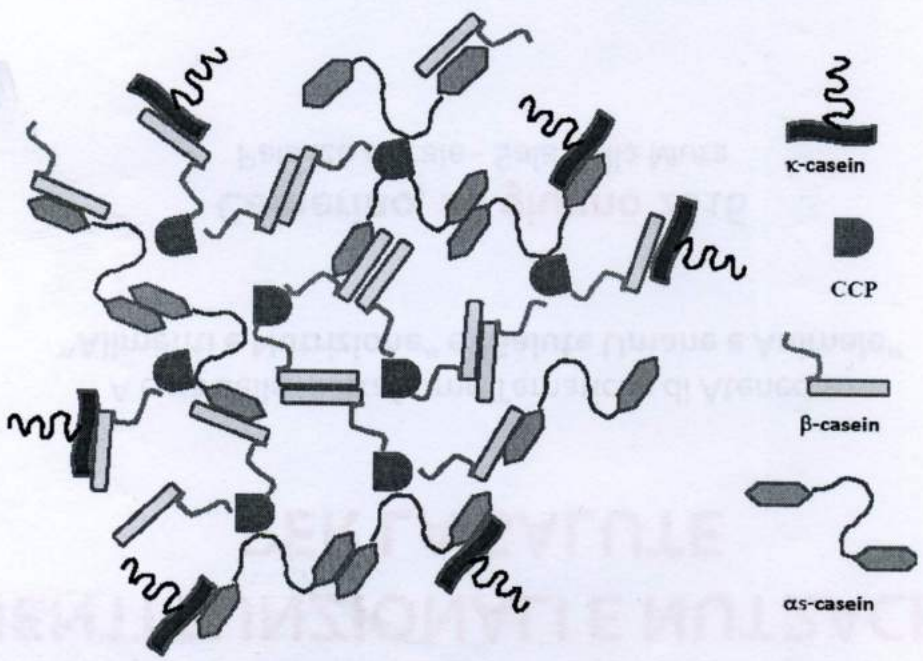


Figure 2. The Horne model, adapted from: Horne, 1998 and Farrel, 2006.

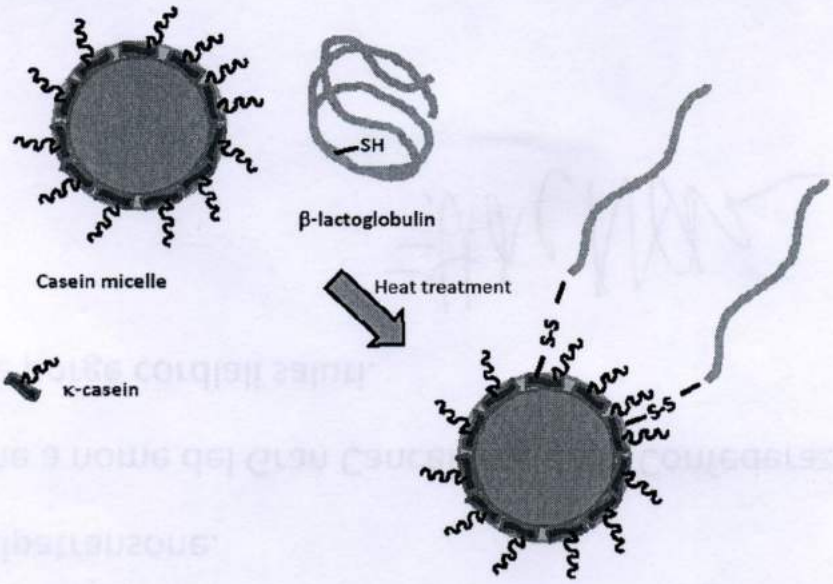


Figure 3. Interaction of β-lactoglobulin with κ-casein after thermal treatment.

In particular β-lactoglobulin, when subjected to heat treatment can interact with casein micelles by a link with the external κ-casein through disulphide links (Figure 3). About 10 polymerized β-lactoglobulin can bind to the casein micelles which gain more surface and new properties (Dalgleish, 1993; Kethireddipalli et al., 2011).

Casein micelles resulted stable to most milk processing procedures, in fact

- In the reconstituted milk powder micelles are not damaged and maintain their properties.
- Milk freezing as little effect on the casein micelle though slow freezing and storage in temperature ranging from -10 to -20°C can destabilize micelle structure.
- Homogenization of milk at pressure up to 20 mPa has very little effect on casein micelle whereas higher homogenization pressure can destabilize the micelle structure.
- The concentration of milk by evaporation or ultrafiltration can decrease the micelle stability because of a closer packing of micelles.
- High temperature short time (HTST) pasteurization (72°C for 15 s) has little effect on casein micelle, but higher temperatures cause the denaturation of β-lactoglobulin and its interaction with k-caseins by disulphide bonds. A further increase of temperature causes.
- dissociation of κ-casein from the micelles, and coagulation (Fox and Brodkorb, 2008).

HUMAN CASEINS

Casein as a class of proteins consists of several subunits which form micelles with Ca^{++} and PO_4^- giving milk its characteristics white appearance (Lönnerdal & Forsum, 1985); the micelles are spherical colloidal particles, in human milk are considerably smaller in size (~50 nm) than those of equine milk (~600 nm), and the presence of α-casein in human milk has not been clearly demonstrated. On a dry weight basis, the micelles contain approximately 94% protein and 6% non-protein species, mainly calcium and phosphate, with smaller amounts of Mg and citrate and traces of other metals (Visser, 1992). These are collectively called colloidal (or micellar) calcium phosphate (CCP or MCP). The milk of most species is supersaturated with calcium phosphate and the insoluble part (CCP) is present in the casein micelles, in which it acts as cementing material: the micelles disintegrate when the CCP is removed. Human milk lacks CCP and its micelles have a rather porous structure (Fox & Kelly, 2012).

The casein micelles scatter light: the white appearance of milk is due mainly to light scattering by the micelles, with a contribution from the fat globules.

Casein-derived phosphorylated peptides, caseinophosphopeptides (CPPs), that enhance vitamin D-independent bone calcification in rachitic infants, were the first bioactive peptides discovered over 50 years ago (Tidona et al., 2009). The ability of CPPs to retain minerals allows the prevention of different diseases caused by their deficiency such as osteoporosis, dental caries, hypertension and anaemia. It is well known that dairy products are a rich source of Ca^{++} that can form with CPPs soluble complexes, enhancing calcium absorption and avoiding the precipitation of insoluble phosphates.

The major constituent of the family of human caseins is β-casein, a highly phosphorylated protein (Greenberg & Groves, 1984). During digestion, phosphopeptides are formed and have been shown to keep Ca^{++} soluble, thus facilitating its absorption. It is therefore likely that phosphopeptides formed from β-casein contribute to the high bioavailability of calcium from human milk (Lönnerdal, 2003). Casein phosphopeptides may also affect the absorption of other divalent cations, such as zinc.

Casein Nutritional Value

In general (Table 2), a distinction is made between "casein" milk (i.e., ruminant milk which is relatively high in casein) and "albumin" milk (i.e., non ruminant milk which has proportionally a higher whey protein content as indicated by the lower casein/whey ratio). The relative proportion of the main milk casein components differ, not only between ruminants and non-ruminants, but also inside ruminant species. Given these different relative proportions, casein micelle characteristics differ as well, in size but also in hydration and mineralization (Claeys et al., 2014). Moreover, the molecular form and amino acid sequence of the milk proteins may differ from one species to another, which additionally affects the protein digestibility, nutritional quality and thermostability.

The nutritional value of milk proteins depends to a great extent on the presence of essential amino acids. A comparison between different species is given in Table 3. Coagulation or curd formation of milk in the stomach delays the degradation of proteins and improve their assimilation. Differences in total protein composition (casein content and casein/whey protein ratio) and in micelle structure (size, casein distribution, mineralization) dteremine the rheological properties of milk rennet and affects as such the milk nutrient uptake. High casein-containing milk, like bovine milk, produces a firm and dense coagulum, while human milk, horse and donkey milk form soft curds in the stomach, which are more easy to digest and are physiologically more adapt for infant nutrition (Malacarne et al. 2002; Uniacke-Lowe et al. 2010).

Table 2. Gross composition of mature milk from different mammals

	Human	Non-ruminants		Ruminants			
		Horse	Donkey	Cow	Sheep	Goat	Buffalo
Total dry matter (g/l)	107-129	93-116	88-117	118-130	181-200	119-163	157-172
Proteins (g/l)	9-19	14-32	14-20	30-39	45-70	30-52	27-47
Casein/whey ratio	0.4-0.5	1.1	1.28	4.7	3.1	3.5	4.6
Fat (g/l)	21-40	3-42	3-18	33-54	50-90	30-72	53-90
Lactose (g/l)	63-70	56-72	58-74	44-56	41-59	32-50	32-49
Ash (g/l)	2-3	3-5	3-5	7-8	8-10	7-9	8-9

Source: adapted by Claeys et al. (2014).

Opioid Peptides in Casein

Consumption of dairy products causes interactions with then nervous system through the action of opioid peptides. Basically they are receptor ligands with agonist or antagonist activities which are located in the nervous, endocrine and immune systems as well as in the gastrointestinal tract of mammals and can intercat with their endogenous ligands (normally synthesized by the organism) or exogenous ligands (introduced by food). The common structural feature of opiod peptides is the presence of a Tyr residue at the N-terminal, coupled with the presence of another aromatic residue, such as Phe or Tyr, in the third or fourth position. This is an important factor that ensures fitting into the binding site of the receptors (Silva & Malcata, 2005).

The major and the first discovered opioid peptides, deriving from milk, are the so called β-casomorphins, which are fragments of β-csein between the 60th and the 70th residues, mainly f60-63, f60-64, f60-65, f60-66 and f60-70 (Smacchi & Gobetti, 2000). The most potent seems to be the pentapeptide f60-64 (Fiat et al., 2003) whose sequence appears similar in β-casein from sheep along with the fragment f60-63 of bovine β-casein called Morphiceptin (Tidona et al., 2009). The fragment f51-54 of human β-casein is also supposed to exert an agonistic opioid activity (Fiat et al., 1993).

Gastric and pancreatic digestion are though to originate those active sequences although their absorption through the intestinal epitelium has not been proven (Silva & Malcata, 2005). During digestion, caseins, because of the acidity of the stomach, spontaneously precipitate. Slowly, they empty the gut in the form of degraded products, including bioactive peptides like β-casomorphins. Therefore the opioid activity id performed only at a peripheral level, whereas β-casomorphins may modulate the absorption of amino acids and the transport of electrolytes by decelerating the intestinal transit time (Meisel & FitzGerald, 2003). As soon as peptides enter the blood stream, they are quickly hydrolysed. On the other hand, β-casein derived peptides may pass through the intestinal mucosa in neonate via passive transport, so babies, thanks to greater intestinal permeability, may become calm and sleepy after milk consumption (Sturner & Chang, 1998).

Opioid antagonists are those peptides that suppress the action of endogenous and exogenous agonistics: known as casoxins, they have been found in both bovine and human k-casein, as well as in αs1-casein (Chiba et al., 1989). Casoxins A and B are opioid receptor ligands of the μ-type, even though they may also bind k-type receptors. Casoxin C is an opioid antagonist

obtained from tryptic dygests of bovine k-casein and possesses the highest biological potency among the casoxins, showing a 50% inhibitory concentration (IC_{50}) of 50.0 μmol/L (Xu, 1998). Casoxin D, composed of 7 residues, was generated from human αs1 casein and was also efficiently produced by using a plasmid hosted by Bacillus brevis (Kato et al., 1995).

Table 3. Amino acid distribution of mature milk from different mammals (mg/100 g milk)

	Human	Non-ruminants		Ruminants			
		Horse	Donkey	Cow	Sheep	Goat	Buffalo
Essential amino acids Histidine	30	56	36	100	167	98	78
Isoleucine	75	90	87	140	338	207	203
Leucine	131	229	135	290	587	314	366
Lysine	81	189	115	270	513	290	280
Methionine	23	35	28	60	155	80	97
Phenylalanine	57	111	68	160	284	155	162
Threonine	60	101	56	150	268	240	182
Tryptophan	23	28	n.a.	50	84	44	53
Valine	78	97	102	160	448	240	219
Non-Essential amino acids Alanine	52	76	55	100	269	118	132
Arginine	52	123	72	110	198	119	114
Aspartate	108	246	140	260	328	210	309
Cysteine	22	14	7	20	35	46	48
Glycine	34	45	19	60	41	50	80
Glutamate	231	474	358	770	1019	626	477
Proline	112	147	138	320	580	368	364
Serine	66	147	98	160	492	181	227
Tyrosine	61	101	58	150	281	179	183
Total Essential amino acids	558	936	627	1380	2844	1668	1640

Source: adapted by Claeys et al., (2014).

Cow Milk Allergy

The word allergy means an altered or abnormal reaction. Such a reaction may occur when occur when theer is contact between a foreign protein "an allergen" and body tissues that are sensitive to it. The allergy may reach the tissues by direct contact with the skin or mucous membranes or through the blood stream after absorption. Cow Milk Allergy (CMA) is clinically an abnormal immunological reaction to cow milk proteins, which may be due to the interaction between one or more milk proteins and one or more immune mechanisms, and resulting in immediate IgE. mediated reactions or delayed non-IgE-mediated (El-Agamy, 2007). On the other hand, reactions not involving the immune system are defined intolerance. The immediate reaction symptoms include anaphylaxis, cutaneous reactions with urticaria and edema, respiratory episodes, and gastro-intestinal distress including vomiting, diarrhea and bloody stools (Pereira, 2014). Similarly, the late-onset phenomenon is also characterized by cutaneous, respeiratiory and gastro-intestinal symptoms, including disorders like atopic dermatitis, milk-induced pulomonary disease, chronic diarrhea and gastro-esophageal reflux disease. These aftereffects can happen 1 h to several days after ingestion of cow milk.

Cow milk is one of the most common food allergies in children (Monti et al., 2012). Although most children out-grow CMA by the age of 4 years, some retain the allergy for life. CMA may occur in adults usually involving immediate allergic reactions or eczema. The incidence of CMA ranges from 0.3 to 7.5% in population-based studies in diffeernt countries (Vincenzetti et al., 2008); the wide range in these estimates may be due mainly to different diagnostic criteria in addition to other factors such as race, age of the tested patients, type of infant feeding, as well as the duration of observations.

Cow milk contains more than 20 proteins (allergens) that can cause allergic reactions; most studies revealed that casein and β-lactoglobulin are the main allergens in cow milk (El-Agamy, 2007). The major allergens of the casein fraction are calcium-sensitive α- and β-caseins (Wal, 1998). They share common structural features; e.g., a dipolar structure with amphipathic properties. The molecules have a globular hydrophobic doamin and a highly solvated and charged domain. The α- and β-casein molecules contain various numbers of proline and hydrophobic residues. They have no rigid tertiary structure but do have a "random coil" conformation which is stabilized by hydrophobic intercations; thus, they are considered poorly immunogenic (Grosclaude, 1988).

As reviewed by Wal (1998), several studies have highlighted the role of the phosphorylated regions of α-caseins in antigenicity, describing six allergenic and/or antigenic fragments, of which the main one is the large peptide 61-123. All the antigenicity of the native molecule of β-casein is found in the sum of the antigenicities of fragments resulting from its hydrolysis by cyanogen bromide and/or trypsin (Otani et al., 1989). This additivity led to the conclusion that at least six antigenic sites exist on the β-casein molecule and that the epitopes are sequential.

The four caseins which constitute the whole-casein fraction are present in the milk of four ruminant species (cow, buffalo, sheep and goat) with high sequence homologies ranging from 80% to more than 90% (Wal, 1998). This could lead to adverse reactions in patients allergic to cow's milk. Several authors have suggested that allergic reactions can be avoided by using milk form non-ruminant species, such as equid milk (Iacono et al., 1992; Carroccio et al., 2000; Monti et al., 2007; Tesse et al., 2009; Vincenzetti et al., 2014).

EQUID CASEINS

Mare's Milk Caseins

The characterization of individual equine caseins, and in particular asinine caseins (see next sub-section) has been poorly studied compared to the number of studies available on the characterization of caseins from bovine milk (see also Table 1). Most likely the equine milk has a great heterogeneity and a high level of post-translational modifications then those of bovine milk and consists in two isoforms of different size, the smallest one derives from splicing processes (Miranda et al., 2004; Uniacke-Lowe and Fox, 2012). From the primary sequence of equine α_{s1}-casein, it is possible to evince that there are six potential phosphorylation sites in serine residues located in very close proximity in order to form a possible phosphorylation cluster. Matéos and coworkers (2009) found a complex electrophoretic pattern for equine α_{s1}-casein, with 36 different variants with several phosphate groups ranging from 2 to 6 or 8. Furthermore it was shown that equine α_{s1}-casein has three hydrophobic domains and it probably has association properties similar to those observed for bovine α_{s1}-casein. As seen in Table 1 equid milk, as well as human milk, has low amount of α_{s1}-casein with respect to bovine milk: this may be responsible, together with the overall low protein content, of soft curd

produced in the stomach of the newborn. As confirmation of this, there is the absence of α_{s1}-casein in the milk of goat which is responsible of the poor coagulation properties compared to other milk containing this class of casein (Uniacke-Lowe and Fox., 2012).

Regarding equid α_{s2}-casein, a study of Ochirkhuyag and coworkers (2000) performed by isoelectric focusing showed the presence of two major band but the complete sequence of this class of casein is still unknown. Recently, by direct sequencing of the equine $CSN1_{S2}$ coding sequence, it was found the presence of 51-bp insertion-deletion polymorphism, which changes the protein sequence since there is a lack or presence of 17-amino acid serine-rich peptide and consequent change of the functional properties of this class of casein (Cieslak et al., 2016).

Equine β-casein showed the presence of a full-length protein and two smaller variants due to splicing processes (Miranda et al., 2004). The primary sequence revealed the presence of seven potential phosphorylation sites at level of the 28 C-terminal serine residue, therefore multiple phosphorylated isoforms of equine β-casein were reported (Matéos et al., 2009). This class of casein may be subjected to spontaneous deamidation reaction at level of Asn-Gly sequence (site of deamidation). This phenomenon is reduced at 10°C while is accentuated by temperature and represent an important modification for equine β-casein but not for bovine β-casein which does not contains deamidation sites in its sequence (Matéos et al., 2009). Furthermore, it has been reported in equine milk the presence of a short-length, highly phosphorylated β-casein resulting from a deletion of a large portion of sequence.

In the previous section has been discussed about the importance of κ-casein in the casein micelles structure since it is κ-casein is the only glycosylated member of the casein family, is located mainly on the surface of the micelles and is responsible for their stability. Regarding the presence of this class of casein in equid milk, in the past it was believed by several authors that this protein is not present in this milk, however, other authors demonstrated the presence of low amount of κ-casein in mare's milk (Malacarne et al., 2000; Iametti et al., 2001; Egito et al., 2002). Equine κ-casein share similar biochemical properties to those of bovine and human κ-casein and its primary sequence revealed sequence homology with κ-casein from camel, pig, human, bovine, ovine and goat. Equine κ-casein showed to be the most conserved casein, followed by β-casein in agreement with the physiological function of these two proteins in micelles formation and with the role of κ-casein in milk coagulation.

Lectin binding studies performed by Iametti and coworkers (2001) indicated that equine κ-casein is glycosylated at level of threonine residue. Very recently, Selvaggi and coworkers (2015) assessed genetic variability in the exon 1 of the κ-casein gene (CSN3) four Italian horse populations and in a sample of Martina Franca donkey, as a result was found the presence of two polymorphisms: 66A > G and 36C > A polymorphism whereas no genetic variability was observed in Martina Franca donkey breed.

It has been shown that equine casein micelles, with an average diameter of about 255 nm, are larger than bovine or human ones (Welsch et al., 1988), electron microscopy studies revealed that the equine milk micelles appear "spongy" with a structure less ordered with respect to bovine micelles, however until now there are not specific studies on the equine micelle sub-structure. From the data obtained the concentration of equine milk micellar calcium and micellar inorganic phosphorus and considering that in this milk casein concentration is less than 0.5 mM, it has been suggested that the micellar calcium: casein ratio >20:1, therefore exceed the calcium capacity of casein. Since both equine α_{s1}- and β-casein contain a phosphorylation centre, it is probable that a formation of nanoclusters of calcium phosphate occurs. Another important consideration is that in equine milk the ratio of micellar calcium: micellar inorganic phosphorus is 2.0 whereas in bovine milk is about 3.9 (Holt & Jenness, 1984), this may indicate that only a small portion of micellar calcium is included into nanoclusters even more corroborating the hypothesis that in the equine milk nanoclusters contain high proportion of casein-bound phosphate.

Furthermore both equine α_{s1}- and β-casein contain also distinct hydrophobic regions through which it is possible to establish hydrophobic interactions between them. Equine α_{s2}-casein shows properties similar to those of α_{s1}-casein (Uniacke-Lowe et al., 2010).

In any case it is important to take into account the fact that equine milk contains very few amounts of κ-casein, therefore the stabilizing role of the micelle could be played by the non-phosphorylated β-casein which may be located at the surface of the micelle (Ochirkhuyag et al., 2000; Doreau and Martin-Rosset, 2002).

In conclusion further studies are necessary to better understand the structure of equid casein micelles since they play an important role in the conversion of milk into a wide variety of dairy products.

Donkey Milk Caseins

Studies performed in order to reveal the casein composition in donkey milk (DM), revealed that in this milk were found mainly β-caseins α_{s1}-casein. DM β-caseins revealed the presence of different variants with pI values ranging from 4.63 to 4.95, close to that found in the equine β-casein (pI = 4.4 to 5.9) but more acidic if compared to human (pI = 4.9 to 5.8) and bovine (pI = 5.20 to 5.85) β-casein (Egito et al., 2002; Poth et al., 2008; Belitz et al., 2009; Vincenzetti et al., 2012). Chianese and coworkers (2010) demonstrated that in DM there is a full-length β-casein and some spliced variants each with 7, 6, 5 bound phosphate groups. This was subsequently confirmed by two-dimensional studies performed by Vincenzetti and coworkers (2012) on DM caseins. In fact in these experiments were found at least three isoforms of the full length form of β-caseins with similar molecular weight (about 33.5 kDa) but different isoelectric points (pIs: 4.72, 4.82, 4.92 respectively) and three (spliced) variant showing smallest molecular weight (about 31.5 kDa) and variable pIs (4.68, 4.80, 4.88). Also DM α_{s1}-casein showed heterogeneity (Chianese et al., 2010; Vincenzetti et al., 2012) due to phosphorylation degree and splicing: three full length, phosphorylated forms (molecular weight: about 31.3 kDa; pIs: 5.15, 5.23, 5.36) and two smallest variants (molecular weight: about 28.0 kDa; pIs: 5.08, 4.92).

Casein dephosphorylation experiments followed by two-dimensional studies confirmed the presence of phosphorylated isoforms of α_{s1}- and β-caseins (Vincenzetti et al., 2013). Phosphorylation of caseins may affect many of its features such as their digestion and the bioavailability of divalent cations. Some authors reported that the α_{S1}-casein in its phosphorylated form plays an important role in the allergenicity of milk and that in general, the α_{S2}- and β-caseins that possess serine-phosphorylated residues can be considered immunoreactive and resistant to digestion (Tezcucano et al., 2007).

Other authors found in DM the presence of low amount of α_{s2}-casein containing 10, 11 and 12 phosphate groups, and very low quantity of κ-casein (11 components) revealed also by specific antibodies (Bertino et al., 2010; Chianese et al., 2010).

Tidona and coworkers (2014) analyzed by photon correlation spectroscopy the size of DM casein micelles and found a wide range of variability in size among different samples of milk. The mean value resulted to be 298.5 ± 18.9 nm measured at pH 6.8, in agreement with the value of 311.5 nm measured for equine milk but higher with respect to those found in bovine

and human milk (183.9 nm and 64 nm, respectively) measured at the same pH value (Malacarne et al., 2002).

The pH value may have effect in determining the size of casein micelles, although the pH value in DM is slightly higher than bovine milk the increase of micelle size in donkey milk appeared relevant and was greater than the bovine one (Tidona et al., 2014).

Furthermore it was found that the variability in micelles size in DM seems to be correlated to the lactation stage contrary to what observed in bovine milk (Tidona et al., 2014).

CONCLUSION

The nutritional richness of milk is unquestionable: it is a good source of high biological value proteins, as well as important vitamins and essentail minerals. Milk proteins are no longer considered merely nutritional components, because they possess encrypted peptides with interesting biological properties. Among milk proteins, caseins represent the prime source of calcium and phosphorus for the neonate. It is widely accepted that the breast milk of a healthy, well-nourished woman is the best nutritional support for the neonate, it offers the most complete nutrition for the newborn, even though it is deficient in iron and vitamin K.

The primary treatment for managing food allergies is eliminating the allergenic food from the diet. The natural course of a cow's milk allergy (CMA) is the acquisition of tolerance spontaneously through an elimination diet, and 85% of patients overcome CMA by the time they are 4-5 years old (Caira et al., 2012). Unfortunately, for children under 12 months of age an elimination diet of dairy foods can have negative consequences in terms of inadequate calcium and vitamin intake. The possibility of using equid milk as a replacer of dairy cows milk in children affected by CMA has been reported as a valid feeding strategy, duie to the high similarity between human milk and equid milk.

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Research and Professional Experience:

1. Molecular and kinetic characterization of enzymes involved in purinic and pyrimidinic nucleosides metabolism in order to set up a chemotherapic strategy by using nucleoside analogues.
2. Single nucleotide polymorphisms of enzymes involved in the pyrimidine metabolism.
3. Studies on the nutritional properties of donkey and sheep milk. In particular proteomic studies (by 2-DE and RP-HPLC techniques) were undertaken in order to characterize the caseinic and whey proteins fraction.

Professional Appointments:

From July 1995 to march 2005: Researcher in Biochemistry and Applied Biology in the University of Camerino, in the School Biosciences and Veterinary Medicine.

From 2005 until now: Associate Professor in Clinical Biochemistry and Clinical Molecular Biology in the School of Biosciences and Veterinary Medicine, University of Camerino.

Honors:

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