



Review

Equine milk proteins: Chemistry, structure and nutritional significance

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ABSTRACT

Equine milk has important nutritional and therapeutic properties that can benefit the diet of the elderly, convalescent or newborn. The protein content of equine milk is lower than that of bovine milk but similar to that of human milk. In this review qualitative and quantitative differences between the caseins and whey proteins of equine, bovine and human milk are discussed. Important biological and functional properties of specific proteins are reviewed and their significance in human nutrition considered. As well as characterizing equine milk proteins in the context of human nutrition and allergology, the potential industrial exploitation of equine milk is explored. Cross-reactivity of proteins from different species is discussed in relation to the treatment of cows' milk protein allergy. While there is some scientific basis for the special nutritional and health-beneficial properties of equine milk based on its protein composition and similarity to human milk, further research is required to fully exploit its potential in human nutrition.

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1. Introduction

Although horses are of minor importance for milk production in comparison with cows, buffalo, sheep and goats, they have been traditionally important dairy animals in Mongolia and in the southern states of the former Soviet Union, e.g., Kazakhstan, Kyrgyzstan and Tajikistan. Because equine milk resembles human milk in many respects and is claimed to have special therapeutic properties (Freudenberg, 1948a, 1948b; Kalliala, Selesté, & Hallman, 1951; Stoyanova, Abramova, & Ladoto, 1988; Lozovich, 1995), it is becoming increasingly important in Europe, especially in France, Italy, Hungary and the Netherlands. Koumiss (fermented equine milk) is used in Russia and Mongolia for the management of digestive and cardiovascular diseases (Levy, 1998; Lozovich, 1995); in Italy, equine milk is recommended as a substitute for bovine milk for allergic children (Curadi, Giampietro, Lucenti, & Orlandi, 2001).

Overall, equine milk is considered to be highly digestible, rich in essential nutrients and possesses an optimum whey protein/casein ratio, making it suitable in paediatric dietetics. Estimates suggest that more than 30 million people worldwide drink equine milk regularly, with that figure increasing significantly annually (Doreau & Martin-Rosset, 2002). However, in comparison with bovine milk, equine milk is very expensive to produce. One of the main constraints of the horse as a dairy animal is the high frequency of milkings required; generally about 5 times per day or every 2 h, with the foal feeding through the night. The capacity of the mare's udder is low, about 2 L, and the foal must be in close proximity during milking (Solaroli, Pagliarini, & Peri, 1993). Unlike dairy cows, mastitis is not a major factor in equine milk production due to the

small udder size that limits exposure of the teats to infection (Doreau & Martin-Rosset, 2002) and despite its high level of unsaturated fatty acids, equine milk has better keeping quality than human or bovine milk (Kalliala et al., 1951). For details on the production of equine milk and the factors that affect it, the reader is referred to the reviews by Doreau (1994), Doreau and Boulot (1989), Doreau, Boulet, Bartlet, and Patureau-Mirand (1990), Doreau and Martin-Rosset (2002) and Park, Zhang, Zhang, and Zhang (2006a).

2. Gross composition of equine milk

Young mammals are born at very different stages of maturity and their maternal milk differs greatly in composition (Table 1), although milk from species in the same taxonomic order, e.g., the equids, tend to be fairly similar (Table 2) (Jenness, 1974a). The composition of equine milk differs considerably from that of the milk of the principal dairying species, i.e., the cow, buffalo, goat and sheep (Table 1). In comparison with bovine milk, equine milk contains less fat, protein, inorganic salts but more lactose, with a concentration close to that in human milk. A mare produces 2–3.5 kg milk per 100 kg live-weight per day to sustain the rapid growth of the foal (Doreau, 1994; Oftedal, Hintz, & Schryver, 1983). A healthy foal will consume 10–25% of its body weight of milk per day (Gibbs, Potter, Blake, & McMullan, 1982; Oftedal et al., 1983; Zicker & Lönnerdal, 1994), which for an average 45 kg foal is 9–13 L of milk daily (Paradis, 2003). The composition and constituents of equine milk have been studied thoroughly; reviews include Doreau and Boulot (1989), Linton (1931), Malacarne,

Table 1
Gross composition of the milk of selected species.

Species	Total solids	Protein	Casein/whey ratio	Fat	Lactose	Ash	Gross energy	References
Man (<i>Homo sapiens</i>) ^a	124.0	9.0	0.4:1	38.0	70.0	2.0	2763 ^c	Hambraeus (1982, 1984); Picciano (2001)
Cow (<i>Bos taurus</i>) ^b	127.0	34.0	4.7:1	37.0	48.0	7.0	2763 ^d	Jenness (1974b)
Horse (<i>Equus caballus</i>) ^a	102.0	21.4	1.1:1	12.1	63.7	4.2	1883 ^c	Malacarne et al. (2002); Schryver, Oftedal, Williams, Soderholm, and Hintz (1986)
Donkey (<i>Equus africanus asinus</i>) ^a	88.4	17.2	1.3:1	3.8	68.8	3.9	1582	Salimei et al. (2004)
Buffalo (<i>Bubalus bubalis</i>) ^a	172.0	46.5	4.6:1	81.4	48.5	8.0	4644 ^c	Jenness and Sloan (1970); Tufarelli, Dario, and Laudadio (2008)
Sheep (<i>Ovis aries</i>) ^b	181.0	55.9	3.1:1	68.2	48.8	10.0	4309 ^d	Raynal-Ljutovac, Lagriffoul, Paccard, Guillet, and Chilliard (2008)
Goat (<i>Capra hircus</i>) ^a	122.0	35.0	3.5:1	38.0	41.0	8.0	2719 ^c	Park, Zhang, Zhang, and Zhang (2006b)
Camel (<i>Camelus dromedarius</i>) ^b	124.7	33.5	1.68:1	38.2	44.6	7.9	2745 ^d	Konuspayeva, Faye, and Loiseau (2009), Vaisman, Reuven, Uzi, Georgi, & Boehm, (2006)
Llama (<i>Llama glama</i>) ^b	131.0	34.0	3.1:1	27.0	65.0	5.0	2673 ^d	Rosenberg (2006)
Yak (<i>Bos grunniens</i>) ^a	160.0	42.3	4.5:1	56.0	52.9	9.1	3702 ^c	Silk, Guo, Haenlein, and Park (2006); Sheng, Li, Alam, Fang, and Guo (2008)

^a Values are expressed as g kg⁻¹ milk.

^b Values are expressed as g L⁻¹ milk.

^c Values are expressed as kJ kg⁻¹ milk.

^d Values are expressed as kJ L⁻¹ milk.

Table 2Gross composition (g kg⁻¹) of the milk of some equid species.

	Total solids	Fat	Protein	Casein/whey ratio	Lactose	Ash	Gross energy (kJ kg ⁻¹)
Donkey ^a	88.9	5.6	16.6	~1.28:1	65.2	4.2	1580
Mountain zebra ^b	100.0	10.0	16.0	—	69.0	3.0	2000
Plains zebra ^b	113.0	22.0	16.0	—	70.0	4.0	2400
Przewalski horse ^b	105.0	15.0	16.0	1.1:1	67.0	3.0	2100
Pony ^b	104.0	15.0	18.0	1.1:1	67.0	5.0	2200

^a Values averaged from Guo et al. (2007), Salimei et al. (2004) and Piccione, Fazio, Caola, and Refinetti (2008), using a value for density of 1029 kg m⁻³ (Chiavari, Coloretto, Nanni, Sorrentino, & Grazia, 2005).

^b Values from Oftedal and Jenness (1988).

Martuzzi, Summer, and Mariani (2002), Nesen, Flade, Heidler, and Steger (1958), Neuhaus (1959), Park et al. (2006a), and Solaroli et al. (1993). This article presents a detailed review of the proteins in equine milk, with comparative data for bovine and human milk, and the nutritional significance thereof.

3. Factors that affect the composition of equine milk

Both genetic and environmental factors affect the gross composition of milk. Including the breed of mammal, individuality of animals, stage of lactation, frequency and completeness of milking, maternal age, health and type of feed. Data in the literature are not conclusive on whether or not the breed of mare has an effect on the concentration of protein in milk. Csapó, Stefler, Martin, Makray, and Csapó-Kiss (1995), Csapó-Kiss, Stefler, Martin, Makray, and Csapó (1995), Doreau et al. (1990) and Kulisa (1977) reported no effect of breed on the concentration of proteins or lipids in equine milk throughout lactation. On the other hand, Boulot (1987), Civardi, Curadi, Orlandi, Cattaneo, and Giangiacomo (2002), Formaggioni, Malacarne, Martuzzi, Summer, and Mariani (2003), and Pelizzola, Contarini, Povolio, and Giangiacomo (2006) reported significant differences in protein content between breeds.

Table 3

Gross composition of the milk of various breeds of mid-lactation, multiparous mares.

Species	Sample size (n)	Total solids	Protein	Ratio of casein/whey	Fat	Lactose	Ash	Reference
Andalusian ^b	18	122.1	19.3	ND ^c	24.0	66.3	ND	Fuentes-García, Abascal, del Castillo Caracuel, Quiles Sotillo, and Vinuesa Silva (1991)
Arabian Mare ^b	—	110.9	20.3	ND	17.0	63.6	ND	Pieszka and Kulisa (2003); Pieszka, Kulisa, Luszeżyński, Długosz, and Jackowski (2004)
Dutch Saddle ^b	39	ND	21.0	ND	6.3	68.4	ND	Smolders, Van der Veen, and Van Polanen (1990)
Finnish Native ^b	15	ND	20.0	ND	10.0	70.0	5.0	Antila, Kylä-Siuro, Uusi-Rauv, and Antila (1971)
Indigenous ^b	—	103.0	22.0	1.1:1	15.0	62.0	3.6	Marconi and Panfili (1998)
Italian ^b	—	—	—	—	—	—	—	—
Murgese ^b	8	ND	18.5	0.86:1.0	10.6	70.4	ND	Caroprese et al. (2007)
Orlov Trotter ^b	—	ND	15.8	0.84:1	ND	ND	ND	Kudryaslov & Krylova (1965)
Quarter Horse ^b	7	104.0	21.0	ND	15.0	ND	ND	Burns, Gibbs, and Potter (1992)
Quarter Horse ^b	14	105.0	21.0	ND	13.0	ND	ND	Gibbs et al. (1982)
Russian Heavy ^b	—	109.5	20.9	1.35:1	18.0	64.0	ND	Stoyanova et al. (1988)
Thoroughbred ^b	10	105.0	19.3	ND	12.9	69.1	ND	Oftedal et al. (1983)
Bretonnes & Comtoises ^a	10	ND	33.8	ND	11.3	62.7	ND	Doreau et al. (1986)
Bretonnes & Comtoises ^a	11	ND	20.6	ND	12.5	67.3	ND	Doreau et al. (1990)
Clydesdale ^a	—	114.1	26.4	ND	19.0	68.5	3.58	Linton (1937)
Haflinger ^a	—	96.8	18.3	1.33:1	9.0	64.7	3.9	Pelizzola et al. (2006)
Haflinger ^a	5	ND	18.2	ND	8.2	67.8	3.9	Mariani et al. (2001)
Lusitano ^a	48	ND	18.4	ND	5.9	60.8	ND	Santos and Silvestre (2008)
Murgese ^a	—	ND	20.5	ND	19.3	63.1	4.2	Di Cagno et al. (2004)
Quarter Horse ^a	—	104.9	23.1	1.42:1	14.0	70.2	5.5	Pelizzola et al. (2006)
Rapid Heavy Draft ^a	—	ND	18.4	1.12:1	11.0	67.8	3.6	Pelizzola et al. (2006)
Sella & Salto ^a	—	ND	26.6	1.63:1	7.0	63.3	5.3	Pelizzola et al. (2006)
Sella & Trotter ^a	11	111.6	23.9	1.13:1	13.6	69.5	4.6	Mariani et al. (1993)
Shetland Pony ^a	—	100.6	17.8	ND	8.0	72.3	2.5	Linton (1937)

^a Values expressed as g kg⁻¹ milk.

^b Values expressed as g L⁻¹ milk.

^c ND, not determined.

Table 3 shows the gross composition of the milk of a number of horse breeds; differences are evident between breeds in the concentration of some milk constituents, especially proteins. Assessment of the effect of breed on milk composition is difficult owing to differences between individuals, as well as between feeding regimens and herd conditions (Martuzzi & Doreau, 2006). The stage of lactation has a marked effect on the composition of equine milk.

In the first few hours after parturition, the mammary gland produces colostrum that is richer in dry matter, protein, fat, vitamins and minerals (except calcium and phosphorus) but poorer in lactose than mature milk. One of the major biological benefits of colostrum is the presence of immunoglobulins, IgA, IgM and IgG, and high levels of several enzymes, including catalase, lipase and proteinase. The transition of colostrum into mature equine milk occurs within 2 days of parturition. The concentration of protein in equine colostrum is very high, >150 g kg⁻¹, immediately post-partum (due primarily to the high concentration of immunoglobulins), but decreases rapidly to <40 g kg⁻¹ within 24 h and to <20 g kg⁻¹ after 4 weeks of lactation (Linton, 1937; Mariani, Martuzzi, & Catalano, 1993). The casein to whey protein ratio in equine milk is 0.2:1 immediately post-partum and changes to 1.2:1 during the first week of lactation (Zicker & Lönnnerdal, 1994). In bovine milk, the protein content decreases during the first 3 months of lactation, but increases subsequently (Fox & McSweeney, 1998; Jenness, 1974b; Tsioulpas, Grandison, & Lewis, 2007).

4. Equine milk proteins

While the protein content of mature equine milk is lower than that of bovine milk, there is a strong qualitative resemblance, the principal classes of proteins, i.e., caseins and whey proteins are similar in both types of milk. However, while the caseins are the predominant class of proteins in bovine milk (~80% of total milk protein), equine milk contains less casein and more whey proteins.

Table 4Concentrations of caseins and whey proteins (g kg⁻¹) in equine, human and bovine milk.^a

Protein	Equine ^b	Human ^c	Bovine ^d
Total casein	13.56	2.4	26.0
α_{s1} -casein	2.4	0.77 ^b	10.7
α_{s2} -casein	0.20	—	2.8
β -casein	10.66	3.87 ^b (>85%) ⁱ	8.6
κ -casein	0.24	(<15%) ⁱ	3.1
γ -casein	—	—	0.8
Total whey protein	8.3 ^e	6.2 ^e	6.3
β -lactoglobulin	2.55	—	3.2
α -lactalbumin	2.37	2.5	1.2
Serum albumin	0.37	0.48	0.4
Proteose peptone	—	—	0.8
Immunoglobulins	1.63 ^e	0.96 ^e	0.80
IgG _{1,2}	0.38 ^f	0.03	0.65
IgA	0.47 ^f	0.96	0.14
IgM	0.03 ^f	0.02	0.05
Lactoferrin	0.58 ^g	1.65	0.10
Lysozyme	0.87 ^g	0.34 ^l	126 × 10 ^{-6j}
NPN	0.381	0.485	0.296 ^k
Casein micelle size (nm) ^h	255	64	182

^a Where necessary values have been adjusted using density values of 1032, 1031 and 1033 kg m⁻³ for equine (Uniacke & Fox, unpublished data), human and bovine milk (Neville & Jensen, 1995), respectively.

^b Adapted from Miranda et al. (2004).

^c Adapted from Hambræus (1984).

^d Adapted from Walstra et al. (2006).

^e Park et al. (2006a).

^f Hurley (2003).

^g Pagliarini et al. (1993).

^h Malacarne et al. (2002).

ⁱ Hambræus and Lönnerdal (2003).

^j El-Agamy et al. (1996).

^k De Peters and Ferguson (1992).

^l Montagne et al. (1998, 2000).

In the past, the proportion of casein in equine milk has been underestimated as determinations were made by acid precipitation at pH 4.6, as in bovine milk, but maximum precipitation of equine casein occurs at pH 4.2 (Egito et al., 2002). The concentrations and distribution of the casein and whey proteins of equine milk are shown in Table 4, with comparative data for human and bovine milk.

4.1. Whey proteins

The major whey proteins in equine milk are β -lactoglobulin (β -Lg), α -lactalbumin (α -La), immunoglobulins (Igs), blood serum albumin (BSA), lactoferrin (Lf) and lysozyme (Lyz) (Bell, McKenzie, Muller, Roger, & Shaw, 1981b), which is similar to bovine milk. Except for β -Lg, all these proteins are also present in human milk. However, the relative amounts of the whey proteins differ considerably between these species (Table 4). Compared with bovine milk, equine milk contains less β -Lg and more α -La and Igs. The principal anti-microbial agent in equine milk is Lyz and to a lesser extent Lf, which predominates in human milk (Table 4). Both Lf and Lyz are very low in bovine milk, in which Igs form the main defense against microbes (Malacarne et al., 2002). Together, IgA, IgG, IgM, Lf and Lyz provide the neonate with immune and non-immune protection against infection (Baldi et al., 2005).

4.1.1. β -Lactoglobulin

β -Lg is the major whey protein in the milk of most ruminants and is also present in milk of monogastrics and marsupials, but is absent from the milk of humans, camels, lagomorphs and rodents. β -Lg is synthesized in the secretory epithelial cells of the

mammary gland under the control of prolactin. Although several biological roles for β -Lg have been proposed, e.g., facilitator of vitamin uptake and an inhibitor, modifier or promoter of enzyme activity, conclusive evidence for a specific biological function of β -Lg is not available (Sawyer, 2003). β -Lg from all species studied binds retinol; β -Lg of many species, but not equine or porcine, binds fatty acids also (Pérez, Puyol, Ena, & Calvo, 1993). During digestion, milk lipids are hydrolysed by lipases, greatly increasing the amount of free fatty acids which could potentially bind to β -Lg, displacing any bound retinol, and implying that fatty acid metabolism, rather than retinol transport, is the more important function of β -Lg (Pérez & Calvo, 1995). Bovine β -Lg is very resistant to peptic digestion and can cause allergenic reactions on consumption. Resistance to digestion is not uniform among species, with ovine β -Lg being far more digestible than bovine β -Lg (El-Zahar et al., 2005). The digestibility of equine β -Lg, which has to our knowledge not been studied, warrants research, particularly considering the potential applications of equine milk as a hypo-allergenic dairy product.

Two isoforms of equine β -Lg have been isolated, β -Lg I and II, which contain 162 and 163 amino acids, respectively. The extra amino acid in equine β -Lg II is a glycine residue inserted after position 116 (Halliday, Bell, & Shaw, 1991). Bovine β -Lg occurs mainly as two genetic variants, A and B, both of which contain 162 amino acids and differ only at positions 63 (Asp in variant A, Gly in variant B) and 117 (Val in variant A, Ala in variant B); a further eleven, less common, genetic variants of bovine β -Lg have also been reported (Sawyer, 2003). The amino acid sequences of equine β -Lg I and II are shown in Fig. 1.

Based on its amino acid sequence, unmodified equine β -Lg I has a molecular mass of 18,500 Da and an isoelectric pH of 4.85, whereas equine β -Lg II, despite having one more amino acid, has a molecular mass of 18,262 Da (ExPASy ProtParam Tool, 2009), and an isoelectric pH of 4.71 (Table 5). Bovine β -Lg A and B have a molecular mass of 18,367 and 18,281 Da, respectively, and an isoelectric pH of 4.76 and 4.83, respectively (Table 5). Using the hydropathy scale proposed by Kyte and Doolittle (1982), equine β -Lg I and II have a grand average hydropathy (GRAVY) score of -0.386 and -0.300, respectively (Table 5). This scale reflects the relative ratio of hydrophobic and hydrophilic amino acid residues in a protein, with a positive value reflecting an overall hydrophobic and a negative value an overall hydrophilic nature of the protein. Bovine β -Lg A and B have a GRAVY score of -0.167 and -0.162, respectively (Table 5), and are, therefore, considered to be less hydrophilic than equine β -Lg I and II. Both equine and bovine β -Lg contain two intra-molecular disulphide bridges, linking Cys₆₆ to Cys₁₆₀ and Cys₁₀₆ to Cys₁₁₉ in equine β -Lg I, Cys₆₆ to Cys₁₆₁ and Cys₁₀₆ to Cys₁₂₀ in equine β -Lg II and Cys₆₆ to Cys₁₆₀ and Cys₁₀₆ to Cys₁₁₉ or Cys₁₂₁ in bovine β -Lg A and B. Unlike bovine β -Lg equine β -Lg lacks a sulphhydryl group, which has large implications for denaturation and aggregation of the protein, as discussed in Section 4.2. Also, unlike bovine β -Lg, equine β -Lg does not dimerize (Sawyer, 2003).

4.1.2. α -Lactalbumin

α -La, a unique protein in the milk of mammals, is homologous with the well-characterized c-type lysozymes. It is a calcium metalloprotein, in which Ca²⁺ plays a crucial role in folding and structure. α -La is synthesized in the rough endoplasmic reticulum, from where it is transported to the Golgi apparatus, where it has a regulatory function in the synthesis of lactose. Together with β -1,4 galactosyltransferase, which is the catalytic component of lactose synthetase, α -La enhances enzymatic affinity for glucose 1000-fold in the final step of lactose synthesis, when glucose is linked to galactose (Brew, 2003; Larson, 1979; Neville, 2009).

Fig. 1. Primary structure of equine β -lactoglobulin I (from Conti, Godovac-Zimmerman, Liberatori, & Braunitzer, 1984) and II (from Godovac-Zimmerman, Conti, Liberatori, & Braunitzer, 1985; Halliday et al., 1991).

Shimazaki, Oota, Nitta, and Ke (1994) purified Lf from equine milk and compared its iron-binding ability with that of human and bovine Lf and with bovine Tf. The iron-binding capacity of equine Lf is similar to that of human Lf but higher than that of bovine Lf and Tf. Various biological functions have been attributed to Lf but the exact role of Lf in iron-binding in milk is unknown and there is no relationship between the concentrations of Lf and Tf and the

^a Primary accession number for the protein in SWISS-PROT database.
^b Grand average hydropathy (GRAVY) score calculated using the scale of Kyte and Doolittle (1982).

Fig. 2. Amino acid sequence of equine α -lactalbumin A (from Kaminogawa, McKenzie, & Shaw, 1984), B and C (from Godovac Zimmerman et al., 1987). Amino acid positions at which the genetic variants differ are shaded.

Bacterial growth is inhibited by the ability of Lf to sequester iron and also to permeabilize bacterial cell walls by binding to lipopolysaccharides through its N-terminus. Lf can inhibit viral infection by binding tightly to the envelope proteins of viruses and is also thought to stimulate the establishment of a beneficial microflora in the gastrointestinal tract (Baldi et al., 2005). Ellison and Giehl (1991) suggested that Lf and Lyz work synergistically to effectively eliminate Gram-negative bacteria; Lf binds oligosaccharides in the outer bacterial membrane, thereby opening 'pores' for Lyz to disrupt glycosidic linkages in the interior of the peptidoglycan matrix. This synergistic process leads to inactivation of both Gram-negative, e.g., *Escherichia coli* (Rainhard, 1986) and Gram-positive bacteria, e.g., *Staphyococcus epidermis* (Leitch & Willcox, 1999). A proteolytic digestion product of bovine and human Lf, i.e., lactoferricin, has bactericidal activity (Bellamy et al., 1992). Bovine and human Lf are reported to have antiviral activity

The amino acid sequence of equine Lf, as reported by [Sharma, Paramasivan, Srinivasan, Yadav, and Singh \(1998\)](#), is shown in [Fig. 3](#). Equine Lf contains 689 amino acid residues, which is similar to bovine Lf and two more than human Lf ([Table 7](#)). Compared with most other milk proteins, Lf has a high isoelectric point, i.e., at pH 8.32, 8.67 or 8.47 for equine, bovine or human Lf ([Table 7](#)). As a result, the protein is positively charged at the pH of milk and may associate with negatively-charged proteins via electrostatic interactions. GRAVY scores are comparable for equine, bovine and human Lf ([Table 7](#)). Equine and human Lf contain 17 and 16 intramolecular disulphide bonds, respectively ([Table 7](#)). The iron-binding capacity of equine, bovine and human Lf is equivalent, although the pH-dependence of the iron-binding capacity of bovine Lf differs from that of equine and human Lf ([Shimazaki et al., 1994](#)).

Species	Variant	Primary accession number ^a	Amino acid residues	Molecular mass (Da)	pI	GRAVY score ^b	Disulphide bridges	Free sulphhydryl groups
Equine	A	P08334	123	14223.2	4.95	−0.416	4 ^c	0
	B	P08896	123	14251.2	4.95	−0.503	4 ^c	0
	C	P08896	123	14249.3	5.11	−0.438	4 ^c	0
Bovine		P00711	123	14186.0	4.80	−0.453	4	0
Human		P00709	123	14078.1	4.70	−0.255	4	0

^a Primary accession number for the protein in SWISS-PROT database.
^b Grand average hydropathy (GRAVY) score calculated using the scale of Kyte and Doolittle (1982).
^c Estimated from structural similarity with bovine and human α -La.

1	Ala-	Pro-	Arg-	Lys-	Ser-	Val-	Arg-	Trp-	Cys-	10	Thr-	Ile-	Ser-	Pro-	Ala-	Glu-	Ala-	Ala-	Lys-	Cys-	20	Ala-
21	Lys-	Phe-	Gln-	Arg-	Asn-	Met-	Lys-	Lys-	Val-	30	Arg-	Gly-	Pro-	Ser-	Val-	Ser-	Cys-	Ile-	Arg-	Lys-	40	Thr-
41	Ser-	Ser-	Phe-	Glu-	Cys-	Ile-	Gln-	Ala-	Ile-	50	Ala-	Ala-	Asn-	Lys-	Ala-	Asp-	Ala-	Val-	Thr-	Leu-	60	Asp-
61	Gly-	Gly-	Leu-	Val-	Tyr-	Glu-	Ala-	Gly-	Leu-	70	His-	Pro-	Tyr-	Lys-	Leu-	Arg-	Pro-	Val-	Ala-	Ala-	80	Glu-
81	Val-	Tyr-	Gln-	Thr-	Arg-	Gly-	Lys-	Pro-	Gln-	90	Thr-	Arg-	Tyr-	Tyr-	Ala-	Val-	Ala-	Val-	Val-	Lys-	100	Lys-
101	Gly-	Ser-	Gly-	Phe-	Gln-	Leu-	Asn-	Gln-	Leu-	110	Gln-	Gly-	Val-	Lys-	Ser-	Cys-	His-	Thr-	Gly-	Leu-	120	Gly-
121	Arg-	Ser-	Ala-	Gly-	Trp-	Asn-	Ile-	Pro-	Ile-	130	Gly-	Thr-	Leu-	Arg-	Pro-	Tyr-	Leu-	Asn-	Trp-	Thr-	140	Gly-
141	Pro-	Pro-	Glu-	Pro-	Leu-	Gln-	Lys-	Ala-	Val-	150	Ala-	Asn-	Phe-	Phe-	Ser-	Ala-	Ser-	Cys-	Val-	Pro-	160	Cys-
161	Ala-	Asp-	Gly-	Lys-	Gln-	Tyr-	Pro-	Asn-	Leu-	170	Cys-	Arg-	Leu-	Cys-	Ala-	Gly-	Thr-	Glu-	Ala-	Asp-	180	Lys-
181	Cys-	Ala-	Cys-	Ser-	Ser-	Gln-	Glu-	Pro-	Tyr-	190	Phe-	Gly-	Tyr-	Ser-	Gly-	Ala-	Phe-	Lys-	Cys-	Leu-	200	Glu-
201	Asn-	Gly-	Ala-	Gly-	Asp-	Val-	Ala-	Phe-	Val-	210	Lys-	Asp-	Ser-	Thr-	Val-	Phe-	Glu-	Asn-	Leu-	Pro-	220	Asp-
221	Glu-	Ala-	Asp-	Arg-	Asp-	Lys-	Tyr-	Glu-	Leu-	230	Leu-	Cys-	Pro-	Asp-	Asn-	Thr-	Arg-	Lys-	Pro-	Val-	240	Asp-
241	Ala-	Phe-	Lys-	Glu-	Cys-	His-	Leu-	Ala-	Arg-	250	Val-	Pro-	Ser-	His-	Ala-	Val-	Val-	Ala-	Arg-	Ser-	260	Val-
261	Asp-	Gly-	Arg-	Glu-	Asp-	Leu-	Ile-	Trp-	Arg-	270	Leu-	Leu-	His-	Arg-	Ala-	Gln-	Glu-	Glu-	Phe-	Gly-	280	Arg-
281	Asn-	Lys-	Ser-	Ser-	Ala-	Phe-	Gln-	Leu-	Phe-	290	Lys-	Ser-	Thr-	Pro-	Glu-	Asn-	Lys-	Asp-	Leu-	Leu-	300	Phe-
301	Lys-	Asp-	Ser-	Ala-	Leu-	Gly-	Phe-	Val-	Arg-	310	Ile-	Pro-	Ser-	Gln-	Ile-	Asp-	Ser-	Gly-	Leu-	Tyr-	320	Leu-
321	Gly-	Ala-	Asn-	Tyr-	Leu-	Thr-	Ala-	Thr-	Gln-	330	Asn-	Leu-	Arg-	Glu-	Thr-	Ala-	Ala-	Glu-	Val-	Ala-	340	Ala-
341	Arg-	Arg-	Glu-	Arg-	Val-	Val-	Trp-	Cys-	Ala-	350	Val-	Gly-	Pro-	Glu-	Glu-	Glu-	Arg-	Lys-	Cys-	Lys-	360	Gln-
361	Trp-	Ser-	Asp-	Val-	Ser-	Asn-	Arg-	Lys-	Val-	370	Ala-	Cys-	Ala-	Ser-	Ala-	Ser-	Thr-	Thr-	Glu-	Glu-	380	Cys-
381	Ile-	Ala-	Leu-	Val-	Leu-	Lys-	Gly-	Glu-	Ala-	390	Asp-	Ala-	Leu-	Asn-	Leu-	Asp-	Gly-	Gly-	Phe-	Ile-	400	Tyr-
401	Val-	Ala-	Gly-	Lys-	Cys-	Gly-	Leu-	Val-	Pro-	410	Val-	Leu-	Ala-	Glu-	Asn-	Gln-	Lys-	Ser-	Gln-	Asn-	420	Ser-
421	Asn-	Ala-	Pro-	Asp-	Cys-	Val-	His-	Arg-	Pro-	430	Pro-	Glu-	Gly-	Tyr-	Leu-	Ala-	Val-	Ala-	Val-	Val-	440	Arg-
441	Lys-	Ser-	Asp-	Ala-	Asp-	Leu-	Thr-	Trp-	Asn-	450	Ser-	Leu-	Ser-	Gly-	Lys-	Lys-	Ser-	Cys-	His-	Thr-	460	Gly-
461	Val-	Gly-	Arg-	Thr-	Ala-	Ala-	Trp-	Asn-	Ile-	470	Pro-	Met-	Gly-	Leu-	Leu-	Phe-	Asn-	Gln-	Thr-	Gly-	480	Ser-
481	Cys-	Lys-	Phe-	Asp-	Lys-	Phe-	Phe-	Ser-	Gln-	490	Ser-	Cys-	Ala-	Pro-	Gly-	Ala-	Asp-	Pro-	Gln-	Ser-	500	Ser-
501	Leu-	Cys-	Ala-	Leu-	Cys-	Val-	Gly-	Asn-	Asn-	510	Glu-	Asn-	Glu-	Asn-	Lys-	Cys-	Met-	Pro-	Asn-	Ser-	520	Glu-
521	Glu-	Arg-	Tyr-	Tyr-	Gly-	Tyr-	Thr-	Gly-	Ala-	530	Phe-	Arg-	Cys-	Leu-	Ala-	Glu-	Lys-	Ala-	Gly-	Asp-	540	Val-
541	Ala-	Phe-	Val-	Lys-	Asp-	Val-	Thr-	Val-	Leu-	550	Gln-	Asn-	Thr-	Asp-	Gly-	Lys-	Asn-	Ser-	Glu-	Pro-	560	Trp-
561	Ala-	Lys-	Asp-	Leu-	Lys-	Gln-	Glu-	Asp-	Phe-	570	Glu-	Leu-	Leu-	Cys-	Leu-	Asp-	Gly-	Thr-	Arg-	Lys-	580	Pro-
581	Val-	Ala-	Glu-	Ala-	Glu-	Ser-	Cys-	His-	Leu-	590	Ala-	Arg-	Ala-	Pro-	Asn-	His-	Ala-	Val-	Val-	Ser-	600	Gln-
601	Ser-	Asp-	Arg-	Ala-	Gln-	His-	Leu-	Lys-	Lys-	610	Val-	Leu-	Phe-	Leu-	Gln-	Gln-	Asp-	Gln-	Phe-	Gly-	620	Gly-
621	Asn-	Gly-	Pro-	Asp-	Cys-	Pro-	Gly-	Lys-	Phe-	630	Cys-	Leu-	Phe-	Lys-	Ser-	Glu-	Thr-	Lys-	Asn-	Leu-	640	Leu-
641	Phe-	Asn-	Asp-	Asn-	Thr-	Glu-	Cys-	Leu-	Ala-	650	Glu-	Leu-	Gln-	Gly-	Lys-	Thr-	Thr-	Tyr-	Glu-	Gln-	660	Tyr-
661	Leu-	Gly-	Ser-	Glu-	Tyr-	Val-	Thr-	Ser-	Ile-	670	Thr-	Asn-	Leu-	Arg-	Arg-	Cys-	Ser-	Ser-	Ser-	Pro-	680	Leu-
681	Leu-	Glu-	Ala-	Cys-	Ala-	Phe-	Leu-	Arg-	Ala-	689												

Fig. 3. Amino acid sequence of equine lactoferrin (from Sharma et al., 1998).

Table 7
Properties of equine, bovine and human lactoferrin. Values were calculated from the amino acid sequences of the mature proteins provided on <http://au.expasy.org/tools>.

Species	Primary accession number ^a	Amino acid residues	Molecular mass (Da)	pI	GRAVY score ^b	Disulphide bridges
Equine	O77811	689	75420.4	8.32	−0.376	17
Bovine	P24627	689	76143.9	8.67	−0.350	16 ^c
Human	P02788	691	76165.2	8.47	−0.415	16

^a Primary accession number for the protein in SWISS-PROT database.

^b Grand average hydropathy (GRAVY) score calculated using the scale of Kyte and Doolittle (1982).

^c Estimated from structural similarity with human lactoferrin.

All Lf types studied to date are glycosylated, but the location and number of potential glycosylation sites, as well as the number of sites actually glycosylated, vary. In bovine Lf, four of five potential glycosylation sites, i.e., Asn₂₂₃, Asn₃₆₈, Asn₄₇₆ and Asn₅₄₅, are glycosylated (Moore, Anderson, Groom, Haridas, & Baker, 1997), whereas in human Lf, two of three potential glycosylation sites, i.e., Asn₁₃₇ and Asn₄₇₈, are glycosylated (Haridas, Anderson, & Baker, 1995). Glycosylation of equine Lf has not been studied, but using the consensus sequence, Asn-Xaa-Ser/Thr (where Xaa is not Pro), for glycosylation, three potential glycosylation sites are likely in equine Lf, i.e., Asn₁₃₇, Asn₂₈₁ and Asn₄₇₆.

4.1.4. Lysozyme

Equine milk contains approximately 0.8–1.1 g kg^{−1} lysozyme (Lyz) (Jauregui-Adell, 1975; Miranda, Mahé, Leroux, & Martin, 2004) (Table 4). Human milk contains ~0.3 g kg^{−1} (Lyz), slightly lower than the level in colostrum (Montagne, Cuillière, Molé, Béné, & Faure, 1998) but bovine milk contains only ~70–130 µg kg^{−1} (Chandan, Shahani, & Holly, 1964; El-Agamy, Ruppanner, Ismail, Champagne, & Assaf, 1996). The concentration of Lyz in human milk increases strongly after the second month of lactation, suggesting that Lyz and Lf play major roles in fighting infection in breast-fed infants during late lactation, and protect the mammary gland (Montagne et al., 1998). Equine milk Lyz is more stable to denaturation than human Lyz during pasteurization at 62 °C for 30 min, but at 71 °C for 2 min or 82 °C for 15 s, the inactivation of both were similar (Jauregui-Adell, 1975). It has been suggested, but research is scarce, that while the composition of breast milk varies widely between well-nourished and poorly-nourished mothers, the amount of Lyz is conserved.

Equine milk Lyz binds calcium, which increases the stability and activity of the enzyme (Nitta, Tsuge, Sugai, & Shimazaki, 1987). The

binding of a Ca²⁺ by Lyz is considered to be an evolutionary linkage between non-Ca²⁺-binding lysozymes and α-La (Chowdhury, Fairman, Bi, Rigotti, & Raleigh, 2004; Tada et al., 2002). The conformation of the calcium-binding loop of equine Lyz is similar to that of α-la (Tada et al., 2002; Tsuge et al., 1992) and both equine Lyz and α-la form stable, partially folded, “molten globule” states under various denaturing conditions (Koshiba, Kobashigawa, Demura, & Nitta, 2001) with that of equine Lyz being considerably more stable than α-la (Lyster, 1992; Morozova-Roche, 2007). However, the molten state of canine Lyz is significantly more stable than that of equine Lyz (Koshiba et al., 2000; Spencer et al., 1999). Equine milk Lyz is very resistant to acid (Jauregui-Adell, 1975) and protease digestion (Kuroki, Taniyama, Seko, Nakamura, & Kikuchi, 1989), and may reach the gut relatively intact.

4.1.5. Immunoglobulins

Three classes of immunoglobulins, which form part of the body's natural defense against infection, are commonly found in milk, immunoglobulin G (IgG), A (IgA) and M (IgM); IgG is often subdivided into two subclasses, IgG₁ and IgG₂ (Hurley, 2003; Madureira, Pereira, Gomes, Pintado, & Malcata, 2007). All monomeric Igs consist of a similar basic structure of four polypeptides, two heavy chains and two light chains, linked by disulphide bridges, yielding a subunit with a molecular mass of ~160 kDa. IgG consists of one subunit, while IgA and IgM consist of two or five subunits, with a molecular mass of ~400 or ~1000 kDa, respectively.

The relative proportions of the Igs in milk differ considerably between species (Table 4). IgG is the principal immunoglobulin in equine colostrum, but IgA is the principal form in equine milk. In bovine milk and colostrum, IgG is the principal immunoglobulin, while IgA is the predominant immunoglobulin in human colostrum

1	Arg	Pro	Lys	Leu	Pro	His	Arg	Gln	Pro	10	Glu	Ile	Ile	Gln	Asn	Glu	Gln	Asp	Ser	Arg	20	Glu
21	Lys	Val	Leu	Lys	Glu	Arg	Lys	Phe	Pro	30	Ser	Phe	Ala	Leu	Glu	Tyr	Ile	Asn	Glu	Leu	40	Asn
41	Arg	Gln	Arg	Glu	Leu	Leu	Lys	Glu	Lys	50	Gln	Lys	Asp	Glu	His	Lys	Asp	Thr	Ser	Asn	60	Glu
61	Ser	Thr	Glu	Glu	Tyr	Leu	Ile	Glu	Asp	70	Pro	Glu	Gln	Gln	Glu	Ser	Ser	Ser	Thr	Ser	80	Ser
81	Ser	Glu	Glu	Val	Val	Pro	Ile	Asn	Thr	90	Glu	Gln	Lys	Arg	Ile	Pro	Arg	Glu	Asp	Met	100	Leu
101	Tyr	Gln	His	Thr	Leu	Glu	Gln	Leu	Arg	110	Arg	Leu	Ser	Lys	Tyr	Asn	Gln	Leu	Gln	Leu	120	Gln
121	Ala	Ile	His	Ala	Gln	Glu	Gln	Leu	Ile	130	Arg	Met	Lys	Glu	Asn	Ser	Gln	Arg	Lys	Pro	140	Met
141	Arg	Val	Val	Asn	Gln	Glu	Gln	Ala	Tyr	150	Phe	Tyr	Leu	Glu	Pro	Phe	Gln	Pro	Ser	Tyr	160	Gln
161	Leu	Asp	Val	Tyr	Pro	Tyr	Ala	Ala	Trp	170	Phe	His	Pro	Ala	Gln	Ile	Met	Gln	His	Val	180	Ala
181	Tyr	Ser	Pro	Phe	His	Asp	Thr	Ala	Lys	190	Leu	Ile	Ala	Ser	Glu	Asn	Ser	Glu	Lys	Tyr	200	Asp
201	Ile	Ile	Pro	Glu	Trp					210												

Fig. 4. Amino acid sequence of equine α_{s1}-casein (from Lenasi et al., 2003). Potential phosphorylation sites are shaded.

Table 8Properties of equine, bovine and human α_{s1} -, β - and κ -caseins.

Protein	Species	Primary accession number ^a	Amino acid residues	MW (Da)	pI	GRAVY ^b	Cysteine residues
α_{s1} -casein	Equine	—	205	24,614.4	5.47	−1.127	0
	Bovine	P02662	199	22,974.8	4.99	−0.704	0
	Human	P47710	170	20,089.4	5.17	−1.013	3
β -casein	Equine	Q9GKK3	226	25,511.4	5.78	−0.415	0
	Bovine	P02666	209	23,583.2	5.13	−0.355	0
	Human	P05814	211	23,857.8	5.33	−0.289	0
κ -casein	Equine	P82187	165	18,844.7	8.03	−0.313	2
	Bovine	P02668	169	18,974.4	5.93	−0.557	2
	Human	P07498	162	18,162.6	8.68	−0.528	1

^a Primary accession number for the protein in SWISS-PROT database.^b Grand average hydropathy (GRAVY) score calculated using the scale of Kyte and Doolittle (1982).

and milk. In some species, e.g., humans, maternal IgG is transferred to the foetus in utero and the intestines are relatively impermeable to proteins in a full-term newborn. In contrast, in ruminants and equids, IgG is not transferred in utero and the newborn depends on colostrum to supply IgG, so that within 2–3 days, the serum level of IgG is similar to adult levels, after which the intestinal tract ‘closes’ and further significant passage of proteins is prevented (Widdowson, 1984).

4.2. Denaturation of whey proteins

Whey proteins are susceptible to heat-induced denaturation. The thermal stability of equine Lf and BSA is comparable with that of their bovine counterparts but equine β -Lg and α -La are more heat stable than corresponding bovine proteins (Bonomi, Iametti, Pagliarini, & Solaroli, 1994). Equine β -Lg is reported to be more thermally stable than equine α -La, which is different from bovine milk, where α -La is the more thermally stable (Civardi, Curadi, Orlandi, Cattaneo, & Giangiacomo, 2007). The high thermal stability of equine β -Lg may be related to its lack of a sulphhydryl group. Thermal denaturation of bovine β -Lg is a two-stage process, unfolding of the polypeptide chain and exposure of the sulphhydryl group, followed by self-association or with other proteins via sulphhydryl-disulphide interchange (Sawyer, 2003). Owing to the lack of a sulphhydryl group, equine β -Lg cannot undergo sulphhydryl-disulphide interchange reactions. Denaturation of α -La is a result of complex formation with β -Lg via sulphhydryl-disulphide interchange and its higher thermal stability may therefore be a result of differences in its environment, rather than its molecular structure. Equine α -La and β -Lg are also less susceptible to high pressure-induced denaturation than their bovine counterparts (Uniacke-Lowe & Huppertz, unpublished data).

4.3. Caseins

Casein micelles are primarily a source of amino acids, calcium, phosphate and bioactive peptides for neonates (Shekar et al., 2006). The lower casein concentration in mature equine milk (~55% of total protein) compared with bovine milk (~80% of total protein) has many implications which will be discussed below. The traditional method for separating caseins from whey proteins is isoelectric precipitation of the caseins at pH ~4.6. The casein fraction of most milk types consists of four gene products: α_{s1} -, α_{s2} -, β - and κ -caseins, of which the first three are calcium-sensitive. All caseins display a distinct lack of secondary structure, which led Holt and Sawyer (1993) to classify them as rheomorphic proteins. The lack of secondary structure may be attributed, at least partially, to the relatively high level of proline residues in casein. As a result, caseins do not denature or associate on heating (Paulson & Dejmek, 1990). The biological function of the caseins lies in their ability to

form macromolecular structures, casein micelles, which transfer large amounts of calcium to the neonate with a minimal risk of pathological calcification of the mammary gland. The following sections will discuss the individual caseins separately and focus on their interactions to form casein micelles and the colloidal stability thereof.

4.3.1. α_{s1} -Casein

The amino acid sequence of equine α_{s1} -casein, derived from its cDNA sequence by Lenasi, Rogelj, and Dovc (2003), is presented in Fig. 4. The protein contains 205 amino acids and has a molecular mass 24,614.4 Da prior to post-translational modification; as such, it is considerably larger than its bovine or human counterpart (Table 8). Two smaller isoforms of α_{s1} -casein have been identified in equine milk, which probably result from the skipping of exons during transcription (Miranda et al., 2004). Equine α_{s1} -casein contains six potential phosphorylation sites (Lenasi et al., 2003; Fig. 4), five of which are in very close proximity (Ser₇₅, Ser₇₇, Ser₇₉, Ser₈₀, Ser₈₁) and can thus form a phosphorylation centre, which is important in the structure of casein micelles. Matêos et al. (2009b) determined the different phosphorylation levels of the native isoforms of equine α_{s1} -casein and identified 36 different isoforms with several phosphate groups ranging from two to six or eight, which, like for equine β -casein, present a complex pattern on 1D and 2D electrophoresis. Bovine α_{s1} -casein contains eight or nine phosphorylation sites (Swaigood, 2003), which form two phosphorylation centres (De Kruif & Holt, 2003). Equine α_{s1} -casein appears to have three domains characterized by a high hydropathy value, i.e., around residues 25–30, 95–105 and 150–205 and therefore probably displays association properties similar to those of bovine α_{s1} -casein which also contains three distinct hydrophobic regions. Furthermore, equine α_{s1} -casein contains two regions with very low hydropathy, i.e., around residues 45–55 and 125–135, and therefore are expected to behave hydrophilically. Human α_{s1} -casein does not appear to have distinct hydrophobic regions on its N-terminus. Overall, equine and human α_{s1} -casein have comparable GRAVY scores, which are lower than that of bovine α_{s1} -casein (Table 8), indicating an overall higher hydrophobicity for the latter.

Prior to the mid-1990's it was generally accepted that human milk contained mainly β - and κ -caseins with little or no α_{s1} -casein (Kunz & Lönnerdal, 1990). A minor casein component has since been identified and is considered to be the human equivalent of α_{s1} -casein although this identification highlights several inconsistencies when compared with the equivalent casein in other species. Uniquely, human α_{s1} -casein appears to contain at least two cysteine residues and exists as a multimer in complex with κ -casein (Cavaletto, Cantisani, Gluffrida, Napolitano, & Coni, 1994; Rasmussen, Due, & Petersen, 1995). Johnsen, Rasmussen, Petersen, and Berglund (1995) identified three cysteine residues in human α_{s1} -casein which

1	Arg-	Glu-	Lys-	Glu-	Glu-	Leu-	Asn-	Val-	Ser-	10	Glu-	Thr-	Val-	Glu-	Ser-	Leu-	Ser-	Ser-	Asn-	20	Glu-
21	Pro-	Asp-	Ser-	Ser-	Ser-	Glu-	Glu-	Ser-	Ile-	30	Thr-	His-	Ile-	Asn-	Lys-	Glu-	Lys-	Leu-	Gln-	40	Phe-
41	Lys-	His-	Gln-	Gly-	Gln-	Gln-	Gln-	Arg-	Gln-	50	Val-	Gln-	Arg-	Gln-	Asp-	<u>Lys-</u>	<u>Ile-</u>	Ser-	Arg-	60	Val-
61	Gln-	Pro-	Gln-	Pro-	Val-	Val-	Tyr-	Pro-	Tyr-	70	Ala-	Glu-	Pro-	Val-	Pro-	Tyr-	Ala-	Val-	Val-	80	Gln-
81	Ser-	Ile-	Leu-	Pro-	Leu-	Ala-	Gln-	Pro-	Pro-	90	Ile-	Leu-	Pro-	Phe-	Leu-	Gln-	Pro-	Glu-	Ile-	100	Met-
101	Val-	Ser-	Gln-	Ala-	Lys-	Glu-	Thr-	Ile-	Leu-	110	Pro-	Lys-	Arg-	Lys-	Val-	Met-	Pro-	Phe-	Leu-	120	Ser-
121	Pro-	Ile-	Val-	Pro-	Phe-	Ser-	Glu-	Arg-	Gln-	130	Ile-	Leu-	Asn-	Pro-	Thr-	Asn-	Gly-	Glu-	Asn-	140	Arg-
141	Leu-	Pro-	Val-	His-	Leu-	Ile-	Gln-	Pro-	Phe-	150	Met-	His-	Gln-	Val-	Pro-	Gln-	Ser-	Leu-	Leu-	160	Thr-
161	Leu-	Met-	Leu-	Pro-	Ser-	Gln-	Pro-	Val-	Leu-	170	Ser-	Pro-	Pro-	Gln-	Ser-	Lys-	Val-	Ala-	Pro-	180	Pro-
181	Gln-	Pro-	Val-	Val-	Pro-	Tyr-	Pro-	Gln-	Arg-	190	Asp-	Thr-	Pro-	Val-	Gln-	Ala-	Phe-	Leu-	Leu-	200	Gln-
201	Asp-	Pro-	Arg-	Leu-	Gly-	Pro-	Thr-	Gly-	Glu-	210	Leu-	Asp-	Pro-	Ala-	Thr-	Gln-	Pro-	Ile-	Val-	220	Val-
221	His-	Asn-	Pro-	Val-	Ile-	Val-															

Fig. 5. Amino acid sequence of equine β -casein (from Lenasi et al., 2003; Girardet et al., 2006). Potential phosphorylation sites are shown grey; the plasmin-sensitive bond is underlined and the Asn residue susceptible to deamidation is in bold.

explains α_{s1} - κ -casein complex formation. Martin, Brignon, Furet, and Leroux (1996) provided definitive evidence for the presence of a functional α_{s1} -casein locus in the human genome which is expressed in the mammary gland during lactation while Sørensen, Møller, Vinther, Petersen, and Rasmussen (2003) reported the phosphorylation pattern of human α_{s1} -casein. In bovine milk, α_{s1} -casein is a major structural component of the casein micelle and plays a functional role in curd formation (Walstra & Jenness, 1984). The relatively low level of α_{s1} -casein in equine milk compared with bovine milk (Table 4) may be significant and, coupled with the low protein content, may be responsible for the soft curd produced in the infant stomach and the foal (Dr. Ursula Fogarty, National Equine Centre, Ireland – personal communication). Goat milk lacking α_{s1} -casein has poor coagulation properties compared with milk containing α_{s1} -casein (Clark & Sherbon, 2000).

4.3.2. α_{s2} -Casein

The complete amino acid sequence of equine α_{s2} -casein is unknown, but Ochirkhuyag, Chobert, Dalgalarrodo, and Haertlé (2000) published the sequence of the N-terminal 15 amino acid residues (Lys-His-Lys-Met-Glu-His-Phe-Ala-Pro-???-Tyr-???-Gln-Val-Leu). Only five of these amino acids (Lys-His-Asn-Met-Glu-His-Arg-Ser-Ser-Ser-Glu-Glu) were confirmed by Miranda et al. (2004).

Isoelectric focusing showed two major bands for equine α_{s2} -casein, with isoelectric points in the pH-range 4.3–5.1 (Ochirkhuyag et al., 2000). Bovine α_{s2} -casein is the most highly phosphorylated casein, usually containing eleven phosphorylated serine residues, with lesser amounts containing ten, twelve or thirteen phosphate groups (Swaisgood, 2003). There are no reports on the presence of α_{s2} -casein in human milk.

4.3.3. β -Casein

The amino acid sequence of equine β -casein, derived from the cDNA, reported by Lenasi et al. (2003), and revised by Girardet, Miclo, Florent, Mollé, and Gaillard (2006) by the insertion of eight amino acids (Glu₂₇ to Lys₃₄), is shown in Fig. 5. The theoretical molecular mass of this 226 amino acid polypeptide is 25,511.4 Da (Table 8). Bovine and human β -casein contain 209 and 211 amino acid residues, respectively (Table 8). Two smaller variants of equine β -casein, which probably result from casual exon-skipping during transcription, were reported by Miranda et al. (2004). The 28 C-terminal amino acids contain seven potential phosphorylation sites (Ser₉, Ser₁₅, Ser₁₈, Ser₂₃, Ser₂₄, Ser₂₅, Ser₂₈) and multiple-phosphorylated isoforms of equine β -casein, containing three to seven phosphoserine residues, have been reported, with an isoelectric point varying from pH 4.74 to 5.30 (Girardet et al., 2006;

1										10										20
Glu- 21	Val-	Gln-	Asn-	Gln-	Glu-	Gln-	Pro-	Thr-	Cys- 30	His-	Lys-	Asn-	Asp-	Glu-	Arg-	Phe-	Phe-	Asp-	Leu-	
Lys- 41	Thr-	Val-	Lys-	Tyr-	Ile-	Pro-	Ile-	Tyr-	Tyr- 50	Val-	Leu-	Asn-	Ser-	Ser-	Pro-	Arg-	Tyr-	Glu-	Pro-	
Ile- 61	Tyr-	Tyr-	Gln-	His-	Arg-	Leu-	Ala-	Leu-	Leu- 70	Ile-	Asn-	Asn-	Gln-	His-	Met-	Pro-	Tyr-	Gln-	Tyr-	
Tyr- 81	Ala-	Arg-	Pro-	Ala-	Ala-	Val-	Arg-	Pro-	His- 90	Val-	Gln-	Ile-	Pro-	Gln-	Trp-	Gln-	Val-	Leu-	Pro-	
Asn- 101	Ile-	Tyr-	Pro-	Ser-	Thr-	Val-	Val-	Arg-	His- 110	Pro-	Cys-	Pro-	His-	Pro-	Ser-	<u>Phe-</u>	<u>Ile-</u>	Ala-	Ile-	
Pro- 121	Pro-	Lys-	Lys-	Leu-	Gln-	Glu-	Ile-	Thr-	Val- 130	Ile-	Pro-	Lys-	Ile-	Asn-	Thr-	Ile-	Ala-	Thr-	Val-	
Glu- 141	Pro-	Thr-	Pro-	Ile-	Pro-	Thr-	Pro-	Glu-	Pro- 150	Thr-	Val-	Asn-	Asn-	Ala-	Val-	Ile-	Pro-	Asp-	140	
Ser- 161	Ser-	Glu-	Phe-	Ile-	Ile-	Ala-	Ser-	Thr-	Pro-	Glu-	Thr-	Thr-	Thr-	Val-	Pro-	Val-	Thr-	Ser-	160	
Val-	Val-	Gln-	Lys-	Leu-																

Fig. 6. Amino acid sequence of equine κ -casein. Potential phosphorylation sites are shaded grey. Potential glycosylation sites are printed in bold font (from Lenasi et al., 2003). The chymosin-sensitive bond is underlined.

Matéos et al., 2009a). Bovine β -casein, which contains four or five phosphorylated serine residues, has an isoelectric point of 5.0–5.5 (Swaisgood, 2003). Human β -casein has up to six phosphorylation levels, i.e., 0, 1, 2, 3, 4 or 5 phosphorylated serine residues (Sood & Slattery, 2000). Equine, bovine and human β -casein have a very hydrophilic N-terminus, followed by a relatively random hydrophathy distribution in the rest of the protein, leading to an amphiphilic protein with a hydrophilic N-terminus and a hydrophobic C-terminus. In equine sodium caseinate, the Lys₄₇–Ile₄₈ bond of equine β -casein is readily hydrolysed by bovine plasmin whereas no cleavage of a corresponding bond, Lys₄₈–Ile₄₉, in bovine β -casein, occurs (Egito et al., 2003). In bovine β -casein, Lys₂₈–Lys₂₉ is readily cleaved by plasmin but the equivalent, Lys₂₈–Leu₂₉, in equine β -casein, is insensitive (Egito et al., 2002). Other plasmin cleavage sites in equine β -casein are Lys₁₀₃–Arg₁₀₄, Arg₁₀₄–Lys₁₀₅ and Lys₁₀₅–Val₁₉₆ (Egito et al., 2002). Equine β -casein is readily hydrolysed by chymosin at Leu₁₉₀–Tyr₁₉₁ (Egito et al., 2001).

Equine β -casein and equine α -la undergo spontaneous deamidation under physiological conditions at Asn₁₃₅–Gly₁₃₆ and Asn₄₅–Gly₄₆, respectively (Girardet et al., 2006, 2004) which has been reported also for canine milk Lyz (Nonaka et al., 2008) and human Lf (Belizy et al., 2001) but not, to our knowledge, for bovine or human β -casein. Recent research has demonstrated that temperature may be an important factor controlling the spontaneous deamidation process and at 10 °C the phenomenon is strongly reduced (Matéos et al., 2009a, 2009b). Spontaneous deamidation represents an important modification of equine milk proteins under certain conditions where bovine milk proteins, which do not contain a potential site for deamidation, remain unaffected. Equine Lf also contains the Asn–Gly sequence and may be susceptible to spontaneous deamidation (Girardet et al., 2006).

Unique to equine milk and apparently absent from the milk of other species, including ruminants, is a low molecular mass multi-phosphorylated β -casein variant which accounts for 4% of the total casein (Miclo et al., 2007). This short protein (94 amino acid residues) is the result of a large deletion (residues 50–181) from full-length equine β -casein. No spontaneous deamidation of this low molecular mass form of β -casein has been found (Miclo et al., 2007).

4.3.4. κ -Casein

The presence of κ -casein in equine milk was an issue of debate for several years, with several authors (Ochirkhuyag et al., 2000; Ono, Kohno, Odagiri, & Takagi, 1989; Visser, Jenness, & Mullin, 1982) reporting its absence. However, other studies (Egito et al., 2001; Iametti, Tedeschi, Oungre, & Bonomi, 2001; Kotts & Jenness, 1976; Malacarne, Summer, Formaggioni, & Mariani, 2000) showed its presence, albeit at a low concentration. The primary structure of equine κ -casein has been derived (Fig. 6) (Iametti et al., 2001; Lenasi et al., 2003; Miranda et al., 2004); it contains 165 amino acids residues, i.e., four less than bovine κ -casein but three more than human κ -casein (Table 8). The

molecular mass of equine κ -casein, prior to post-translational modification, is 18,844.7 Da. Equine and human κ -casein have a considerably higher isoelectric pH than bovine κ -casein (Table 8), and they have a net positive charge at physiological pH, whereas bovine κ -casein has a net negative charge. The GRAVY score of bovine κ -casein is considerably lower than that of equine κ -casein (Table 8), indicating that equine κ -casein is less hydrophilic than its bovine counterpart. Bovine κ -casein is characterized by a hydrophilic C-terminus, which is very important for the stabilization of bovine casein micelles, but a comparison of the hydrophathy distribution of bovine and equine κ -caseins indicates that the C-terminus of equine κ -casein is far less hydrophilic, particularly as a result of the absence of a strong hydrophilic region at residues 110–120. Equine κ -casein appears to be more like human than bovine κ -casein in terms of the distribution of hydrophathy along the polypeptide chain.

4.3.5. Glycosylation of κ -casein

κ -Casein is the only glycosylated member of the casein family and exhibits micro-heterogeneity due to the level of glycosylation (Saito & Itoh, 1992). Oligosaccharides consisting of N-acetylneuraminic acid (NANA), galactose and N-acetylgalactosamine are attached to κ -casein via O-glycosidic linkages to threonine residues in the C-terminal portion of the molecule (glycomacropeptide, GMP, region). Although no direct information is available, lectin-binding studies indicate that equine κ -casein is glycosylated (Iametti et al., 2001), possibly at residues Thr₁₂₃, Thr₁₂₇, Thr₁₃₁, Thr₁₄₉ and Thr₁₅₃ (Fig. 6; Lenasi et al., 2003), although these glycosylation sites are not fully in agreement with those proposed by Egito et al. (2001). About two-thirds of bovine κ -casein molecules are glycosylated at one of six threonyl residues, i.e., Thr₁₂₁, Thr₁₃₁, Thr₁₃₃, Thr₁₃₅, Thr₁₃₆ (only in bovine κ -casein variant A) or Thr₁₄₂ (Pisano, Packer, Redmond, Williams, & Gooley, 1994); Ser₁₄₁ is also a potential glycosylation site (Kanamori, Doi, Ideno, & Ibuki, 1981). Human κ -casein has seven glycosylation sites, Thr₁₁₃, Thr₁₂₃, Thr₁₂₈, Thr₁₃₁, Thr₁₃₇, Thr₁₄₇ and Thr₁₄₉ (Fiat, Jollès, Aubert, Loucheux-Lefebvre, & Jollès, 1980). To date, no non-glycosylated κ -casein has been identified in equine milk (Martuzzi & Doreau, 2006).

κ -Casein is located mainly on the surface of the casein micelles and is responsible for their stability (Walstra, 1990). The presence of a glycan moiety in the C-terminal region of κ -casein enhances its ability to stabilize the micelle, by electrostatic repulsion, and may increase the resistance by the protein to proteolytic enzymes and high temperatures (Dziuba & Minkiewicz, 1996; Minkiewicz, Dziuba, & Muzińska, 1993). Biologically, NANA residues have antibacterial properties and act as a bifidogenic factor (Dziuba & Minkiewicz, 1996). κ -Casein is thought to play a major role in preventing adhesion of *Helicobacter pylori* to human gastric mucosa (Strömqvist et al., 1995). It is likely that heavily glycosylated κ -casein provides protection due to its carbohydrate content and breast-feeding infants is thought to provide some protection, especially as *H. pylori* infection is occurring at an increasingly younger age (Lönnnerdal, 2003).

4.3.6. Hydrolysis of κ -casein

The hydrolysis of bovine κ -casein by chymosin at Phe₁₀₅–Met₁₀₆ leads to the production of the hydrophobic C-terminal para- κ -casein and the hydrophilic N-terminal caseinomacropeptide (CMP) (Walstra & Jenness, 1984). Calf chymosin hydrolyses the Phe₉₇–Ile₉₈ bond of equine κ -casein (Egito et al., 2001) and slowly hydrolyses the Phe₁₀₅–Ile₁₀₆ bond of human κ -casein (Plowman, Creamer, Liddell, & Cross, 1999). However, as summarized in Table 9, the CMPs released from equine or human κ -casein are considerably less hydrophilic than bovine CMP. The sequence 97–116 of κ -casein is highly conserved across species, suggesting that the limited

Table 9
Properties of equine, bovine and human para- κ -casein and caseinomacropeptide.

Protein	Species	Residues	Amino acid residues	Mw (Da)	pI	GRAVY ^a
Para- κ -casein	Equine	1–97	97	11,693.3	8.96	–0.675
	Bovine	1–105	105	12,285.0	9.33	–0.617
	Human	1–105	97	11,456.9	9.63	–1.004
CMP	Equine	98–165	68	7169.3	4.72	0.203
	Bovine	106–169	63	6707.4	4.04	–0.370
	Human	106–162	65	6723.7	4.24	0.182

^a Grand average hydrophathy (GRAVY) score calculated using the scale of Kyte and Doolittle (1982).

proteolysis of κ -casein and subsequent coagulation of milk are of major biological significance (Mercier, Chobert, & Addeo, 1976).

A grouping system for mammals based on κ -casein structure and the site of cleavage by chymosin has been suggested (Mercier et al., 1976; Nakhasi, Grantham, & Gullino, 1984). Group I species (cow, goat, sheep, buffalo) have a higher content of dicarboxylic amino acids and low hydrophobicity and carbohydrate content and κ -casein is cleaved at Phe₁₀₅-Met₁₀₆, while Group II species (human, mouse, pig, rat) have a high proline content, less dicarboxylic amino acids and a much higher hydrophobicity and carbohydrate content and are cleaved at Phe₉₇-Ile₉₈ or Phe₁₀₅-Leu₁₀₆. Marsupial κ -casein appears to form a separate group, with a putative chymosin cleavage site of Phe-Ala, which is different from that in eutherian mammals (Stasiuk, Summers, & Demmer, 2000). Cleavage of equine milk at Phe₉₇-Ile₉₈, as well as other characteristics of its κ -casein, place the horse in Group II. The divergence between species into groups I and II could account for differences in the clotting mechanisms of ruminant and non-ruminant milk (Herskovitis, 1966).

In addition to the differences in cleavage site, the grouping system also divides species based on the number of O-glycosylation sites. As equine and human κ -caseins are considerably more highly glycosylated than bovine κ -casein and non-glycosylated κ -casein has not been found in equine milk (Egito et al., 2001), equine and human κ -casein belong to the same group. The level of glycosylation does not affect micelle structure but it does affect the susceptibility of κ -casein to hydrolysis by chymosin, with susceptibility decreasing as the level of glycosylation increases (Addeo, Martin, & Ribadeau-Dumas, 1984; Doi, Ibuki, & Kanamori, 1979; Van Hooydonk, Olieman, & Hagedoorn, 1984; Vreeman, Visser, Slangen, & van Riel, 1986; Zbikowska, Dziuba, Jaworska, & Zaborniak, 1992). Therefore, equine milk probably has a different chymosin-induced clotting mechanism than bovine milk.

5. Equine casein micelles

In the milk of all species studied in sufficient detail, the caseins exist predominantly as micelles, which are hydrated spherical structures with dimensions in the sub-micron region. The dry matter of casein micelles consists predominantly (>90%) of proteins, with small amounts of inorganic matter, collectively referred to as micellar calcium phosphate (MCP). The structure and sub-structure of bovine casein micelles has been studied in detail and reviews include De Kruif and Holt (2003), Farrell, Malin, Brown, and Qi (2006), Fox and Brodtkorb (2008), Holt and Horne (1996), Horne (1998, 2006), Phadungath (2005), Qi (2007). The casein micelles are best described as sterically stabilized association colloids (De Kruif & Holt, 2003). MCP exists as nanometer-sized clusters of amorphous calcium phosphate which are stabilized by a shell of caseins (α_{s1} -, α_{s2} - and β -caseins). This core-and-shell structure is commonly referred to as a nanocluster. Such nanoclusters may associate to form particles of colloidal dimensions, either via cross-linking through caseins which contain more than one phosphorylation centre, or through solvent-mediated association of the proteins of the shell of the nanoclusters. Growth of casein micelles is terminated by the solvent-mediated adsorption of κ -casein onto the micellar surface. The hydrophilic C-terminal region of κ -casein or 'brush' protrudes from the surface of the micelles and sterically stabilizes them against aggregation (De Kruif & Zhulina, 1996).

Equine casein micelles have an average diameter of ~ 255 nm (Buchheim, Lund, & Scholtissek, 1989; Welsch, Buchheim, Schumacher, Schinko, & Patton, 1988) while bovine casein micelles have an average diameter of ~ 180 nm (Table 4). Human casein micelles are considerably smaller, with an average diameter of 60–80 nm (Table 4).

Electron microscopy shows a 'spongy' appearance for equine and bovine micelles, although bovine micelles appear more ordered and equine micelles 'looser'; human micelles are considerably looser than equine micelles (Jasińska & Jaworska, 1991). Such a loose open structure may affect the susceptibility to hydrolysis by pepsin. Jasińska and Jaworska (1991) reported that human micelles were much more susceptible to peptic hydrolysis than either equine or bovine micelles.

The sub-structure of equine casein micelles has not been studied in detail but some information may be derived from comparison with bovine milk. Equine milk contains ~ 10.1 mmol L⁻¹ micellar calcium and ~ 2.6 mmol L⁻¹ micellar inorganic phosphorus, compared with ~ 20.2 and 9.7 mmol L⁻¹ in bovine milk (Holt & Jenness, 1984). Considering that equine milk contains <0.5 mmol casein L⁻¹, this suggests a micellar calcium: casein molar ratio of $>20:1$, which far exceeds the calcium-binding capacity of equine casein. Hence, it may be assumed that like bovine micelles, equine micelles contain nanoclusters of calcium phosphate.

Both equine α_{s1} -casein (residues 75–81; Fig. 4) and β -casein (residues 23–28; Fig. 5) contain a phosphorylation centre, which is required for the formation of nanoclusters (De Kruif & Holt, 2003); furthermore, both proteins also contain distinct hydrophobic regions through which solvent-mediated protein–protein interactions may occur. Equine α_{s2} -casein may have similar properties, pending further characterization. The ratio of micellar calcium: micellar inorganic phosphorus is 2.0 in equine milk, but ~ 3.9 in bovine milk (Holt & Jenness, 1984) and might indicate that either a smaller proportion of micellar calcium is incorporated into nanoclusters in equine milk, or that equine nanoclusters contain a higher proportion casein-bound phosphate. The latter would imply smaller nanoclusters, since the casein-bound phosphate can participate only at the surface of the core. However, unlike bovine κ -casein, equine κ -casein does not have a distinctly hydrophilic C-terminal domain; thus, it is unclear if this part of the protein is capable of protruding from the micellar surface to sterically stabilize the micelle.

Doreau and Martin-Rosset (2002) and Ochirkhuuyag et al. (2000) concluded that the steric stabilization of equine casein micelles by κ -casein may be aided by non-phosphorylated β -casein on the surface of the micelle, thus compensating for the low κ -casein content. A similar conclusion was reported by Dev, Satish, Sood, De Wind, and Slattery (1994) for the stabilization of human casein micelles which also have a very low content of κ -casein. Further research is required to elucidate the structure of the equine casein micelle as destabilization of the micelles is the basis for the successful conversion of milk into a range of dairy products, e.g., cheese or yoghurt.

5.1. Colloidal stability of casein micelles

Coagulation of milk occurs when colloidal stability is destroyed and may be desirable or undesirable. Coagulation is desirable in the manufacture of yoghurt and cheese. Coagulation of milk is also important from a nutritional point of view, as clotting of the caseins in the stomach, and the type and structure of the resultant coagulum, strongly affects digestibility. In contrast, heat-induced coagulation of casein micelles, which can occur at a temperature >120 °C, e.g., during the retort sterilization of liquid products for infant or clinical nutrition, is undesirable. In this section, common types of micellar instability will be described.

As outlined above, bovine casein micelles are sterically stabilized by a brush of predominantly κ -casein, which protrudes from the micelle surface. Coagulation of casein micelles can occur only following collapse of the brush, which occurs on acidification of milk, i.e., in the manufacture of yoghurt or on removal of the brush which occurs on rennet-induced coagulation of milk. The combined

process of enzyme and acid-induced coagulation is likely to contribute to coagulation of casein micelles in the stomach.

5.2. Enzymatic coagulation of equine milk

Enzymatic coagulation of milk is the first step in the manufacture of most cheese varieties and also plays a role in the flocculation of casein micelles in the stomach. For cheese manufacture, the process involves the addition of a milk-clotting enzyme, e.g., chymosin, to the milk, followed by incubation at a temperature $>30^{\circ}\text{C}$. During incubation of bovine milk with rennet, chymosin hydrolyses the Phe₁₀₅-Met₁₀₆ bond in κ -casein, leading to the formation of two fragments, the hydrophobic N-terminal fragment, f1-105, which remains attached to the casein micelles and is referred to as para- κ -casein, and the hydrophilic C-terminal fragment, f106-169, which is released into the milk serum and is referred to as the caseinomacropetide (CMP). As a result, the micelles lose steric stabilization and become susceptible to aggregation, particularly in the presence of Ca^{2+} . Since equine κ -casein is hydrolysable by chymosin at the Phe₉₇-Ile₉₈ bond (Egito et al., 2001), albeit slowly (Kotts & Jenness, 1976) and without gel formation (Uniacke-Lowe & Fox, unpublished data), it appears that either the chymosin-sensitive bond of equine κ -casein is located in the micelle in a manner which renders it is inaccessible by chymosin, or the equine casein micelle derives colloidal stability from constituents other than κ -casein. The high degree of glycosylation may also affect the ability of chymosin to hydrolyse equine κ -casein.

5.3. Acid-induced flocculation of equine milk

When bovine milk is acidified to a pH below 5.0, flocculation of casein micelles occurs, leading ultimately to gel formation. This process is the basis of the manufacture of yoghurt, in which acidification is induced by the production of lactic acid by lactic acid bacteria and also occurs at the low pH of the stomach. Equine casein micelles are considerably less susceptible to acid-induced flocculation than bovine casein micelles and equine casein has a lower isoelectric pH (4.2) than bovine casein (pH 4.6) due to the slightly more acidic character of equine β - and κ -caseins (Egito et al., 2001, 2002). Acidification of equine milk causes a relatively small increase in viscosity (Di Cagno et al., 2004) compared with that of acidified bovine milk and, in the case of the former, is probably indicative of micellar flocculation rather than gelation. Differences in acid-induced flocculation between equine and bovine casein micelles may be related to differences in the mechanism by which they are sterically stabilized. Acid-induced flocculation of bovine casein micelles is believed to result from a reduction in the solvency of the κ -casein brush on the micellar surface due to protonation of the negatively-charged carboxylic acid groups of Glu and Asp. Elucidation of the mechanism of steric stabilization of equine casein micelles is likely to shed further light on this subject.

5.4. Heat-induced coagulation of equine milk

Heat-induced coagulation of milk occurs when milk is heated sufficiently long at $>120^{\circ}\text{C}$. Unconcentrated bovine milk, usually assayed at 140°C , displays a typical profile, with a heat coagulation time (HCT) maximum (~ 20 min) at pH ~ 6.7 and a minimum at pH ~ 6.9 (O'Connell & Fox, 2003). In contrast, the HCT of unconcentrated equine milk at 140°C increases with pH, i.e., it has a sigmoidal pH-HCT profile, with a mid-point around pH 6.7. HCT at pH 7 is ~ 60 min (Uniacke-Lowe, Huppertz & Fox, unpublished data). Differences in heat stability between equine and bovine milk may be related to differences in steric stabilization of the micelles; in addition, heat-induced complexing of β -Lg with κ -casein greatly

Table 10

The free amino acids ($\mu\text{M L}^{-1}$) of equine, bovine and human milk.

Amino acid	Equine ^a	Bovine ^a	Human ^b
Alanine	105	30.0	227.5
Arginine	14.0	10.0	35.4
Aspartic acid	40.0	15.0	183.2
Cystine	2.0	21.0	(56) ^a
Glutamic acid	568.0	117.0	1184.1
Glutamine	485.0	12.0	284.8
Glycine	100.0	88.0	124.6
Histidine	46.0	9.0	7.7
Isoleucine	8.0	3.0	33.4
Leucine	16.0	3.0	55.6
Lysine	26.0	15.0	39.0
Methionine	~ 0	~ 0	8.8
Phenylalanine	5.0	3.0	23.6
Proline	1.61	-	64.3
Serine	175	23.0	273.7
Taurine	32.0	13.0	301.1
Threonine	137.0	16.0	97.6
Tyrosine	3.0	0.3	2.5
Valine	45.0	5.0	72.7
Total	~ 1960	578	3019.7

^a Rassin et al. (1978).

^b Agostini et al. (2000).

affects the heat stability of bovine milk (O'Connell & Fox, 2003), but is unlikely to occur in equine milk due to lack of a sulphhydryl group in equine β -Lg. Finally, the lower casein concentration in equine milk is also likely to contribute to its higher heat stability.

6. Non-protein nitrogen of equine milk

The non-protein-nitrogen (NPN) of milk consists primarily of urea, peptides, amino acids and ammonia. NPN constitutes 10–15% of the total nitrogen in mature equine milk which is intermediate between the values for human milk and ruminant milk, 25 and 5%, respectively (Atkinson, Schnurr, Donovan, & Lönnerdal, 1989; Hambræus, 1984; Oftedal et al., 1983; Walstra, Wouters, & Geurts, 2006). In equine milk, NPN increases from $<2\%$ of total nitrogen at parturition to $>10\%$ after two weeks (Zicker & Lönnerdal, 1994). The components of the NPN in human milk have been characterized (see Atkinson & Lönnerdal, 1995; Atkinson et al., 1989; Carratù, Boniglia, Scalise, Ambruzzi, & Sanzini, 2003), but the NPN of equine milk has not been studied in detail.

6.1. Bioactive peptides

Both caseins and whey proteins are believed to contribute to human health through latent biological activity after enzymatic hydrolysis by digestive enzymes, fermentation with specific starter cultures or enzymatic hydrolysis by enzymes derived from micro-organisms or plants (Phelan, Aherne, Fitzgerald, & O'Brien, 2009). Some of the peptides released, termed bioactive peptides, are capable of modulating specific physiological functions: anti-hypertensive, opioid, mineral-binding, anti-bacterial and immunomodulatory activities have been reported for casein-derived bioactive peptides (Abd El-Salam, El-Shibinyand, & Buchheim, 1996; Baldi et al., 2005; Brody, 2000; Dziuba & Minkiewicz, 1996; Malkoski et al., 2001; Michaelidou, 2008; Silva & Malcata, 2005; Thomä-Worringer, Sørensen, & López-Fandiño, 2006) and whey-protein derived peptides (Chatterton, Smithers, Roupas, & Brodtkorb, 2006; Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005; Hernández-Ledesma, Recio, & Amigo, 2008; Mullally, Meisel, & Fitzgerald, 1996; Nagaoka, Kanamaru, & Kuzuya, 1991; Pellegrini, Dettling, Thomas, & Hunziker, 2001; Yamauchi, Wada, Yamada, Yoshikawa, & Wada, 2006).

The topic bioactive peptides is outside the scope of this article but general reviews include: Dziuba and Darewicz (2007), Haque, Chand, and Kapila (2009), Phelan et al. (2009), Shah (2000), and Shahidi and Zhong (2008). Research on the bioactive peptides from equine milk is very limited but it has been reported that peptides derived from equine β -casein may have a positive affect on human health (Doreau & Martin-Rosset, 2002).

6.2. Free amino acids in equine milk

The free amino acid content of equine, bovine and human milk are 1960, 578 and 3020 $\mu\text{M L}^{-1}$, respectively (Agostini, Carratù, Boniglia, Riva, & Sanzini, 2000; Rassin, Sturman, & Gaull, 1978) (Table 10). Glutamine, glutamate, glycine, alanine and serine are the most abundant free amino acids in equine, bovine and human milk while taurine is also exceptionally high in human milk (Carratù et al., 2003; Rassin et al., 1978; Sarwar, Botting, Davis, Darling, & Pencharz, 1998). Taurine is as an essential metabolite for the human infant and may be involved in the structure and function of retinal photoreceptors (Agostini et al., 2000). Compared with bovine milk, equine milk has an appreciable amount of taurine although it is 10 times less than that of human milk (Table 10). By contrast to total amino acid composition, which is essentially similar between equine, bovine and human milk, free amino acids show a pattern characteristic for each species (Table 10) which may be important for early post-natal development in different animals. Free amino acids are more easily absorbed than protein-derived amino acids and glutamic acid and glutamine, which comprise >50% of the total free amino acids of human milk, are a source of ketoglutaric acid for the citric acid cycle and also act as neurotransmitters in the brain (Agostini et al., 2000; Levy, 1998).

7. Digestibility of equine protein

In vivo digestion of milk proteins is initiated in the stomach by pepsin and, in young animals, by chymosin. Coagulation of milk in the stomach delays the degradation of proteins and allows for their better assimilation by the body. Degradation of casein is slow but extensive and while β -Lg is relatively resistant to gastric proteolysis, α -lactalbumin is readily hydrolysed when the gastric pH is ~ 3.5 (Savalle, Miranda, & Pélissier, 1988). The structure of the coagulum formed in the stomach depends on the casein content of ingested milk; high-casein milk yields firm, tough clots. Species that nurse their young at frequent intervals, e.g., horses and humans, produce dilute milk with a casein content <60% of the total protein and the coagulum formed in the stomach is soft and disintegrates quickly (Ofstedal, 1980).

The physico-chemical differences between human and bovine caseins result in the formation of different types of curd in the stomach (Hambræus, 1984) and because the protein profile of equine milk is quite similar to that of human milk, equine milk may be more appropriate in human nutrition than bovine milk. Turner (1945) compared the digestibility of equine, human and bovine milk based on the average percentage conversion of acid-insoluble protein to acid-soluble protein during digestion. Equine and human milk have a much lower buffering capacity than bovine milk and, while equine milk is very digestible, it was slightly less so than human milk but significantly better than bovine milk. Turner (1945) concluded that both equine and human milk form soft curds in the stomach which pass through the digestive tract more quickly than bovine milk curd. Kalliala et al. (1951) also reported that the overall digestibility of equine and human milk (by in-vitro experiments) appeared quite similar and both were easier to digest compared with bovine milk. Human milk forms fine, soft flocs in the stomach with an evacuation time

of 2–2.5 h, whereas bovine milk forms compact hard curds with a digestion time of 3–5 h.

8. Equine milk in human nutrition

The human infant has a much slower growth rate than other animals, except other primates (Emmett & Rogers, 1997), and the concentration of nutrients in human milk reflects this (Table 1). Equine milk, with a composition close to that of human milk, may be a good nutritional source for the neonate when breast milk is unavailable. Bovine milk or milk products are used traditionally as substitutes for human milk in infant nutrition. However, bovine milk is considerably different from human milk in terms of its macronutrients and micronutrients and the absorption rates of vitamins and minerals from the two milks are different, which can be problematic for infants. Bovine milk has lower concentrations of lactose, polyunsaturated fatty acids, free amino acids and vitamins A, D and K than human milk. Lf and Tf, while present in both types of milk, occur in different ratios, which may alter their effectiveness (Emmett & Rogers, 1997).

8.1. Cow milk protein allergy

Cows' milk allergy (CMA) is an IgE-mediated type I allergy, which may be life-threatening, and is defined as a set of immunologically-mediated adverse reactions that occur following the ingestion of milk. CMA is important because bovine milk is the first foreign antigen ingested in large quantities in early infancy (Hill & Hosking, 1996). CMA is the subject of many articles and reviews including: Apps & Beattie, 2009; Bindslev-Jensen, 1998; El-Agamy, 2007; Hill, 1994; Høst, 1988, 1991; Taylor, 1986; Wal, 2002, 2004.

Because β -Lg is absent from human milk, it has commonly been considered to be the most important cows' milk allergen (Ghosh, Malhotra, & Mathur, 1989; Goldman et al., 1963) although other whey proteins (Jarvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001) and caseins (Restani et al., 1995; Savilahti & Kuitunen, 1992) have also been implicated in allergic reactions. The resistance of β -Lg to proteolysis allows the protein to remain intact after digestion with the possibility of the being absorbed across the gut mucosa (Wal, 2002). Ingested β -Lg has been detected in human milk and could be responsible for colic in breast-fed infants and the sensitization of infants, predisposing them to allergies (Axelsson, Jakobsson, Lindberg, & Benediktsson, 1986; Jakobsson & Lindberg, 1978; Jakobsson, Lindberg, Benediktsson, & Hansson, 1985; Kilshaw & Cant, 1984; Stuart, Twiselton, Nicholas, & Hide, 1984).

The choice of substitute for cows' milk in cases of CMA depends on two major factors, i.e., nutritional adequacy and allergenicity; cost and taste must also be taken into account (Businco, 1993). Many soy or hydrolysate (casein-based and more recently whey protein-based) formulae are available for treatment of CMA but they can themselves induce allergic reactions (Eastman, 1989; Hill, Heine, Cameron, Francis, & Bines, 1999; Restani et al., 1999). Heat treatment of milk may destroy heat-labile proteins, especially BSA and Igs, and change the antigenic properties of other whey proteins, such as β -Lg and α -la although caseins need severe heat treatment ($121^\circ\text{C} \times 20\text{ min}$) to reduce sensitizing capacity (Hill, 1994). Enzymatic treatment of milk proteins has resulted in products with unacceptable taste due to bitterness arising from the production of peptides and amino acids and such peptides may, in fact, be allergenic (El-Agamy, 2007; Schmidt, Meijer, Slangen, & van Beresteijn, 1995; Sélo et al., 1999).

Many clinical studies have been carried out on the use of the milk of different species in infant nutrition, e.g., goat, sheep (Restani, Beretta, Fiocchi, Ballabio, & Gali, 2002), camel (El-Agamy, 2007), buffalo (El-Agamy, 2007), horse and donkey (Businco et al.,

Table 11Relationships (%) between proteins from milk of different species and their human homologues.^a

Casein	% Identity to closest		
	Primary accession number ^b	Bovine homologue	Human homologue
α_{S1} -Casein			
Cow	P02662	100	29
Goat	Q8M1H4	88	29
Sheep	P04653	88	28
Horse	Q8SPR1	39	44
Human	P47710	29	100
Camel	O97943	41	36
α_{S2} -Casein			
Cow	P02663	100	16
Goat	P33049	88	17
Sheep	P04654	89	17
Camel	O97944	56	11
β -Casein			
Cow	P02666	100	53
Goat	Q712N8	91	54
Sheep	P11839	91	54
Horse	Q9GKK3	56	58
Human	P05814	53	100
Camel	Q9TVD0	66	58

^a From Jenkins et al. (2007).^b Primary accession number for the protein in SWISS-PROT database.

2000; Carroccio, Cavataio, & Iacono, 1999; El-Agamy, Abou-Shloue, & Abdel-Kader, 1997; Iacono et al., 1992; Monti et al., 2007). Results on the benefits of such milk types are conflicting and infants with CMA may suffer allergic reactions to buffalo, goat, sheep, donkey and mare milk proteins due to the presence of positive immunological cross-reaction with their counterparts in cow's milk (Bellioni-Businco et al., 1999; Businco, 1993; El-Agamy, 2007). Lara-Villoslada, Olivares, and Xaus (2005) found that the balance between caseins and whey proteins may be an important factor in determining the allergenicity of bovine milk proteins in humans and that modification of this balance may reduce the allergenicity of bovine milk. Presumably, equine milk, with a ratio of whey proteins to casein close to that in human milk, would potentially be a good substitute for human milk.

The difference between CMA and lactose intolerance is of particular interest to many people and is an area which causes much confusion. CMA is a food allergy, an adverse immune reaction to a food protein that is normally harmless to the non-allergic individual. Lactose intolerance is a non-immunological adverse reaction, due to a lack of the enzyme, β -galactosidase, required to digest the predominant sugar, lactose, in milk. Lactose intolerance manifests as abdominal symptoms and chronic diarrhoea after ingestion of milk (Bindslev-Jensen, 1998). Lactose intolerance is not a disease; 70% of the world's population is lactose-intolerant (for reviews: Ingram & Swallow, 2003; Ingram, Mulcare, Ital, Thomas, & Swallow, 2009). Adverse effects of lactose intolerance occur at much higher levels of milk consumption than milk protein allergy. From a positive viewpoint, lactose is thought to have a major affect on bone mineralization during the first few months after birth as it stimulates the intestinal absorption of calcium (Wasserman & Langemann, 1960). With high lactose content similar to human milk, equine milk would seem to be suitable for infant nutrition, especially as lactose intolerance is uncommon in infants and children under two years of age.

8.2. Cross reactivity of proteins

Cross-reaction occurs when two food proteins have similar amino acid sequences or when the three-dimensional

conformation makes two molecules similar in their capacity to bind specific antibodies (Restani et al., 2002). Cross reactivity of proteins from different species generally reflects the phylogenetic relations between animal species, e.g., homologous proteins from vertebrates often cross-react.

A comprehensive study on the subject by Jenkins, Breiteneder, and Mills (2007) highlights some interesting points, especially concerning the potential allergenicity of caseins from different species. The authors set out to determine how closely a foreign protein had to resemble a human homologue before it actually loses its allergenic affect. A high degree of similarity to human homologues would, presumably, imply that a foreign animal food protein would be much less likely than a protein with little or no similarity to its human homologue to be immunogenic in human subjects. In addition, the study of potential animal allergens must take into account the capability of the human immune system to discriminate between its own proteins and those from another species (i.e., an autoimmune response) that have a high similarity, i.e., how closely does a foreign protein have to resemble a human homologue before it loses its ability to act as an allergen (Jenkins et al., 2007; Spitzauer, 1999)?

Table 11 gives the percentage identity of α_{S1} -, α_{S2} - and β -caseins from different species to bovine and human homologues. Known allergens are less than 53% identical to human sequences (Jenkins et al., 2007). Natale et al. (2004) found that 90% of a group of infants with CMA had serum IgE against bovine α_{S2} -casein, 55% against bovine α_{S1} -casein and only 15% against bovine β -casein, which is closest to human β -casein. Caprine and ovine milk proteins are more closely related to each other than either is to bovine milk proteins, thus explaining why an individual allergic to goat's cheese may exhibit high IgE cross-reactivity with sheep's milk proteins but could, in fact, tolerate cow's milk and its products (Jenkins et al., 2007).

Allergy to equine milk appears to be rare and, to date, only two documented cases have been reported. Fanta and Ebner (1998) reported on the case of an individual who experienced sensitization to horse dander allergen and subsequently produced IgE antibodies on ingestion of equine milk, which was prescribed to 'strengthen' her immune system. Horse serum albumin has been identified as an allergen in horse serum, while dander, hair and skin have been found to be allergenic (Spitzauer, 1999). IgE antibodies to animal-derived proteins are known to occur in about 2% of the population and in about 40% of atopic individuals making any animal an important source of inhalant allergens (Spitzauer, 1999). Gall, Kalveram, Sick, and Sterry (1996) demonstrated the existence of an IgE-mediated equine milk allergy in one patient, caused by low molecular mass heat-labile proteins, most likely α -La and β -Lg, without cross-reaction to the corresponding whey proteins from bovine milk. Presumably, the above cases are not isolated incidents and as the consumption of equine milk and its products increases, it is likely that further cases will be reported.

Bevilacqua et al. (2001) tested the capacity of goats' milk with low and high α_{S1} -casein content to induce milk protein sensitization in guinea pigs and found significantly less sensitization in milk with low α_{S1} -casein. This may represent an important attribute of the low α_{S1} -casein content of equine milk for use in human allergology.

9. Products from equine milk

9.1. Koumiss

Unlike other milk, cheese is not produced from equine milk as no curd is formed on addition of rennet. It forms a weak coagulum under acidic conditions and this is exploited in the production of yoghurt-type products, especially in the Netherlands, where it is generally flavoured with concentrated fruit extract. Koumiss

(Kumys), a fermented equine milk product, is widely consumed in Russia, Western Asia (e.g., Kazakhstan), primarily for its therapeutic value. In Mongolia, koumiss, called Airag, is the national drink and distilled koumiss, Arkhi, is also produced (Kanbe, 1992; Ørskov, 1995). Koumiss and kefir belong to the yeast-lactic fermentation group where alcoholic fermentation using yeasts is used in combination with lactic acid fermentation (Tamine & Marshall, 1984).

In traditional Koumiss manufacture, part of the previous days batch is used to inoculate fresh mares' milk and fermentation takes place over three to eight hours with the indigenous microbial population that includes *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus casei*, *Lactococcus lactis* subsp. *lactis*, *Kluyveromyces fragilis* and *Saccharomyces unisporus* (Litopoulou-Tzanetaki & Tzanetakis, 2000). Koumiss is still manufactured in remote areas of Mongolia by this traditional method but with increased demand elsewhere, it is now produced under carefully controlled and regulated conditions. A starter for the production of Koumiss was patented in 1990 in the former USSR; it consists of *Lc. lactis* subsp. *lactis*, *Lb. delbrueckii* subsp. *bulgaricus* and the yeast *Torula* spp (Surono & Hosono, 2003).

Koumiss contains about 900 g kg⁻¹ moisture, 21 g kg⁻¹ protein (12 g kg⁻¹ casein and 9 g kg⁻¹ whey proteins), 55 g kg⁻¹ lactose, 12 g kg⁻¹ fat and 3 g kg⁻¹ ash, as well as the end-products of microbial fermentation, i.e., lactic acid, 18 g kg⁻¹, ethanol, 6–25 g kg⁻¹ and CO₂, 5–9 g kg⁻¹ (Doreau & Boulot, 1989; Litopoulou-Tzanetaki & Tzanetakis, 2000; Lozovich, 1995; Pagliarini, Solaroli, & Peri, 1993). Good quality koumiss is produced when lactic and alcoholic fermentations proceed simultaneously so that the products of fermentation occur in definite proportions (Berlin, 1962). During fermentation, 0.7–1.8% lactic acid, 0.6–2.5% ethanol and 0.5–0.9% CO₂, volatile acids and other compounds are formed that are important for aroma and taste. Up to 10% of the equine milk proteins are hydrolysed after 96 h but the fat remains unchanged (Berlin, 1962; Tamine & Marshall, 1984).

Koumiss is a milky, grey, fizzy liquid with a sharp alcohol and acidic taste (Berlin, 1962; Tamine & Marshall, 1984). Products with varying amounts of lactic acid and ethanol are produced and generally 3 categories of koumiss, mild, medium and strong, are known. Koumiss is thought to be more effective than raw equine milk in the treatment of various illnesses due to the additional peptides and bactericidal substances from microbial metabolism (Doreau & Martin-Rosset, 2002). Nowadays, the main interest in fermented foods such as koumiss is their apparent ability to promote functions of the human digestive system in a positive way, i.e., to have a probiotic effect (Sahlin, 1999).

9.2. Processing of equine milk products

As sales of equine milk have increased considerably during recent years, research is now focused on the development of new products or new methods for extending the shelf-life, while maintaining some of the unique components of equine milk. The ability of milk to withstand relatively high processing temperatures is very important from a technological point of view. Whey proteins in equine milk are much more thermo-stable than those of bovine milk, making equine milk less sensitive to thermal processing. Heat treatment at 80 °C × 80 s caused only a 10–15% decrease in non-casein nitrogen, with a marked decrease evident only when the temperature was increased above 100 °C (Bonomi et al., 1994). Lactoferrin and equine BSA appeared to be the most sensitive but were not completely denatured until the temperature reached 130 °C. β-Lg and α-La are almost completely denatured at temperatures over 130 °C and lysozyme at temperatures greater than 110 °C. The latter is in agreement with work by Jauregui-Adell (1975) who found 68% residual lysozyme activity after heating at 82 °C for 15 min.

The presence of a high level of thermo-stable lysozyme in equine milk may interfere with the microbial activity of starter cultures in the production of fermented products (Jauregui-Adell, 1975) and thus cause technological problems in the processing of equine milk. Di Cagno et al. (2004) heated equine milk to 90 °C for 3 min to inactivate lysozyme and, using yoghurt cultures of *Lb. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, produced an acceptable fermented product. They found that fortification of equine milk with Na caseinate (1.5 g 100⁻¹ g), pectin (0.25 g 100⁻¹ g) and threonine (0.08 g 100⁻¹ g) enhanced the rheological and sensory properties of fermented products made from equine milk. The resultant products had good microbiological, rheological and sensory characteristics after 45 days at 4 °C. Fermented unmodified equine milk alone had an unacceptable viscosity and scored very low in comparison with fortified products for appearance, consistency and taste. Addition of sucrose and Na caseinate had a positive effect on the rheological properties of a fermented equine milk product due to strengthening of the protein network.

In health food shops and some pharmacies in Western Europe, equine milk is sold frozen or as capsules of lyophilised milk. Other products from equine milk include frozen or lyophilised colostrum which is used mostly in the high-value horse industry to feed orphaned foals. It is claimed that many of the products relieve metabolic and intestinal problems while having a gut-cleansing effect coupled with 'repair' of intestinal flora. Relief from stomach ulcers, high blood pressure, high cholesterol and liver problems are also reported and equine milk is recommended as an aid in the treatment of cancer patients. The recommended amount of equine milk is 250 mL per day. The use of equine milk in the production of cosmetics is relatively new and includes soaps, creams and moisturisers (Doreau & Martin-Rosset, 2002).

10. Conclusions

The characteristics of equine milk of interest in human nutrition include an exceptionally high concentration of polyunsaturated fatty acids, low cholesterol content, high lactose and low protein contents (Solaroli et al., 1993), as well as high levels of vitamins A, B and C. The renal load of equine milk, based on levels of protein and inorganic substances, is equal to human milk, providing further indication of its suitability as an infant food (Iacono et al., 1992).

The claimed invigorating effect of equine milk is thought to be, at least in part, due to its immuno-stimulating ability. Lys, Lf and ω-3 fatty acids have long been associated with the regulation of phagocytosis of human neutrophils in vitro (Ellinger, Linscheid, Jahnecke, Goerlich, & Endbergs, 2002). The concentration of these compounds is exceptionally high in equine milk and the consumption of frozen equine milk significantly inhibits chemotaxis and respiratory burst, two important phases of the phagocytic process (Ellinger et al., 2002). This result suggests a potential anti-inflammatory effect by equine milk.

To be successful as a substitute for human milk in infant nutrition, equine milk must be capable of performing many biological functions associated with human milk. The presence of high concentrations of lactoferrin, lysozyme, ω-3 and ω-6 fatty acids in equine milk are good indicators of its potential role. However, the lack of research must be addressed to develop the potential of equine milk in the health and nutritional markets. Scientific studies are required to bring the health claims for equine milk out of the realms of regional folklore. It seems reasonable that equine milk could be marketed as a dietary aid where the immune system is already depleted, i.e., as a type of 'immuno-boost'. Data suggest that more than 30% of customers who purchase equine milk in the Netherlands are patients undergoing chemotherapy, and find

equine milk helpful in counteracting the effects of cancer treatment. The composition of equine milk suggests a product with interesting nutritional characteristics with potential use in dietetics and therapeutics, especially in diets for the elderly, convalescent and newborns.

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