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## Comparison of the principal proteins in bovine, caprine, buffalo, equine and camel milk

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Proteomic analysis of bovine, caprine, buffalo, equine and camel milk highlighted significant interspecies differences. Camel milk was found to be devoid of  $\beta$ -lactoglobulin, whereas  $\beta$ -lactoglobulin was the major whey protein in bovine, buffalo, caprine, and equine milk. Five different isoforms of  $\kappa$ -casein were found in camel milk, analogous to the micro-heterogeneity observed for bovine  $\kappa$ -casein. Several spots observed in 2D-electrophoretograms of milk of all species could tentatively be identified as polypeptides arising from the enzymatic hydrolysis of caseins. The understanding gained from the proteomic comparison of these milks may be of relevance both in terms of identifying sources of hypoallergenic alternatives to bovine milk and detection of adulteration of milk samples and products.

**Keywords:** Milk protein, bovine milk protein allergy, 2-DE, MALDI-ToF.

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Bovine milk has long been the primary milk source for human consumption. However, hypersensitivity to bovine milk proteins is a major food allergy, which affects primarily infants, but may also persist throughout adulthood and can be very severe (Lara-Villoslada et al. 2005). Most children suffering from bovine milk protein allergy synthesise antibodies principally against the  $\alpha_s$ -caseins and  $\beta$ -lactoglobulin (Restani et al. 1999; Bevilacqua et al. 2001; Ametani et al. 2003). Human milk has long been recognised as the optimal form of nutrition for the neonate (Work Group on Breastfeeding, 1997) and it is well known that the allergenicity of its primary alternative, i.e., infant formula is primarily based on its bovine milk-derived protein fraction. At present, the most common hypoallergenic alternative to conventional bovine milk-based formula is formula containing protein hydrolysates, containing peptides with a molecular mass around 3 kDa, as these do not normally act as allergens (El-Agamy, 2007). A drawback of this approach is that formulae containing milk protein hydrolysates often suffer from bitterness and require the inclusion of additional emulsifiers.

In recent years, there has been an increasing focus on the use of proteins from non-bovine milks as an alternative protein source for hypo-allergenic bovine milk alternatives.

There are several reports that equine, camel and caprine milk might be preferable to bovine milk with respect to allergenicity, particularly for infants and elderly people. Sanz Caballos et al. (2009) showed that caprine milk protein is more digestible and more tolerable than bovine milk protein, while Egito et al. (2002) suggested that equine milk may be a curative agent for digestive and cardiovascular diseases.

The gross composition of the protein fraction of milks of common dairying species have been characterised, but nutritional suitability is not only related to gross composition, but is also strongly affected by the microheterogeneity of the protein fraction; hence, further stages in the evaluation of the potential suitability of non-bovine milks in human/infant nutrition include a detailed characterisation of their proteinaceous constituents. Proteomic techniques are ideally suited to obtaining such information and have been applied to the study of bovine milk and milk products (Holt & Zeece, 1987; Chin & Rosenberg, 1998; Holland et al. 2004; Lindmark-Månsson et al. 2004; Fong et al. 2008; Pappa et al. 2008). However, only a few studies have examined non-bovine milks using the proteomic approach. The aim of the present study was to apply proteomic tools, i.e., two-dimensional electrophoresis (2-DE) followed by MALDI-ToF mass spectrometry, to characterise the protein system in buffalo, caprine, equine, camel and bovine milk. The outcomes of these investigations can be applied as an

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initial step to evaluate the suitability of non-bovine milk proteins as suitable source for hypoallergenic nutritional products.

## Material and Methods

### *Milk supply*

Raw fresh bovine milk was obtained from a local dairy company. Caprine milk was obtained from a commercial source. Raw fresh buffalo milk was obtained from North Wales Buffaloes (Midlist Farm, Halkyn, Holywell, Flintshire, UK). Raw fresh camel milk was obtained from Kamelenmelkerij Smits (Cromvoirt, The Netherlands). Raw fresh equine milk was obtained from Orchid's Paardemelkerij, (Zeeland, NBr., The Netherlands).

### *One- and two-dimensional gel electrophoresis*

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Urea-PAGE, were performed as described by Hinz et al. (2007) and Larsen et al. (2010), respectively. 2-DE was performed as described by Larsen et al. (2010). Stained gels were then scanned using a calibrated GS-800 densitometer (Bio-Rad, Hercules, CA, USA)

### *In-gel digestion and matrix-assisted-laser-desorption/ionization time-of-flight (MALDI-ToF) analysis*

Relevant protein spots were excised from the 2D-gels and 50  $\mu$ l acetonitrile were added to each gel spot and left at room temperature for 15 min. Acetonitrile was then removed and 25  $\mu$ l of 25 mM ammonium bicarbonate was added for 10 min to rehydrate the spots. This procedure was repeated twice more, after which the spots were dehydrated with 50  $\mu$ l acetonitrile. The acetonitrile was subsequently removed and the spots were dried at 37 °C for 20 min in a heating block.

Sequencing-grade modified trypsin (Promega Corporation, Madison, WI, USA) was dissolved in 25 mM ammonium bicarbonate at a final concentration of 10 ng per  $\mu$ l. A 3  $\mu$ l aliquot of trypsin solution was added to each spot and this was allowed to absorb fully into the spot, before further 3  $\mu$ l aliquots of trypsin were added until the spot was fully rehydrated. Subsequently, 10  $\mu$ l of 0.5 mM ammonium bicarbonate was added to cover the spots. Each gel spot was kept on ice during rehydration with trypsin to prevent trypsin autodigestion peaks. Gel spots were digested in a Discover Microwave (CEM Corporation, Matthews, NC, USA) at 50 W at 55 °C for 15 min. Digests were allowed to cool and the supernatant was transferred to a fresh vial. To each gel spot, 10  $\mu$ l of 25 mM ammonium bicarbonate in 50% acetonitrile was added, after 15 min this was added to the original supernatant.

Mass spectrometry was performed on the gel spot extracts with an Axima ToF<sup>2</sup> MALDI-ToF mass spectrometer (Shimadzu Biotech, Manchester, UK). A 0.5  $\mu$ l aliquot of matrix solution (10 mg/ml,  $\alpha$ -cyano 4-hydroxy cinnamic acid, in 50% (v/v) acetonitrile-0.1% (v/v) trifluoroacetic acid) was deposited onto the target and left for 5 s before being removed. The residual solution was allowed to air-dry and 0.5  $\mu$ l of the sample solution was deposited onto the pre-coated sample spot. A 0.5  $\mu$ l aliquot of matrix solution was added to the deposited sample and allowed to air-dry. The sample was subsequently analysed in positive-ion reflectron mode.

Protein identification was carried out via peptide mass fingerprinting (PMF) using the Mascot search engine (<http://www.matrix-science.com>). The monoisotopic, positive ion data  $\pm 0.25$  Da, was searched using the following parameters: NCBIInr database or Swiss Prot, taxonomy mammalian, trypsin digest with one missed cleavage. Variable modifications including cysteine modified by carbamidomethylation and methionine modified by oxygen were also checked.

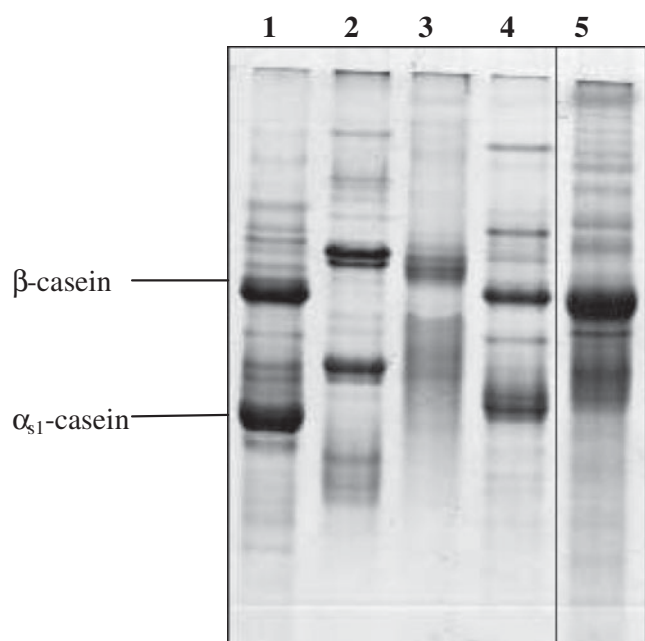
Tandem mass spectrometry (MS/MS) was carried out on peaks from spots that did not score well using PMF. The MS/MS positive ion averaged data,  $\pm 0.8$  Da, was again searched using the Mascot engine and the following parameters: NCBIInr database or Swiss Prot, taxonomy mammalian trypsin digest with one missed cleavage or semi trypsin with one missed cleavage; variable modifications including cysteine modified by carbamidomethylation, methionine modified by oxygen and acetyl N-terminal were also checked.

## Results and Discussion

### *SDS-PAGE and Urea-PAGE analysis of milk samples*

Urea-PAGE patterns of proteins from the five types of milk are shown in Fig. 1. Bovine  $\beta$ - and  $\alpha_{s1}$ -casein had about the same migration position as their counterparts in caprine or buffalo milk, whereas the migration of equine  $\beta$ - and  $\alpha_{s1}$ -casein was slightly slower. The slowest migration for  $\beta$ - and  $\alpha_{s1}$ -casein was found in camel milk. Since separation of proteins by Urea-PAGE is determined primarily by the net-negative charge of the proteins, the results presented in Fig. 1 suggest that equine and camel caseins may have a lower net-negative charge than their bovine, buffalo and caprine counterparts.

SDS-PAGE gels of bovine, buffalo, caprine, camel and equine milk casein (Fig. 2) highlighted a unique electrophoretic pattern for the milk of each species. The principal bands in all samples corresponded to  $\alpha_{s1}$ -casein,  $\beta$ -casein and, with the exception of equine milk,  $\kappa$ -casein (Merin et al. 2001; Park, 2001; Haenlein, 2004; El-Agamy et al. 2009). The migration positions of  $\alpha_{s1}$ -,  $\beta$ - and  $\kappa$ -casein in bovine, buffalo, caprine and equine milk were comparable, but a slower migration rate was observed for the camel milk



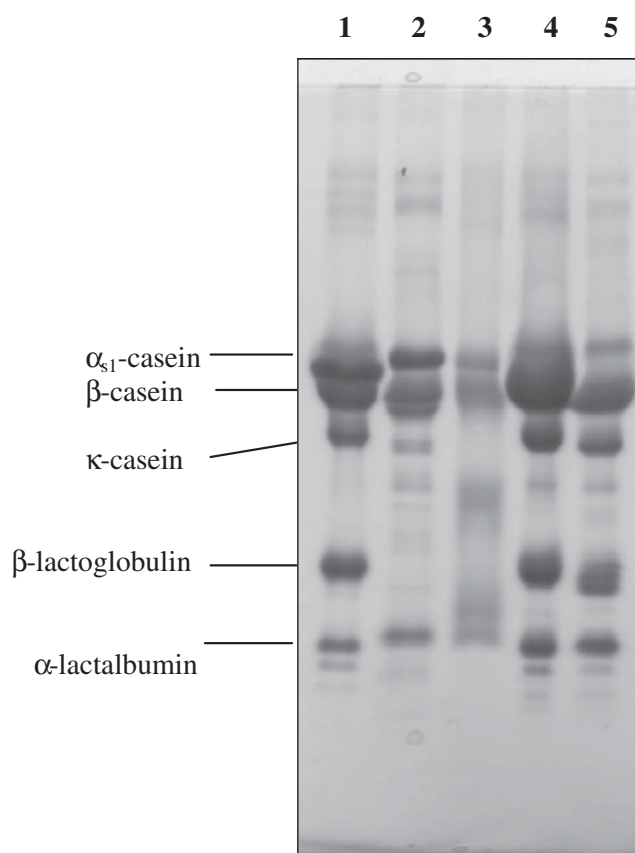
**Fig. 1.** Urea-PAGE electrophoretogram of (1) bovine milk; (2) camel milk; (3) equine milk; (4) buffalo milk or (5) caprine milk. Protein identification is based on the bovine sample lane.

caseins. Bovine, buffalo, caprine and equine milk showed a band for  $\beta$ -lg, while camel milk did not contain a band at the expected position of  $\beta$ -lg.  $\alpha$ -Lactalbumin was observed in all samples.

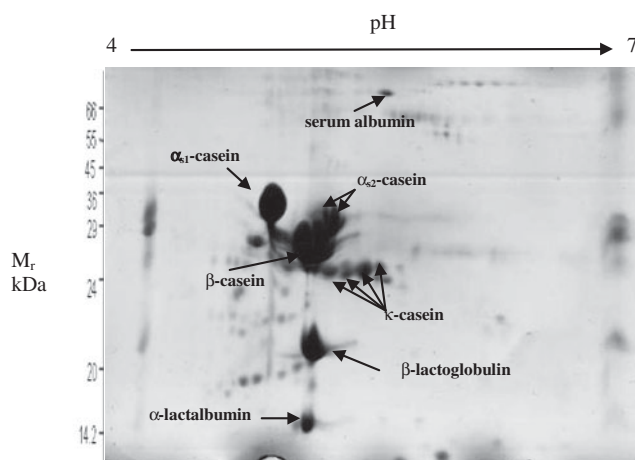
#### Comparison of the 2-DE patterns and identification of protein spots from different milks

The protein patterns of each milk sample were also analysed by 2-DE. A typical 2-DE electrophoretogram of bovine milk is shown in Fig. 3; as expected the most abundant proteins of bovine milk were the caseins  $\alpha_{s1}$ -casein,  $\alpha_{s2}$ -casein,  $\beta$ -casein and  $\kappa$ -casein and the whey proteins  $\beta$ -lg,  $\alpha$ -la and bovine serum albumin. There were clear differences between the 2D electrophoretograms of buffalo, caprine, camel and equine milk (Fig. 4), compared with that of bovine milk. Mass spectrometry was performed on peptic digests of spots excised from these gels to identify the principal proteins, as well as proteolysis products in these milks.

Mass spectrometry identifications of the relevant spots highlighted in Figs 4a–d are shown in Table 1. For camel milk, 13 spots were identified, arising from  $\beta$ -casein (spots 4A, 4A, 5A and 7A),  $\alpha_{s1}$ -casein (spots 2A, 8A and 11A)  $\kappa$ -casein (spots 9A, 10A, 11A, 12A and 13A),  $\alpha$ -la (spots 6A) and serum albumin (spot 1A). The camel  $\kappa$ -caseins were identified in the pI region 4.1–4.6, which has not been reported previously. Indeed, some studies have not detected bands for  $\kappa$ -casein at all in SDS-PAGE electrophoretograms (Farah & Farah-Riesen, 1985) but, in this study five isoforms of  $\kappa$ -casein were identified using 2-DE. Several isoforms of  $\kappa$ -casein are known to be present in 2-DE

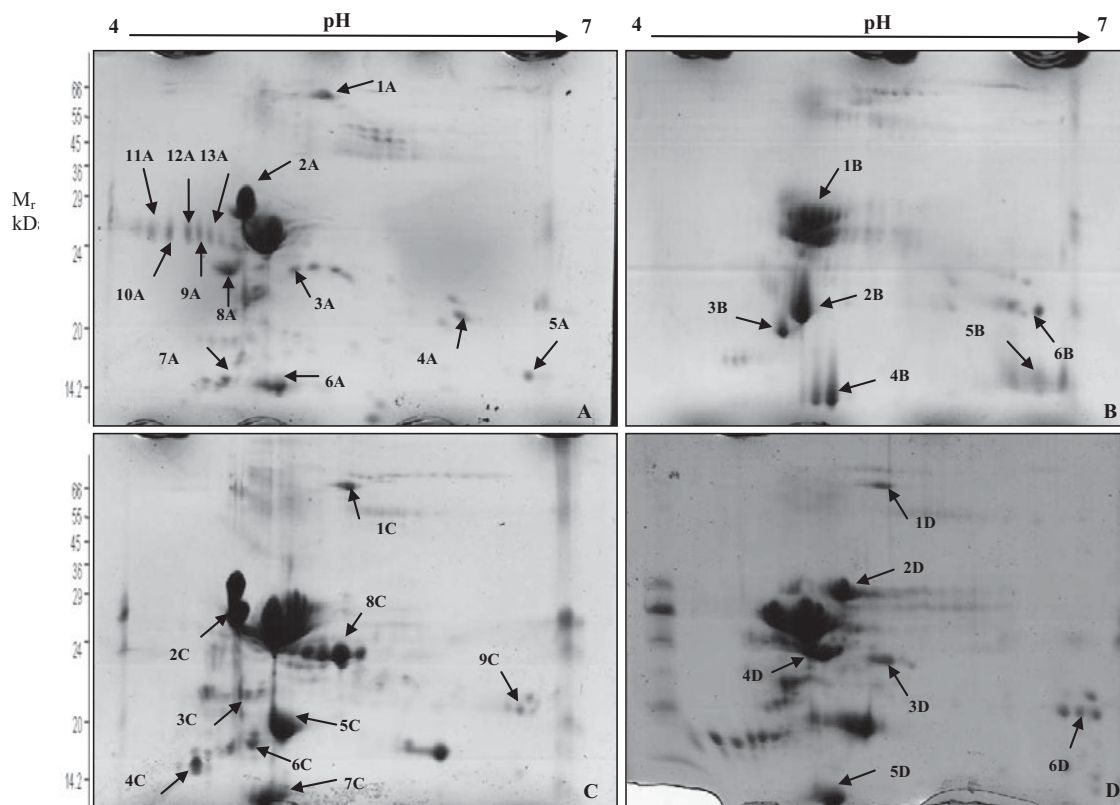


**Fig. 2.** SDS-PAGE electrophoretogram of (1) bovine milk; (2) camel milk; (3) equine milk; (4) buffalo milk or (5) caprine milk. Protein identification is based on the bovine sample lane.



**Fig. 3.** Two-dimensional gel electrophoretogram of bovine milk. The gels show molecular mass ( $M_r$ ) and IEF at pH 4 to 7.

electrophoretograms of bovine milk (Holland et al. 2004). The low pI region where the camel  $\kappa$ -caseins were observed is not in agreement with their theoretical pI of 8.03 (Table 1). However, it should be taken into account that the theoretical



**Fig. 4.** Two-dimensional gel electrophoretograms of (a) camel milk; (b) equine milk; (c) buffalo milk or (d) caprine milk. The gels show molecular mass ( $M_r$ ) and IEF at pH 4 to 7.

pI values in Table 1 are based on the primary structure of the protein only and do not take into account post-translational modification.  $\kappa$ -Casein, however, is a phosphoglycoprotein, and the carbohydrate groups are attached to  $\kappa$ -casein via *O*-glycosidic linkages to serine and threonine residues within the C-terminal portion of the molecule (West 1986; Rasmussen et al. 1999). Glycosylation occurs post-translationally, and a possible explanation for the lower-than-predicted pI of camel  $\kappa$ -casein could be a strong negative charge introduced through glycosylation and phosphorylation; this has not been studied to date, and requires experimental conformation. The observation that a decrease in pI for the 5  $\kappa$ -casein spots in Fig. 4a is accompanied by increases in molecular mass, and that the molecular mass is considerably higher than that predicted based on its amino acid sequence (Table 1) support the possibility of strong glycosylation of camel  $\kappa$ -casein. Studies have also shown that human  $\kappa$ -casein is more highly glycosylated than the bovine protein and that carbohydrate residues may account for up to 55% of the weight of the molecule (Ginger & Grigor, 1999).

$\beta$ -Lg was not found in camel milk (Fig. 4a), which is in agreement with the SDS-PAGE electrophoretogram (Fig. 2a) and previous reports (Kappeler et al. 2003, El-Agamy et al. 2009). It should be noted that, unlike the other common milk proteins, camel  $\beta$ -lg is not included in the Swiss Prot and

UniProt database, as it has not been possible to confirm the presence of this protein in milk and thus allow its isolation and primary sequence determination if present. However, unlike serum albumin (see below), for which the sequence of the camel variant has thus far not been established either, no spot showing a high degree of sequence homology to  $\beta$ -lg from other species was found in camel milk. As  $\beta$ -lg is a common allergen in bovine milk and is absent from human milk, camel milk has been suggested to be a good substitute for people who suffer from bovine milk allergy, as this is free of  $\beta$ -lg; in addition, the ratio of casein to whey protein is low, which results in soft curds and therefore easier digestibility (El-Agamy, 2006). The molecular weight of camel serum albumin estimated in this study was similar to data reported by Farah (1986) and Ereifej et al. (2011), who found that its molecular weight was 66 kDa. However, there seems to be a close taxonomic proximity between camel serum albumin and bovine serum albumin.

When comparing the masses identified by electrophoresis, to those of the parent proteins, it is apparent that for some spots, the observed mass was considerably lower than that of the full protein. This indicates that some of the spots, i.e., 3A, 4A, 5A, 7A and 8A in the case of camel milk (Fig. 4a), represent proteolysis products rather than intact proteins. Identification of the peptides covered in the MALDI-ToF and MS/MS spectra of the tryptic digests of

**Table 1.** Identification of spots from the 2D gels of milk from different animal species, by peptide mass fingerprinting (PMF) using MALDI-To/ToF

Spot no.	Protein name	Ref. Swiss Prot	Mol. mass (kDa) theor/obs	pI theor/obs	Score	% Cov.	Peptides	Species
1A	Serum albumin	P0279	66.4/66.0	5.6/5.4	99	19	12	<i>Bos taurus</i>
2A	$\alpha_{s1}$ -casein	O97943	25.3/27.3	4.8/4.5	130	58	13	<i>Camelus dromedarius</i>
3A	$\beta$ -casein $\ddagger$	Q9TVD0	24.6/23.5	5.2/5.1	61	26	5	<i>Camelus dromedarius</i>
4A	$\beta$ -casein	Q9TVD0	24.6/20.6	5.2/6.4	55	25	3	<i>Camelus dromedarius</i>
5A	$\beta$ -casein $\ddagger$	Q9TVD0	24.6/21.5	5.2/6.8	60	25	2	<i>Camelus dromedarius</i>
6A	$\alpha$ -lactalbumin	P00710	14.4/14.2	5.0/5.0	64	24	4	<i>Camelus dromedarius</i>
7A	$\beta$ -casein	Q9TVD0	24.6/15.5	5.2/4.6	62	25	3	<i>Camelus dromedarius</i>
8A	$\alpha_{s1}$ -casein	O97943	25.3/23.7	4.8/4.7	95	26	7	<i>Camelus dromedarius</i>
9A	$\kappa$ -casein	P79139	18.2/25.9	8.0/4.6	70	25	4	<i>Camelus dromedarius</i>
10A	$\kappa$ -casein	P79139	18.2/25.9	8.0/4.3	55	24	3	<i>Camelus dromedarius</i>
11A	$\kappa$ -casein	P79139	18.2/25.9	8.0/4.1	36	32	4	<i>Camelus dromedarius</i>
12A	$\kappa$ -casein	P79139	18.2/25.9	8.0/4.2	75	25	4	<i>Camelus dromedarius</i>
13A	$\kappa$ -casein	P79139	18.2/25.9	8.0/4.6	80	25	4	<i>Camelus dromedarius</i>
1B	$\beta$ -casein	Q9GKK3	25.5/27.2	5.7/5.3	76	37	7	<i>Equus caballus</i>
2B	$\beta$ -lactoglobulin	P0278	18.5/21.2	4.8/5.1	121	44	9	<i>Equus caballus</i>
3B	$\beta$ -lactoglobulin	P0278	18.5/19.9	4.8/5.1	41	49	7	<i>Equus caballus</i>
4B	$\alpha$ -lactalbumin	P08334	14.2/14.1	4.9/5.3	125	95	20	<i>Equus caballus</i>
5B	$\alpha_{s1}$ -casein	Q8SPR1	25.3/15.4	6.0/6.6	107	52	4	<i>Equus caballus</i>
6B	$\beta$ -casein	Q9GKK3	25.5/21.1	5.7/6.7	76	37	7	<i>Equus caballus</i>
1C	Serum albumin	P0279	66.4/69.3	5.6/5.5	108	27	16	<i>Bos taurus</i>
2C	$\alpha_{s1}$ -casein	Q62823	22.7/27.2	4.7/4.7	95	32	7	<i>Bubalus bubalis</i>
3C	$\alpha_{s1}$ -casein	Q62823	22.7/21.8	4.7/4.7	16	16	3	<i>Bubalus bubalis</i>
4C	$\beta$ -casein	Q9TSI0	23.5/15.5	5.1/4.4	45	25	2	<i>Bubalus bubalis</i>
5C	$\beta$ -lactoglobulin	P02755	18.2/19.9	4.8/5.1	74	39	9	<i>Bubalus bubalis</i>
6C	$\alpha$ -lactalbumin	Q9TSN6	14.2/18.4	4.7/4.8	54	7	1	<i>Bubalus bubalis</i>
7C	$\alpha$ -lactalbumin	Q9TSN6	14.2/14.1	4.7/5.1	75	26	5	<i>Bubalus bubalis</i>
8C	$\kappa$ -casein	P11840	19.1/23.8	6.3/5.4	77	23	4	<i>Bubalus bubalis</i>
9C	$\beta$ -casein	Q9TSI0	23.5/20.9	5.1/6.7	80	15	3	<i>Bubalus bubalis</i>
1D	Serum albumin	B3VHM9	63.3/69.2	9.1/5.4	81	55	5	<i>Capra hircus</i>
2D	$\alpha_{s2}$ -casein	P33049	24.7/29.0	7.8/5.3	75	42	8	<i>Capra hircus</i>
3D	$\beta$ -casein $\ddagger$	P33048	23.3/23.4	5.1/5.5	21	8	1	<i>Capra hircus</i>
4D	$\kappa$ -casein	P02670	19.1/23.6	5.3/5.2	80	26	5	<i>Capra hircus</i>
5D	$\beta$ -lactoglobulin	P02754	18.1/14.1	5.2/5.2	30	25	3	<i>Capra hircus</i>
6D	$\beta$ -casein $\ddagger$	P33048	23.3/21.0	5.1/6.8	16	8	1	<i>Capra hircus</i>

† Protein reference (Ref.) correspond to the Swiss-Prot/NCBI accession number; % cov. refers to sequence coverage. Theoretical molecular mass and isoelectric point (pI) of proteins are as according to the amino acid primary sequence and without consideration and/or degradation modifications. Observed molecular mass and isoelectric point (pI) are as observed with the position of the corresponding spots on the 2-DE gels

‡ Tandem mass spectrometry (MS/MS) was carried out on peaks from spots that did not score well using PMF

the spots is shown in Table 2. Using these peptides, the minimum part of the parent protein covered in the peptide found in each spot can be reconstructed. As outlined in Table 2, for the peptide spots found in the 2D-gel of camel milk, the 4 peptides derived from  $\beta$ -casein all included its C-terminus. Plasmin is known to be present in camel milk, and hydrolysis of camel casein by plasmin results in the formation of hydrolysis products which are probably homologous to the  $\gamma$ -caseins in bovine milk (Baer et al. 1994). Hence, it is possible that the  $\beta$ -casein peptides identified in the 2-DE electrophoretogram of camel milk (Fig. 4a, Tables 1 & 2) represent peptides analogous to the  $\gamma$ -caseins produced in bovine milk as a result of plasmin-induced hydrolysis of  $\beta$ -casein. The  $\alpha_{s1}$ -casein peptide identified in camel milk (spot 8A) covered part of the middle part in the amino acid sequence (Table 2).

In equine milk, 6 spots were identified, as  $\beta$ -casein (spots 1B and 6B),  $\alpha_{s1}$ -casein (spot 5B),  $\beta$ -lg (spots 2B and 3B) and  $\alpha$ -la (spot 4B). In contrast to the other milks investigated,  $\kappa$ -casein was not identified in equine milk. Spots 5B and 6B were identified as peptides originating from the C-terminus of equine  $\alpha_{s1}$ -casein and  $\beta$ -casein, respectively. The two polypeptide fragments derived from  $\alpha_{s1}$ - and  $\beta$ -casein may be proteolysis products arising from the action of plasmin, as reported by Egito et al. (2002). Matéos et al. (2009) reported that equine  $\beta$ -casein presents a complex 2-DE pattern, arising from variable degrees of phosphorylation, alternative splicing, and probable instability resulting from non-enzymatic deamidation, but this microheterogeneity was not observed in this study (Fig. 4b).

The 2-DE pattern of buffalo milk (Fig. 4c) is similar to that of bovine milk. The nine spots identified in buffalo milk

**Table 2.** Peptide identification for selected spots from 2-DE gels by peptide mass fingerprinting (PMF) by using MALDI-ToF

Spot no.	Parent protein	Species	Peptides found	Minimum protein coverage
3A	$\beta$ -casein	<i>Camelus dromedarius</i>	36–49; 171–184; 174–184; 185–203; 204–217	36–217
4A	$\beta$ -casein	<i>Camelus dromedarius</i>	171–184; 185–203; 204–215	171–217
5A	$\beta$ -casein	<i>Camelus dromedarius</i>	171–184; 204–217	171–217
7A	$\beta$ -casein	<i>Camelus dromedarius</i>	171–184; 185–203; 204–217	171–217
8A	$\alpha_{s1}$ -casein	<i>Camelus dromedarius</i>	85–95; 96–102; 96–105; 108–115; 108–118; 125–136; 137–150	85–150
5B	$\alpha_{s1}$ -casein	<i>Equus caballus</i>	104–116; 121–139; 140–148; 197–212	104–212
6B	$\beta$ -casein	<i>Equus caballus</i>	106–120; 112–120; 121–132; 168–181; 182–195; 196–218	106–218
3C	$\alpha_{s1}$ -casein	<i>Bubalus bubalis</i>	59–79; 104–124; 133–151	59–151
9C	$\beta$ -casein	<i>Bubalus bubalis</i>	49–68; 184–202; 184–209	49–209
6D	$\beta$ -casein	<i>Capra hircus</i>	114–132	114–132

consisted of  $\beta$ -casein (spots 4C and 9C),  $\alpha_{s1}$ -casein (spots 2C and 3C),  $\kappa$ -casein (spot 8C),  $\beta$ -lg (spot 5C),  $\alpha$ -la (spots 2C and 3C) and serum albumin (spot 1C). Peptide spot 9C in buffalo milk covered the C-terminus of  $\beta$ -casein; Madkor & Fox (1991) reported that the breakdown of buffalo casein by plasmin was generally similar to that in bovine milk, so it is possible that spot 9C represents a buffalo  $\gamma$ -casein. Spot 3C covered a large part of the middle of the amino acid sequence of buffalo  $\alpha_{s1}$ -casein. Serum albumin was identified by the database search as bovine serum albumin which suggests that buffalo serum albumin has a close taxonomic analogy to bovine serum albumin (Satija et al. 1979; Buffoni et al. 2011).

Six spots were identified in caprine milk, i.e.,  $\beta$ -casein (spots 3D and 6D),  $\alpha_{s2}$ -casein (spot 2D),  $\kappa$ -casein (spot 4D) and serum albumin (spot 1D).  $\beta$ -Casein is the major fraction in caprine casein, which is similar to human casein and different from bovine casein (El-Agamy, 2006). Caprine milk lacks  $\alpha_{s1}$ -casein and this may explain why caprine milk is less allergenic than bovine milk (Lara-Villoslada et al. 2005). The appearance of low molecular weight products in Fig. 4d are polypeptide fragments derived from  $\beta$ -casein (spots 6D). Caprine milk contains the entire plasmin system: plasmin, plasminogen, plasmin inhibitors, plasminogen activators, and inhibitors of plasminogen activators (Trujillo et al. 1997). Santillo et al. (2009) reported that plasmin mainly contributed to hydrolysis of  $\beta$ -casein in caprine milk, analogously for bovine milk.

Two-DE studies have also been undertaken on the protein system in human milk (Armaforte et al. 2010). While caseins are the principal class of protein in milk from most species, human milk has a higher proportion of whey proteins, and  $\beta$ -lg is absent. The comparison of 2-DE gels of human milk with those of bovine, buffalo, camel, goat and equine milk showed that the protein expression of human milk and bovine/buffalo differs remarkably, whereas human milk is more similar to that of goat, camel and equine milk (D'Auria et al., 2005; Armaforte et al. 2010). While the most abundant proteins in the bovine milk proteome are  $\alpha_{s1}$ -casein,  $\alpha_{s2}$ -casein,  $\beta$ -casein and the two genetic variants of  $\beta$ -lg, with  $\alpha$ -la and high molecular mass proteins present in low

concentrations, the most abundant protein in human milk was  $\alpha$ -la, followed by groups of isoforms of  $\beta$ -caseins and  $\alpha$ -caseins; moreover, high molecular mass proteins represented an important fraction of the total protein content. Several isoforms of  $\kappa$ -casein are known to be present in bovine milk, and several isoforms were found in camel milk, while only one spot was identified as  $\kappa$ -casein in human milk (Armaforte et al. 2010). Armaforte et al. (2010) also identified a number of breakdown products in pre-term human milk, which derived mainly from  $\alpha_{s1}$ - and  $\beta$ -casein. The study also reported a proteomic comparison between bovine milk and infant formulae and, as expected, the qualitative protein profile of powdered infant formula was very similar to that of bovine milk.

## Conclusion

A proteomic approach showed the differences in the protein composition of the milk of 5 different milk species. Several studies have evaluated the use of milk from different animals like camel, buffalo, goat and horse. The available data in the literature may suggest the use of animal milk as alternatives to human milk. However, proteomic tools highlighted significant differences in the protein profile between the animal species and can help further to find alternatives to human milk due to hypoallergenic properties of their proteins. The findings of the present study provide further evidence that the protein profile of bovine milk appears to be quite different, i.e., from camel, caprine, equine or buffalo milk. Camel milk might be a promising new protein source for children allergic to bovine milk protein, as  $\beta$ -lg the most dominant bovine milk allergen, could not be found in camel milk. These results could thus be useful in the further improvement of infant formulae. Another potential application of these results relates to adulteration of expensive milks (horse, camel, buffalo, goat) with cheaper milk types, which is not uncommon in various parts of the world and remains an issue of concern. In this respect, 2D electrophoresis in tandem with MS could prove a rapid way to identify the origin of individual proteins.

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## References

- Ametani A, Sakurai T, Katakura Y, Kuhara S, Hirakawa H, Hosoi T, Dosako S & Kaminogawa S 2003 Amino acid residue substitution at T-cell determinant-flanking sites in beta-lactoglobulin modulates antigen presentation to T-cells through subtle conformational change. *Bioscience, Biotechnology, Biochemistry* **67** 1507–1514
- Armaforte E, Curran E, Huppertz T, Ryan AC, Caboni MF, O'Connor PM, Ross RP, Hirtz C, Sommerer N, Chevalier F & Kelly AL 2010 Proteins and proteolysis in pre-term and term human milk and possible implications for infant formulae. *International Dairy Journal* **20** 715–723
- Baer A, Ryba I & Farah Z 1994 Plasmin activity in camel milk. *Lebensmittel Wissenschaft und Technologie* **27** 595–598
- Bevilacqua C, Martin P, Candalh C, Fauquant J, Piot M, Roucayrol AM, Pilla F & Heyman M 2001 Goat's milk of defective  $\alpha_{s1}$ -casein genotype decreases intestinal and systemic sensitization to  $\beta$ -lactoglobulin in guinea pigs. *Journal of Dairy Research* **68** 217–227
- Buffoni JN, Bonizzi I, Paciullo A, Ramunno L & Feligini M 2011 Characterization of the major whey proteins from milk of Mediterranean water buffalo (*Bubalus bubalis*). *Food Chemistry* **127** 1515–1520
- Chin HW & Rosenberg M 1998 Monitoring proteolysis during cheddar cheese ripening using two-dimensional gel electrophoresis. *Journal of Food Science* **63** 423–428.
- D'Auria E, Agostoni C, Giovannini M, Riva E, Zetterstroem R, Fortin R, Greppi F, Bonizzi L, Roncada P 2005 Proteomic evaluation of milk from different mammalian species as a substitute for breast milk. *Acta Paediatrica* **94** 1708–1713
- Egito AS, Miclo L, López C, Adam A, Girardet JM & Gaillard JL 2002 Separation and characterization of mares' milk  $\alpha_{s1-7}$ ,  $\beta$ -,  $\kappa$ -caseins,  $\gamma$ -casein-like, and proteose peptone component 5-like peptides. *Journal of Dairy Science* **85** 697–706
- El-Agamy EI 2006 Camel milk. In *Hand Book of Milk of Non-Bovine Mammals* (Ed. Haenlan Park). USA: Blackwell
- El-Agamy EI 2007 The challenge of cow milk protein allergy. *Small Ruminant Research* **68** 64–72
- El-Agamy EI, Nawar M, Shamsia SM, Awad S & Haenlein GFW 2009 Are camel milk proteins convenient to the nutrition of cow milk allergic children. *Small Ruminant Research* **82** 1–6
- Ereifej KI, Alu'datt H, AlKhalidy HA, Alli I & Rababah T 2011 Comparison and characterisation of fat and protein composition for camel milk from eight Jordanian locations. *Food Chemistry* **127** 282–289
- Farah Z 1986 Effect of heat treatment on whey proteins of camel milk. *Milchwissenschaft* **41** 763–765
- Farah Z & Farah-Riesen M 1985 Separation and characterization of major components of camel milk casein. *Milchwissenschaft* **40** 669–671
- Fong BY, Norris CS & Palmano KP 2008 Fractionation of bovine whey proteins and characterization by proteomic techniques. *International Dairy Journal* **18** 23–46
- Ginger MR & Grigor MR 1999 Review Comparative aspects of milk caseins. *Comparative Biochemistry and Physiology Part B* **124** 133–145
- Haenlein GFW 2004 Goat milk in human nutrition. *Small Ruminant Research* **51** 155–163
- Hinz K, Huppertz T, Kulozik U & Kelly AL 2007 Influence of enzymatic cross-linking on milk fat globules and emulsifying properties of milk proteins. *International Dairy Journal* **17** 289–293
- Holland JW, Deeth HC & Alewood PF 2004 Proteomic analysis of  $\kappa$ -casein micro-heterogeneity. *Proteomics* **4** 743–752
- Holt DL & Zeece MG 1987 Two dimensional electrophoresis of bovine milk proteins. *Journal of Dairy Science* **71** 2044–2050
- Kappeler SR, Farah Z & Puhani Z 2003 5'-flanking regions of camel milk genes are highly similar to homologue regions of other species and can be divided into two distinct groups. *Journal of Dairy Science* **86** 498–508
- Larsen LB, Hinz K, Jørgensen ALW, Møller HS, Wellnitz O, Bruckmaier RM, & Kelly AL 2010 Proteomic and peptidomic study of proteolysis in quarter milk after infusion with lipoteichoic acid from *Staphylococcus aureus*. *Journal of Dairy Science* **93** 5613–5626
- Lara-Villoslada F, Olivares M & Xaus J 2005 The balance between caseins and whey proteins in cow's milk determines its allergenicity. *Journal of Dairy Science* **88** 1654–1660
- Lindmark-Månsson H, Timgren A, Aldén G & Paulsson M 2004 Two-dimensional gel electrophoresis of proteins and peptides in bovine milk. *International Dairy Journal* **15** 111–121
- Madkor SA & Fox PF 1991 Plasmin activity in buffalo milk. *Food Chemistry* **39** 139–156
- Matéos A, Girardet JM, Molle D, Dary A, Miclo L & Gaillard JL 2009 Two-dimensional cartography of equine  $\beta$ -casein variants achieved by isolation of phosphorylation isoforms and control of the deamidation phenomenon. *Journal of Dairy Science* **92** 2389–2399
- Merin U, Bernstein S, Bloch-Damti A, Yagil R, van Creveld C, Lindner P, Gollop N 2001 A comparative study of milk serum proteins in camel and bovine colostrums. *Livestock Production Science* **67** 297–301
- Pappa EC, Robertson JA, Rigby NM, Mellona F, Kandarakis I & Mills ENC 2008 Application of proteomic techniques to protein and peptide profiling of Telème cheese made from different types of milk. *International Dairy Journal* **18** 605–614
- Park YW 2001 Proteolysis and lipolysis of goats milk cheese. *Journal of Dairy Science* **84** 84–92
- Rasmussen LK, Johnsen LB, Tsiora A, Sørensen ES, Thomsen JK & Nielsen NC 1999 Disulphide-linked caseins and casein micelles. *International Dairy Journal* **9** 215–218
- Restani P, Gaiaschi A, Plebani A, Beretta B, Cavagni G, Fiocchi A, Poisei C, Velona T, Ugazio AG & Galii CL 1999 Crossreactivity between milk proteins from different animal species. *Clinical and Experimental Allergy* **29** 997–1004
- Sanz Caballos L, Morales ER, de la Torre Adarve G, Diaz Castro J, Perez Martinez L & Sanz Sampelayo MR 2009 Composition of goat and cow milk produced under similar conditions and analyzed by identical methodology. *Journal of Food Composition and Analysis* **22** 322–329
- Santillo A, Kelly AL, Palermo C, Sevi A & Albenzio M 2009 Role of indigenous enzymes in proteolysis of casein in caprine milk. *International Dairy Journal* **19** 655–660
- Satija KC, Rajpal S, Pandey R & Sharma VK 1979 Electrophoresis of buffalo (*Bos bubalis*) serum proteins including immunoglobulins. *Infection and Immunity* **24** 567–570
- Trujillo AJ, Guamis B & Carretero C 1997 Hydrolysis of caprine  $\beta$ -casein by plasmin. *Journal of Dairy Science* **80** 2258–2263
- West DW 1986 Structure and function of the phosphorylated residues of casein. *Journal of Dairy Research* **53** 333–352
- Work Group on Breastfeeding 1997 American Academy of Pediatrics: breastfeeding and the use of human milk. *Pediatrics* **100** 1035–1039