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A study of polymorphism in milk proteins from local and imported dairy sheep in Australia by capillary electrophoresis

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Abstract

Casein and whey protein fractions of milk obtained from 47 ewes of five breeds or crossbreeds (Awassi, Merino, East Friesian × Merino, Awassi × Merino and Awassi × East Friesian) were analysed by capillary electrophoresis (CE). The experiments were performed on a Beckman P/ACE^{TM} system 5510 with an uncoated fused-silica capillary and a low pH buffer containing urea and a polymeric additive. The four major caseins (α_{s1}-, α_{s2}-, β- and κ-casein) in an acid precipitate were well separated, as were the two whey proteins, α-lactalbumin (α-La) and β-lactoglobulin (β-Lg). The electromigration of the proteins was in the order of α-La, β-Lg, α_{s2}-CN, α_{s1}-CN, κ-CN and β-CN. The milk samples were composed of the same variant of α-La and two different genotypes (A and B) of β-Lg while the β-Lg AB genotype was evident in the milk of some animals. The α_{s1}-CN fractions displayed considerable heterogeneity with at least 4 different peaks, representing 4 different variants. A fifth peak, corresponding to the Welsh variant (or α_{s1}-CN D), was present in 90% of the ewes' milk samples. The κ-CN fraction was resolved as a single peak, while the β-CN revealed significant heterogeneity with 3 variants. It appears that the presence of the α_{s1}-CN Welsh variant in Merino ewe and its crosses with Awassi and East Friesian ewes adversely affected milk composition and yield.

Keywords: Milk; Proteins; Caseins; Sheep; Capillary electrophoresis

1. Introduction

Milk protein is divided into two major groups, the caseins and the whey (serum) proteins. Of these two, the caseins are the most important, constituting about 76–86% of the total protein (Swaisgood, 1992). The caseins comprise four main fractions: alpha s-$_1$ (α_{s1}-CN), alpha s-$_2$ (α_{s2}-CN), beta (β-CN) and kappa (κ-CN). Each of them has a number of genetic polymorphs (Table 1) (Amigo, Recio, & Ramos, 2000), which are due to amino acid substitution or deletion, different phosphorylation levels and glycosylation differences. The whey proteins are composed of two main fractions, called α-lactalbumin (α-La) and β-lactoglobulin (β-Lg).

The Australian sheep milking industry is still relatively small, but it is growing rapidly. In the last decade years, Australia has imported Awassi and East Friesian sheep that are reported to be the highest milk producers in the world (Epstein, 1985; Anifantakis, 1986). Milk protein polymorphism was reported in Australian sheep by Thomas et al (Thomas, Dawe, & Walker, 1989), but it is not known what variants were present in the Awassi and East Friesian sheep that were imported. The fact that different genetic polymorphs of the casein are likely to be present in local Australian dairy sheep and their crosses with imported breeds requires further investigation.

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Table 1
Positions and amino acid differences in polymorphs of ovine milk proteins (Amigo et al., 2000)

Protein	Variant	Position and amino acid in the protein						
α_{s1}-CN (199)		12	13	64	66	68		
	A	SerP	Ser	SerP	SerP	SerP		
	B[a]							
	C	Ser	Pro					
	D[b]			Ser	Ser	Asn		
	E[a]							
α_{s2}-CN (207)		49						200
	A	Asn						Lys
	B	Asp						Asn
	Fast[a]							
β-CN (209)		2						
	A	Glu						
	B[a]							
	C	Gln						
β-Lg (162)		20					148	
	A	His					Gln	
	B	Tyr						
	C						Arg	

[a]No sequence data are available yet.
[b]Also known as the Welsh variant.

There is currently no information on the type of polymorphs in these animals, either pure or crossbreed.

Genetic polymorphism observed in milk proteins is a consequence of mutations, which change the nucleotide sequence of the particular gene involved and, therefore, different amino acid sequences result. Genetic differences in milk protein polymorphisms are of great interest to the cheese maker because of correlations to curd firmness, coagulation time, casein content and cheese yield. Many studies on cow and goat milk proteins (Marziali & Ng-Kwai-Hang, 1986a, b; Aleandri, Schneider, & Buttazzoni, 1989; Paterson, Otter, & Hill, 1995) have shown that genetic selection for polymorphisms can have advantages for cheese processing, but much less is known for sheep milk (Haenlein, 2001). Since ewe's milk is mainly used for cheese making, a better characterization of ovine milk is important in order to allow cheese producers to optimize the process according to the technological properties of the milk.

Capillary electrophoresis (CE) is an alternative separation technique that has great potential for the analysis of milk proteins. It has been used to identify the different protein fractions and genetics variants of milk proteins from goats' (Recio, Ramos, & Amigo, 1997b), cows' (Chen & Zang, 1992; Paterson et al., 1995) and sheep milk (Recio et al., 1997b). De Jong, Visser, and Olieman (1993) established a CE method using a hydrophilically coated capillary, which allowed the simultaneous separation of caseins and whey proteins, including some polymorphs. This method was later optimized by Recio and Olieman (1996) in order to provide a quantitative determination of the proteins separated in the electropherograms.

The objective of the present study was to analyse, by CE, the different polymorphs of proteins present in milk of different breeds of sheep (Awassi, Merino, East Friesian and their crosses).

2. Materials and methods

2.1. Population of animals investigated

Imported high milk yielding sheep breeds (Awassi and East Friesian) were crossed with some local breeds at the Shenton Park Research Station of The University of Western Australia, WA Australia. The ewes lambed over a period of 8 weeks between June and August. Animals of comparable age, stage of lactation and which were managed under similar conditions were used. The ewes were removed from the dairy and their lactation was considered complete when the milk yield fell below 200 g/day. A flock of 47 animals was available for this study with the following breed allocation: 15 Awassi (A), 7 Merino (M), 5 Awassi × Merino (AM), 11 East Friesian × Merino (EFM) and 9 East Friesian × Awassi (EFA).

2.2. Management of the animals

2.2.1. Housing and nutrition

During the experimental periods, animals were kept in communal paddocks where they grazed irrigated pasture composed predominantly of Kikuyu and subterranean clover and had meadow hay available ad libitum. They received up to 1 kg of lupins/head (392 g/kg DM protein, 21 MJ/kg DM energy) daily and at each milking, they were given approximately 300 g of a mixture of 700 g/kg oaten chaff, 300 g/kg lupins and 5 g/kg hi-cal/salt preparation.

2.2.2. Milking

The ewes were milked twice a day on a 12 bay rapid exit milking parlour (Prattley, Temuka, NZ) with an Alfa Laval milking machine that had a pulsation rate of 120/min and vacuum pressure of 40 kPa. At the end of each milking the teats were disinfected with an iodine based commercial preparation (Alfadyne Teat Sanitiser, Australia).

2.3. Measurements of production and composition of milk

Milk production was measured with Tru Test milk meters (Tru Test Distributors, Auckland, New Zealand). Samples of milk were collected from the Tru Test

jars and stored at 1–4 °C. Milk production and composition were recorded over 21 weeks (14 samplings) between August and December 2001.

To collect samples for the CE the sheep were milked twice a day by hand to avoid any contamination of the samples. Samples of milk were stored at 1–4 °C and analysed for composition within 24 h. Each milk sample was first filtered through glass wool (Asia Pacific Speciality Chemicals 1755-500G) to eliminate the crude impurities, then defatted by centrifugation at 5000*g* for 15 min at 4 °C (Beckmann Avanti^{TM} J-25, USA). The samples were analysed at 40 °C for protein, fat, lactose, total solids (TS) and solids nonfat (SNF) with a Milko Scan 133 (Foss Electric, Denmark) calibrated for sheep milk with standard methods for each component. The pH of the skim milk was measured with a portable pH meter (PHM 201, Radiometer, Copenhagen).

2.4. Capillary electrophoresis

The method used for this study was based on the procedure of De Jong et al. (1993). All solutions were based on highly purified water (Ropure ST, Barnstead|Thermolyne, USA).

2.4.1. Separation of milk components for CE

The skim milk, heated to 35 °C, was separated into whey proteins (α-La and *β*-Lg) and whole casein (αs_1-, αs_2-, *β* and *κ*-caseins) by isoelectric precipitation of the caseins at pH 4.6 with 1 mol/l HCl. After centrifugation (5000*g*, 15 min, 30 °C), the supernatant (whey proteins), was collected while the precipitated caseins were washed twice with acidified distilled water (pH 4.6). Both fractions were then freeze-dried (Heto FD4, Denmark).

2.4.2. Preparation of electrophoresis (run) buffer

A 10 mmol/l phosphate solution (pH 2.50 ± 0.02) was prepared daily by dissolving in a 25 ml volumetric flask, 307.1 mg of 1 mol/l sodium dihydrogen orthophosphate solution (Univar 471-500G), 12.5 mg hydroxy-propyl-methyl-cellulose (HMPC) (Sigma H-9262) and 18.8 ml of 8 mol/l urea (Univar 817-500G). The pH was adjusted to 2.50 with 4 mol/l phosphoric acid (BDH AnalaR 101736U) and the volume was made up with water. Before use, the buffer was filtered through a 0.45 μm filter (Advantec MFS 25CS045AN) and degassed by sonication for 20 s (Kristiansen, Otte, Zakora, & Qvist, 1994).

2.4.3. Preparation of (sample) reduction buffer

The sample reduction buffer (pH 8.00 ± 0.02) was prepared by dissolving in a 250 ml volumetric flask 365 mg of tri-sodium citrate dihydrate (Univar 467), 190 mg of DL-dithiothreitol (DTT) (Sigma D-0632) and 187.5 ml of 8 mol/l urea (Univar 817-500G). The pH was adjusted to 8.00 with 1 mol/l NaOH and the volume was made up with water.

2.4.4. Sample preparation for CE

Freeze-dried casein or whey protein samples (Section 2.6.1) were dissolved in sample reduction buffer (1:100, w/w) in a 1.5 ml Micro tube (Sarstedt 72.690). The suspension was mechanically shaken and sonicated for 3 min and incubated for at least 1 h at room temperature. During that time, the vials were occasionally shaken to enhance dissolution.

2.4.5. Capillary electrophoresis separation

CE was performed on a Beckman P/ACE^{TM} system 5510 (Beckman Instruments, Gladesville, NSW, Australia) using an uncoated fused-silica capillary column (eCAP^{TM} Capillary Tubing 338472, Beckman Instruments, Gladesville, NSW, Australia), 570 mm total length × 50 μm ID × 375 μm OD. The distance between the detection window and the outlet was 80 mm, resulting in an effective capillary length of 490 mm.

The separations were performed at 20 kV with a final current of about 70 μA. Sample solutions were injected for 10 s at 20 psi (1 psi = 6894.76 Pa). Detection was performed on the column at 214 nm. Before each sample injection, the capillary was flushed with purified water for 2 min and then with the run buffer for 3 min. After 4 injections a complete wash of the capillary was conducted with 1 mol/l NaOH (Univar 2534) for 2 min, purified water for 3 min, 1 mol/l HCl (Selby-Biolab, 1789) for 2 min, purified water for 5 min and with the run buffer for another 3 min. Each sample was analysed in triplicate and typical electropherograms were reported.

2.4.6. Statistical analyses

The individual electropherograms were compared by using the Supercompare program (P/ACE Station Supercompare, Beckman Instruments, USA). The protein variants were identified by reference to literature (Cattaneo, Nigro, & Greppi, 1996; De Jong et al., 1993; Molina, Defrutos, & Ramos, 2000; Recio, Perez-Rodriguez, Amigo, & Ramos, 1997a; Recio et al., 1997b; Recio, Péres-Rodriguez, Ramos, & Amigo, 1997c).

The statistical analysis of the milk production and composition was done by least-squares analysis of variance and effects were assumed to be significant when the level of probability was 5% or less ($P < 0.05$).

All results presented are means plus or minus their standard error.

3. Results and discussion

The individual caseins and whey proteins were well separated by the CE method, indicating that this method is suitable for the determination of protein fractions in ovine milk. The electromigration of sheep milk proteins were in the order of α-La, β-Lg, α_{s2}-CN, α_{s1}-CN, κ-CN and β-CN (Figs. 1 and 2). The whey proteins were the first peaks to migrate. α-La had the fastest migration time (17.5 min) followed by two peaks of β-Lg (18–19 min). The α_{s2}-CN was separated into three peaks migrating between 19 and 20.5 min, while α_{s1}-CN was composed of at least four peaks migrating between 22.5 and 26.5 min. The κ-CN peak migrated before three peaks of β-CN, which appeared between 27 and 30 min. The ovine casein sample in Fig. 2 contained the α_{s1}-CN Welsh variant while Fig. 3 shows an electrophoretic comparison of the protein profile of one animal from each breed or crossbreed. Fig. 4 represents the results of experiments carried out to evaluate the occurrence of different polymorphs between the animals of the same breed.

The five breeds or crossbreeds all had the same variant of α-La, as a minor peak with the shortest migration time. The β-lactoglobulin was present in two genotypes, A and B. The EFA and the Merino mainly had the β-Lg A genotype with an average of 78% and 71% of the animals respectively. Each breed analysed contained some animals with the β-Lg AB genotype and the average for the whole flock was 40%. The EFM crossbreeds, however, had the highest percentage of β-Lg AB at 73%. The α_{s1}-CN fractions displayed considerable heterogeneity with at least 4 different peaks. A fifth (Welsh) variant, was present in 90% of the ewes, which was a high percentage compared with literature values (Amigo et al., 2000). Furthermore, this variant was most prominent in the milk obtained from the Merino ewes. Recently, Chianese et al. (1996) have called this variant α_{s1}-CN D. This variant has a frequency that varies from 2.2% (Chiofalo & Micari, 1987) to 22% (Caroli, Bolla, Pagnacco, & Fraghi, 1989) and it provokes a reduction in casein content, a worsening of milk clotting properties in homozygous animals and to a lesser extent, in heterozygous animals (Piredda, Papoff, Sannas, & Campus, 1993). The fact that this variant was present in most of the animals

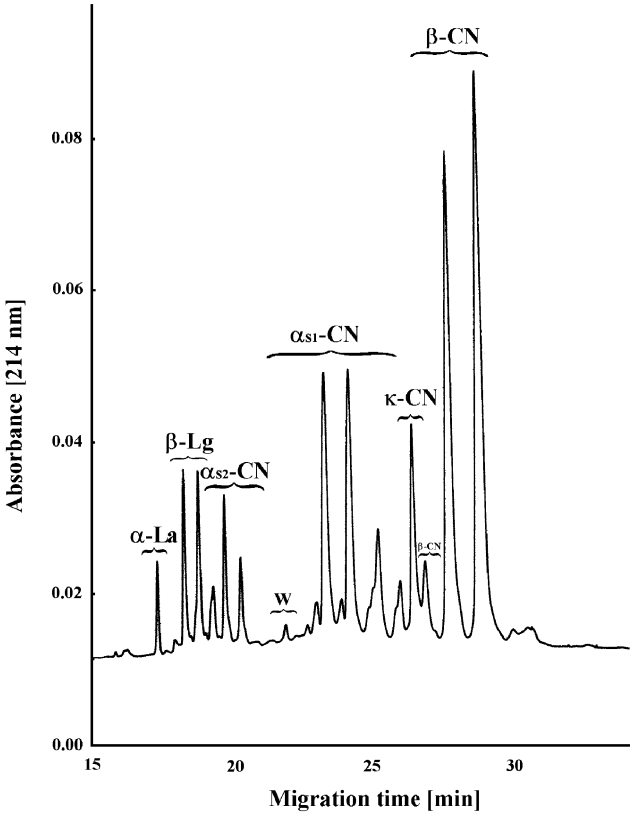

Fig. 1. Typical capillary electropherogram of individual ovine casein and whey proteins sample. See text for details of abbreviations and CE method.

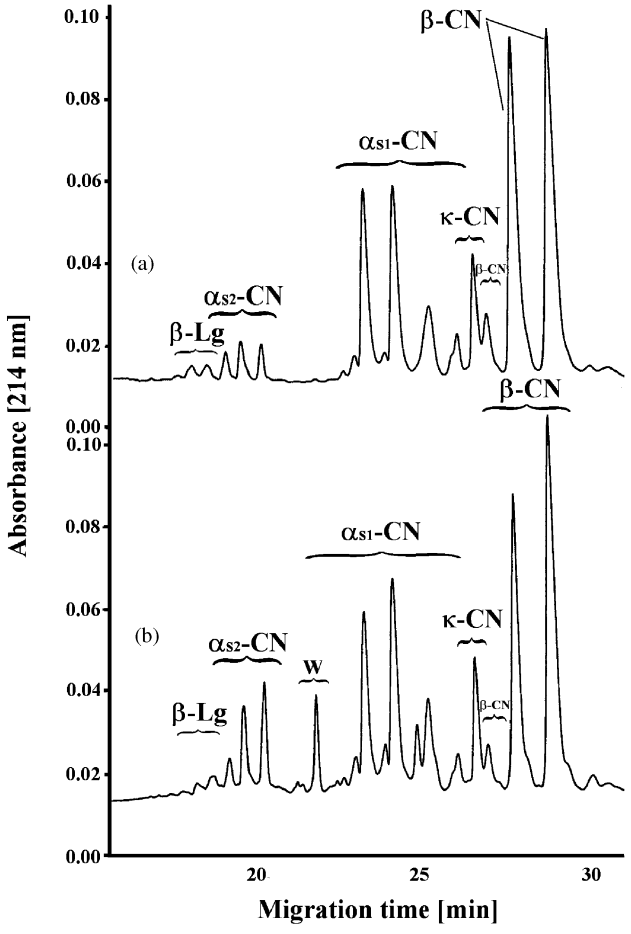

Fig. 2. Capillary electropherograms of two individual ovine casein samples (a) without the Welsh α_{s1}-CN variant; (b) with the Welsh α_{s1}-CN variant (W). Protein abbreviations as in Fig. 1.

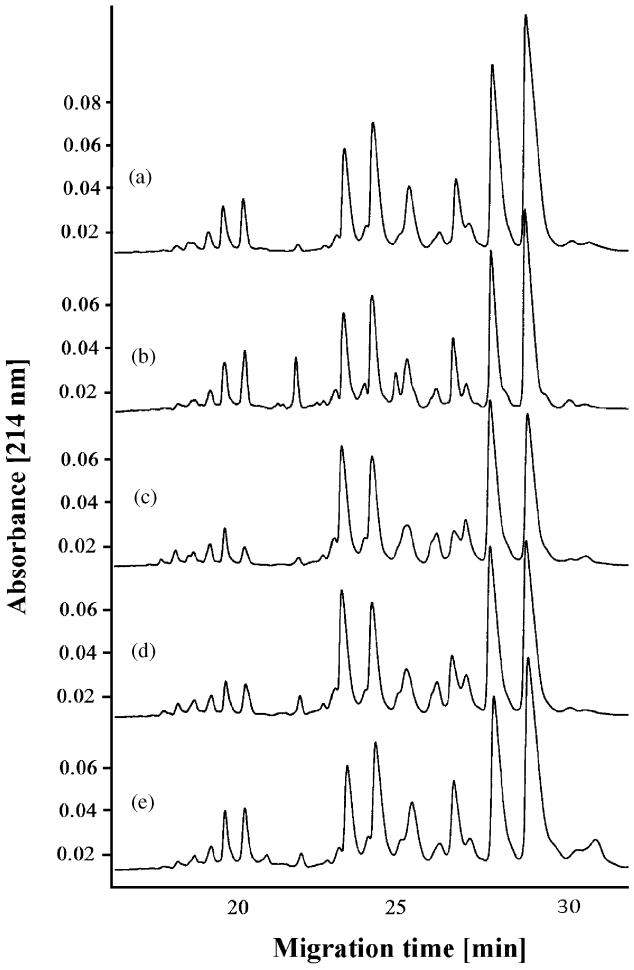

Fig. 3. Typical capillary electropherograms of individual ovine casein fractions of (a) Awassi breed; (b) Merino breed; (c) Awassi–Merino crossbreed; (d) East Friesian–Merino crossbreed; (e) East Friesian–Awassi crossbreed.

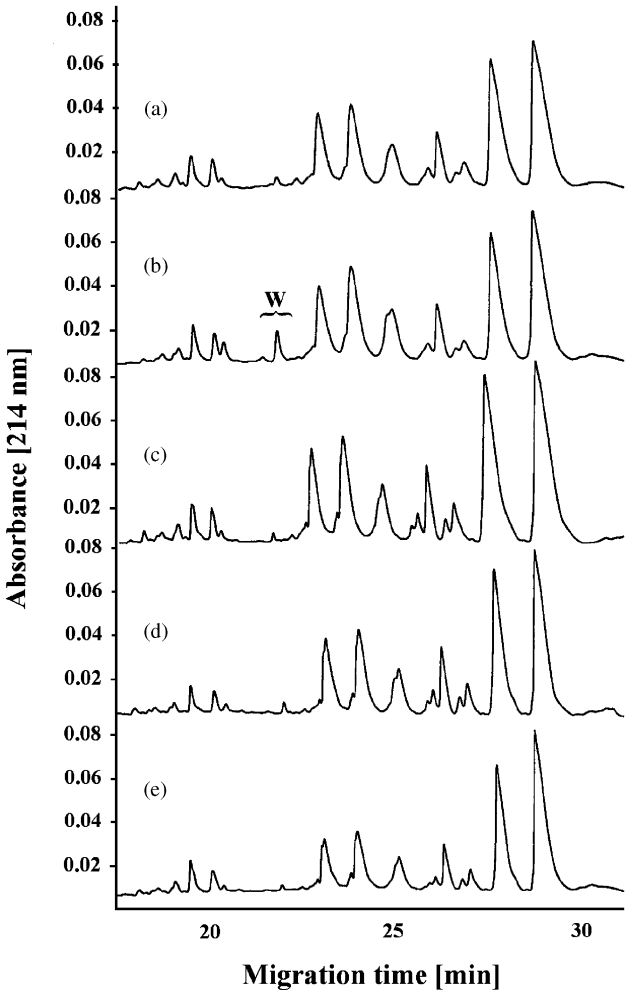

Fig. 4. Capillary electropherogram of pooled casein fractions from: (a) 15 Awassi; (b) 7 Merino; (c) 5 Awassi–Merino crossbreeds; (d) 11 East Friesian–Merino crossbreeds; (e) 9 East Friesian–Awassi crossbreeds. Note the prominence of the Welsh variant of α_{s1}-CN in the trace (b).

should be of concern for the newly established Australian sheep milking industry. Each animal analysed contained three fractions of the α_{s2}-CN while the κ-CN fraction was resolved by CE as one major peak migrating between the α_{s1}-CN fractions and the small β-CN peak.

Analysis of the β-CN revealed an important heterogeneity where two major peaks, β_1- and β_2-casein, and another minor peak with a shorter migration time, could be observed. This had also been found using conventional isoelectric focussing (IEF) by Chianese et al. (1997) who attributed this heterogeneity to multiple phosphorylation of the polypeptide chain. The major peak with shorter migration time was assigned to β_2-CN, which contains five phosphate groups, while the β_1-CN fraction contains an extra phosphorylation site in Thr_{12} residue (Richardson & Creamer, 1976). The potential of CE for the separation of different phosphorylated forms has already been demonstrated (Recio et al., 1997c).

When comparing each individual ovine electropherogram pattern (Fig. 3), it can be seen that there were the same polymorphs for each protein, the main difference being related to the quantity of each variant. Even by mixing different samples together to evaluate the presence of a different protein polymorph between the animals of the same breed (Fig. 4) and between the 47 animals of the whole flock (figure not shown), no major difference was observed. These results seem to indicate that the 5 breeds or crossbreeds assessed (A, M, AM, EFM, EFA), all contained the same polymorphs of proteins.

Table 2 shows the average values obtained for the milk samples of each breed and crossbreed. The EFA and Awassi ewes produced significantly more milk than the other breeds ($P<0.05$). This resulted in significantly greater yields of protein, fat, lactose and TS (Table 3). The East Friesian and Awassi sheep have been imported into Australia to help the establishment of a sheep

Table 2
Average composition of skimmed sheep milk samples used for this project depending on the breed (mean ± standard error)

Variables	Whole flock	Awassi	Merino	AM	EFM	EFA
Protein (g/100 g)	5.59 ± 0.11	5.79 ± 0.16	6.02 ± 0.41	5.85 ± 0.21	5.31 ± 0.25	5.20 ± 0.21
Lactose (g/100 g)	5.01 ± 0.03	5.01 ± 0.06	4.87 ± 0.07	4.95 ± 0.05	5.01 ± 0.07	5.15 ± 0.06
SNF (g/100 g)	11.39 ± 0.10	11.59 ± 0.15	11.68 ± 0.40	11.59 ± 0.16	11.12 ± 0.25	11.14 ± 0.18
TS (g/100 g)	12.25 ± 0.10	12.46 ± 0.16	12.52 ± 0.40	12.46 ± 0.14	11.97 ± 0.24	11.99 ± 0.18
pH (-)	6.55 ± 0.03	6.48 ± 0.07	6.68 ± 0.07	6.60 ± 0.02	6.60 ± 0.03	6.43 ± 0.01

Table 3
Average composition and yields of sheep milk samples

Variables	Whole flock	Awassi	Merino	AM	EFM	EFA
Milk yield (g/day)	621 ± 51	656 ± 94	352 ± 43	413 ± 51	513 ± 81	1047 ± 106
Protein (g/100 g)	4.88 ± 0.05	5.01 ± 0.07	4.97 ± 0.15	5.10 ± 0.12	4.74 ± 0.11	4.68 ± 0.09
Protein yield (g/day)	30 ± 2	33 ± 5	18 ± 2	21 ± 2	24 ± 3	49 ± 4
Fat (g/100 g)	5.77 ± 0.11	6.24 ± 0.19	5.55 ± 0.47	5.61 ± 0.19	5.48 ± 0.14	5.71 ± 0.19
Fat yield (g/day)	38 ± 3	43 ± 7	21 ± 4	25 ± 3	29 ± 4	62 ± 6
Lactose (g/100 g)	4.88 ± 0.03	4.90 ± 0.04	4.81 ± 0.06	4.80 ± 0.09	4.83 ± 0.07	5.01 ± 0.07
Lactose yield (g/day)	31 ± 2	32 ± 5	17 ± 2	20 ± 3	25 ± 4	53 ± 6
TS (g/100 g)	16.34 ± 0.14	16.89 ± 0.22	16.19 ± 0.57	16.33 ± 0.20	15.90 ± 0.22	16.23 ± 0.24
TS yield (g/day)	103 ± 9	112 ± 17	59 ± 9	68 ± 8	82 ± 12	172 ± 17

milking industry in this country, and the present results confirm that they are superior to the local Merino sheep for their dairy potential.

Analysis of the relationship between polymorphism and milk properties of different breeds showed that, in general, the Merino breed had the highest content of α_{s1}-CN Welsh variant with about three times as much as the other breeds. Comparatively, the crossbreeds AM and EFA presented a lower quantity of Welsh variant. The milk yield and the protein content were significantly affected by the amount of Welsh variant, which agrees with previous studies (Richardson & Creamer, 1976; Caroli et al., 1989). Indeed, Bolla et al. (1989) found that the α_{s1}-CN Welsh variant was associated with lower fat and protein content in the milk. The observed trend was the more Welsh variant, the less milk yield and lactose content. However, the milk yield was affected by the type of breed with East Friesian–Awassi crossbreed being clearly the highest milk producers of all breeds and throughout the lactation period, producing about twice as much milk as the other breeds. There was no significant effect of the β-Lg genotype on either milk yield or composition.

4. Conclusions

CE using an uncoated fused-silica capillary column and a low pH buffer, containing urea and a polymeric additive, has allowed the identification of the different protein polymorphs of ewe's milk. The CE method made possible the simultaneous separation of each polymorph in a single run and was well suited to analyse ewe's milk casein and whey protein fractions. Ovine casein fractions were satisfactorily resolved in less than 30 min.

However, the application of CE was restricted to the resolution of proteins with differing net charges, caused by the substitution of one amino acid for another, and it was only possible to achieve semi-qualitative results. It is possible to improve the current method by performing two different electrophoretic separations and testing each sample at alkaline and acidic pH values in the presence of urea and reducing agent. As a consequence, the whole spectrum of genetic polymorphism for α-La, β-Lg, α_{s1}-CN, α_{s2}-CN, κ-CN and β-CN could be established. The silent variants, variants due to amino acid substitution that do not lead to a change in the net charge of the proteins, can not be detected by the CE method. They can be detected successfully by a fractionation of the casein fractions using reversed-phase HPLC (Visser, Slangen, & Rollema, 1991), electrophoresis in the presence of SDS or IEF.

Although it appears that protein polymorphism significantly ($P < 0.05$) affected milk yield and composition, research on its influence on ovine milk clotting and cheese making ability continues.

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