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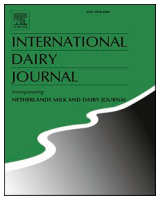

Research paper

Effect of protein polymorphisms on milk composition, coagulation properties, and protein profile in dairy sheep

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ABSTRACT

The objective of this study was to quantify the effect of protein polymorphisms on milk composition, coagulation properties, and protein profile in dairy sheep from a New Zealand flock. A total of 470 test-day records, from 147 lactating ewes, were used in the statistical analyses. Protein polymorphisms observed in the RP-HPLC were self-named for purposes of the present study. Data were analyzed using a mixed linear model, including the fixed effects of ewe age, litter size, coat-colour, and stage of lactation, and, as a covariate, deviation from the median lambing date of the flock. The effects of protein polymorphisms were added to the model, one at a time. Protein polymorphisms were significantly ($p < 0.05$) associated with milk composition and protein profile. Polymorphisms of β-lactoglobulin were significantly associated with milk heat stability, being AB type more heat stable than AA. The other processability traits were not significantly affected by protein polymorphisms. Further studies are required to confirm the protein variants and the properties of individual protein polymorphisms.

1. Introduction

Milk casein and whey proteins occur in several different phenotypes, also called protein polymorphisms. These are determined either by biochemical modifications in the proteins, such as alterations in the amino acid sequence, or by differences in phosphorylation levels, variations in the degree of glycosylation, and differences in chain lengths (Martin, Cebo, & Miranda, 2013). The protein polymorphisms are a result of genetic polymorphisms (single nucleotide polymorphisms, SNPs), mutations in the DNA that result in the presence of more than one type of allele in a locus (Moioli, Pilla, & Tripaldi, 1998), and a result of post-translational modifications, which are also influenced by genetics and other factors (Fang et al., 2016).

Polymorphisms of casein and whey proteins have been linked both directly and indirectly (due to changes in the proportion of protein fractions) with milk coagulation properties (MCP) in cows, sheep, and goats (Frederiksen et al., 2011; Garzon & Martinez, 1992; Ketto, Abdelghani, Johansen, Skeie, & Øyaas, 2019; Piredda, Papoff, Sanna, & Campus, 1993; Zhang et al., 2023). With the advancement of molecular genetics, this area remains under active investigation. Furthermore, protein polymorphisms may affect human health (Ali et al., 2022). Hence, there is potential to incorporate genetic variants of proteins that are favorable for milk technological properties or human health in animal breeding programs.

Different laboratory techniques are available for determining protein polymorphisms (Amigo, Recio, & Ramos, 2000; Bonfatti, Tuzzato, Chiarot, & Carnier, 2014). High-performance liquid chromatography (HPLC) offers the benefit of identification of silent protein variants, with a shorter total analysis and preparation time compared to alternative methods, allowing for routine analysis of many individual milk samples. However, reports on protein polymorphism using HPLC applied to ovine milk are scarce (De Pascale et al., 2022; Moatsou, Samolada, Katsabeki, & Anifantakis, 2004; Trujillo, Casals, & Guamis, 2000), while it is quite usual for cow milk (Vigolo, Niero, Penasa, & De Marchi, 2022).

In ovine milk, several phenotypes for caseins and whey proteins have been reported, but amino acid sequences have been determined only for

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some variants (Chianese, Caira, Garro, & Addeo, 2007; Ferranti et al., 1995; Picariello et al., 2009; Richardson & Mercier, 1979). In addition, there is still no official protein nomenclature report for sheep milk like there is for cow milk (Farrell Jr et al., 2004). It is possible to confirm the genetic variants of milk protein polymorphims with the combined use of DNA PCR-RFLP, and the polymorphisms of α-lactalbumin (α-LA); β-lactoglobulin (β-LG); and α_{s1}-, α_{s2}-, β-, and κ-casein (α_{s1}-, α_{s2}-, β-, and κ-CN) proteins have been widely associated with the genes LALBA, PAEP, CSN1S1, CSN1S2, CSN2, and CSN3, respectively (Suárez-Vega, Gutiérrez-Gil, Klopp, Tosser-Klopp, & Arranz, 2017).

This study aimed to investigate the effect of self-named polymorphisms of casein and whey proteins observed from HPLC of sheep milk on milk composition, protein composition, coagulation traits, and heat stability, and therefore highlight the need for additional research and analytical advancements in this field concerning sheep milk. Further genomic studies are necessary to confirm the genetic variants of the different protein polymorphisms determined by reverse-phase (RP)-HPLC chromatograms as commercial ovine milk protein standards are unavailable. Secondarily, this study investigated the associations between protein composition (quantity of protein fractions) and milk processability.

2. Materials and methods

Ethics approval was obtained for this study (Massey University Animal Ethics Committee Protocol 21/45). A total of 470 herd-test records were obtained from 147 ewes at 50–182 days of lactation, milked once a day (November 2021–January 2022). The ewes belonged to a small flock located in Masterton, New Zealand. The farm operates on a pasture-based system with low supplementation. Milk collection started after the full weaning of lambs at around four weeks of age. Description of the animals and the farm has been previously reported (Marshall, Lopez-Villalobos, Loveday, Ellis, & McNabb, 2023). The development of the breed started over 27 years ago using mainly East Friesian genetics.

Each ewe had a minimum of two records. The milk yield of individual ewes was manually recorded from the total volume taken from individual test buckets, and a representative milk sample was taken for compositional, processability, and protein profile analyses. The milk samples were immediately refrigerated for transportation to Palmerston North within 4 h of collection and had sodium azide added upon arrival (at a final concentration of 0.025%). The samples were kept refrigerated for compositional and processability analyses. The aliquot for protein profile analyses was kept frozen (from −20 °C to −80 °C) and was sent to the laboratories of the University of Padova (Legnaro, Italy).

2.1. Milk composition

An aliquot was analyzed using a Combi FOSS FT6000 instrument (Foss Analytics). The milk composition analyses included fat (%), protein (%), lactose (%), and somatic cell count (SCC, cells mL^{-1}). The analyses for casein (%) and urea (mg 100 mL^{-1}) were performed using a Fourier-transform Infrared (FTIR) milk analyser MilkoScan FT6000 (Foss Analytics).

Milk samples were submitted to a contract laboratory (Massey Nutrition Lab) for analysis of total calcium content (mg 100 mL^{-1}) using the Arsenazo III method (Randox reagent kit Ca8309) and the RX Daytona Plus clinical analyser.

2.2. Milk processability

Measures of milk processability traits were described in Marshall, Lopez-Villalobos, Loveday, Weeks, and McNabb (2024). The traditional MCP were obtained using a Formagraph instrument (Foss Analytics). The parameters obtained from the Formagraph included rennet coagulation time (RCT, min), time to reach curd firmness of 20 mm (K_{20}, min), and curd firmness at 30 min (A_{30}, mm) (McMahon & Brown, 1982).

Milk pH was measured at 31 °C, using a calibrated pH meter (Eco-Scan Model pH5) on the same day as MCP were obtained.

A smaller set of a total of 315 samples, due to feasibility reasons, was processed for measurement of individual laboratory cheese yield (ILCY), as per Othmane, Carriedo, De La Fuente Crespo, and San Primitivo (2002). Individual laboratory cheese yield (%) was the relative weight of the centrifuge residue (g) on the initial weighed milk (g).

The heat coagulation time (HCT, min) of whole raw milk was defined as the time of milk coagulation after submerging the sample in an oil bath set at 140 °C, as described by Cole and Tarassuk (1946).

2.3. Determination of proteins

The measurement of protein composition of the individual sheep milk samples was performed through RP-HPLC. The RP-HPLC equipment consisted of an Agilent 1260 Infinity II LC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary pump (Agilent 1260 Infinity II, G7111B) and a diode array detector (Agilent 1260 Infinity II, G7115A). Protein separations were performed on a reversed-phase analytical column C8 (Zorbax 300SB-C8 RP, Agilent Technologies) with silica-based packaging (3.5 μm, 300 Å, 150 mm × 4.6 mm i.d.). A Security Guard Cartridge System (300SB-C8 Guard Cartridges 4.6 mm × 12.5 mm, 4/PK, Agilent Technologies), was used as pre-column.

The sample preparation step followed the method proposed by Bobe, Beitz, Freeman, and Lindberg (1998). Frozen sheep milk aliquots (500 μL) were thawed at room temperature, and treated with an aqueous solution of guanidine (Gdn) HCl (6 M Gdn-HCl, 0.1 M BisTris buffer, 5.37 mM sodium citrate, and 19.5 mM dithiothreitol) in a 1:1 ratio (v/v). Each milk sample was vortexed for 10 s, incubated at room temperature for 1 h to promote protein solubilization, and centrifuged for 10 min at room temperature at 13,000×*g* for fat separation. After centrifugation, the fat layer was discarded, and the remaining solubilized sample was diluted with a solution containing 4.5 M GdnHCl diluted in a solvent mixture consisting of water, acetonitrile, and trifluoroacetic acid (100:900:1; v/v/v), in a 1:3 ratio (v/v). Gradient elution was carried out with a mixture of solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.1% trifluoroacetic acid in acetonitrile). The separation of milk protein fractions and the chromatographic conditions were described in Bonfatti, Grigoletto, Cecchinato, Gallo, and Carnier (2008) and Vigolo, Niero, et al. (2022), respectively. External standards of bovine κ-CN, α-CN, β-CN, α-LA, β-LG A, and β-LG B (Merck KGaA) of the highest purity available were employed for instrument calibration. Agilent OpenLab 2 CDS software (Agilent Technologies) was used for data acquisition and analysis.

The identification of separated protein fractions was achieved by comparing the elution times with those reported by Trujillo et al. (2000). In particular, the elution times and the characteristics of the corresponding peaks of κ-CN, α_{s1}-CN, α_{s2}-CN, β-CN, α-LA, and β-LG were comparable with those reported by Trujillo et al. (2000). Moreover, separate analysis of whey fractions in a smaller set of samples confirmed its distinct elution from the casein fractions. Finally, the results were expressed as area (mAU) underneath the peaks of κ-CN, αs1-CN, αs2-CN, β-CN, α-LA, and β-LG, which represents the quantity of each protein fraction. A strong linear relationship ($r^2 > 0.90$) was observed between protein concentration in milk (g L^{-1}) and combined peak areas of protein fractions (total area), which has also been confirmed previously by Bonfatti et al. (2008). The percentage of each protein in total protein (% TP) was calculated as the area of the respective fraction divided by the total area. Additionally, the method allowed for the observation of protein polymorphisms, with self-named nomenclature being given based on peak characteristics in the chromatogram. Further genomic studies are required for confirmation of protein genetic variant (allele genotype discrimination).

2.4. Statistical analyses

Descriptive statistics (mean, standard deviation, minimum and maximum values, and coefficient of variation) for milk yield, milk composition, protein composition, milk processability (RCT, K_{20}, A_{30}, ILCY, HCT), pH, and somatic cell score (SCS) were obtained in SAS version 9.4 software (SAS Institute Inc., Cary, NC, USA). Analyses of variances were performed using the MIXED procedure with a linear model that included the fixed effects of ewe coat-colour as an indicator of genetic variety within the breed (categorical variable with two levels: black or white), litter size (categorical variable with two levels: 1 lamb or 2 lambs and greater), ewe age (categorical variable with four levels: 1, 2, 3, and 4 years and older), and stage of lactation as a categorical variable with three levels: 1, 2, and 3, representing the different ranges of days in milk (date of milk sampling – date of lambing) from $\geq$50 to $<$ 95, $\geq$95 and $<$ 140, $\geq$140 and $\leq$ 182, respectively.

The fixed effects of self-named protein polymorphisms were included in the model, one at a time, these included the effect of aggregated caseins (categorical variable with two levels: AA/AA/AA or AB/AB/AB), α-LA (categorical variable with three levels: AA, AB, or BB), and β-LG (categorical variable with two levels: AA or AB). Polymorphisms that represented less than 7% of the population were not considered as an effect. As a covariate, deviation from the median lambing date of the flock (dmld, days) was added to the model. Random effects included a random effect of ewe and random residual error. Least squares means and standard errors for each class of the fixed effect of self-named protein polymorphism were obtained and used for mean comparisons using Fisher's least significant difference. The GLM procedure was used to investigate partial correlations between the content of protein fractions (% TP) and the processability traits, using the same animal model, as described above.

3. Results

3.1. Individual chromatograms

The RP-HPLC chromatograms from two individual milk samples are provided in Fig. 1, to illustrate the self-named protein polymorphisms observed in the flock, and included as fixed effects in the model. In the present study, κ-CN was monomorphic, and the polymorphisms of casein proteins (α_{s1}-, α_{s2}-, β-CN) seemed to be inherited together as a haplotype. It was observed that α_{s1}-CN occurred in duplicate, for some ewes, with two very distinct fractions that eluted separately (19 min vs 20 min), these were named AB and are suggested to be a heterozygous form of α_{s1}-CN. The ewes with only one fraction of α_{s1}-CN are likely to be homozygous for α_{s1}-CN, and this form was named AA (20 min). Both heterozygous (AB) and homozygous (AA) forms of α_{s1}-CN had a minor peak eluting after a major peak. The least frequent form (named BB) was the single fraction (also with a minor peak) that eluted earlier than AA (19 min).

The α_{s2}-CN and β-CN were also present as a single peak (named AA), with two distinct peaks (named AB), or as a single peak (named BB) that eluted earlier than AA. The given polymorphism names do not necessarily reflect heterozygous or homozygous forms of the same genetic variant, and they could be distinct genetic variants of different phosphorylation levels.

The minor peaks observed for α_{s1}-and α_{s2}-CN, in the same individuals, were sometimes with a broad rather than a sharp shape, and of heights that fluctuated throughout the lactation/season. For α_{s1}-CN there was a clear trend, the minor peaks became wider and less sharp with the advancement of the lactation/season. In addition, the double peaks of β-CN AB became sharper and taller with the advancement of the season/lactation.

For the whey proteins, α-LA and β-LG also occurred as either a double peak (AB), a single peak (AA) of later elution time, or a single peak of earlier elution time (BB). For α-LA and β-LG less variation is noted in the literature, and the observable variants of β-LG are either A or B, with C being uncommon.

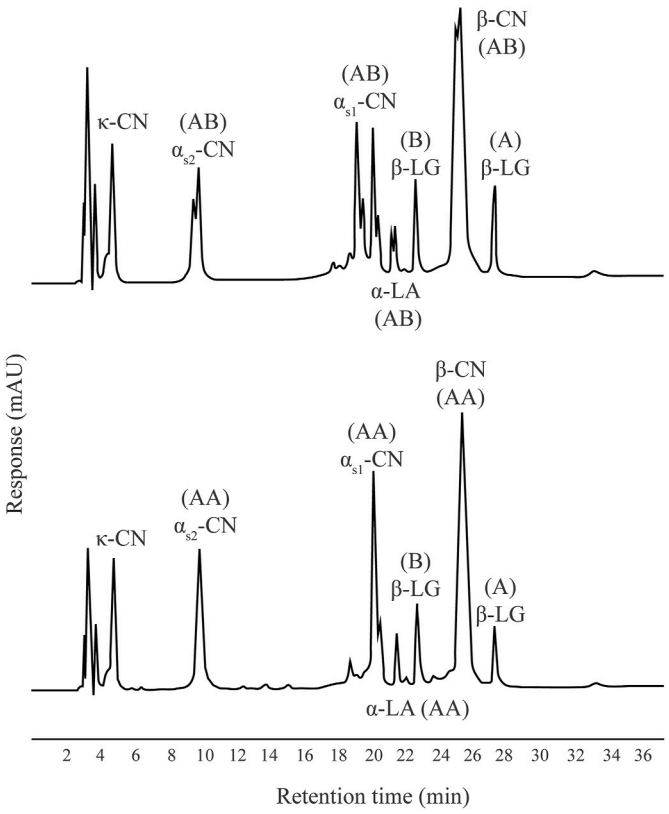

Fig. 1. Two individual RP-HPLC chromatograms illustrate all the possible self-named milk protein polymorphisms observed in the studied dairy sheep population. The protein fractions are represented by the response or peak area (measured as the intensity of absorbance, mAU) plotted as a function of retention time (min). Top chromatogram: κ-casein (monomorphic, approx. retention time between 4 and 5 min), followed by one fraction of α_{s2}-casein with double peak, named AB (9–10 min); two fractions of α_{s1}-casein with double peak, named AB (19–21 min); α-lactalbumin with two fractions, named AB (21–22 min); β-lactoglobulin named B (22–23 min); β-casein with a double peak, named AB (25–26 min); and β-lactoglobulin named A (27–28 min). Bottom chromatogram: κ-casein (monomorphic), followed by one fraction of α_{s2}-casein with a single peak, named AA (9–10 min); one fraction of α_{s1}-casein with a double peak, named AA (19–21 min); α-lactalbumin with one fraction, named AA (21–22 min); β-lactoglobulin named B (22–23min); β-casein with a single peak, named AA (25–26 min); and β-lactoglobulin named A (27–28 min).

3.2. Frequency of polymorphisms

The frequencies of the milk protein polymorphisms observed in the studied flock are presented in Supplementary Table S1.

3.3. Average protein composition

Descriptive statistics of milk yield and composition, protein composition (% TP), SCS, milk coagulation, cheese yield, heat coagulation time, and milk pH are summarised in Table 1.

The κ-CN, α_{s1}-CN, α_{s2}-CN, and β-CN constituted on average 7.3, 27.6, 12.8, and 40.1 % TP, respectively. The α-LA and β-LG constituted 1.9% and 10.4% TP, respectively. Coefficients of variation were largest for the content of α-LA (32%), followed by the content of κ-CN (18%). The lowest coefficient of variation was for content of β-CN, with only 5% of variation.

Table 1
Means, standard deviations (SD), minimum and maximum values, and coefficients of variation (CV) for milk production, milk composition, protein composition, SCS, milk coagulation properties, cheese yield, heat coagulation time, and milk pH of dairy sheep milked once-a-day during the 2021–2022 production season.

Trait[a]	N	Mean	SD	Minimum	Maximum	CV (%)
Milk yield (L day^{-1})	470	0.57	0.25	0.10	1.80	44
Milk composition						
Fat (%)	470	6.32	1.26	1.87	11.24	20
Protein (%)	470	5.52	0.68	4.09	8.80	12
Lactose (%)	470	4.72	0.27	3.66	5.34	6
Casein (%)	470	4.21	0.50	3.08	6.28	12
Casein:Protein	470	0.77	0.02	0.54	0.82	3
Calcium (mg 100 mL^{-1})	470	185.5	25.3	104.6	269.4	14
Urea (mg 100 mL^{-1})	470	39.64	7.61	9.39	63.13	19
SCS (Log_2SCC)	470	16.16	1.96	9.97	23.83	12
SCC × 10^3 (cells mL^{-1})	470	275	1090	1	14906	400
Protein composition (% TP)						
κ-casein	470	7.28	1.30	2.60	13.80	18
α_{s1}-casein	470	27.58	1.78	20.60	32.80	6
α_{s2}-casein	470	12.78	1.58	5.90	17.80	12
β-casein	470	40.06	1.96	32.50	45.50	5
α-lactalbumin	470	1.94	0.61	0.09	4.60	31
β-lactoglobulin	470	10.36	1.25	6.20	14.30	12
Milk coagulation properties						
RCT (min)	451	13.3	3.2	6.86	26.0	24
K_{20} (min)	447	2.7	1.1	1.30	10.2	41
A_{30} (mm)	451	53.3	9.5	11.60	75.4	18
ILCY (%)	315	44.20	8.97	23.4	78.8	20
HCT (min)	295	1.41	0.81	0.07	5.00	57
pH	470	6.59	0.09	6.34	6.90	1

[a] Casein:Protein = ratio of casein to protein, calculated as casein (%) divided by protein (%); SCS = somatic cell score (Log_2 SCC); SCC = somatic cell count; TP = total protein; RCT = rennet coagulation time; K_{20} = time to reach curd firmness of 20 mm; A_{30} = curd firmness at 30 min post rennet addition; ILCY = individual laboratory cheese yield; HCT = heat coagulation time; CV = coefficient of variation.

Table 2
F-values for effects of self-named protein polymorphisms on milk yield and composition, contents of calcium and urea, SCS, protein composition, milk coagulation properties, cheese yield, heat coagulation time, and milk pH of dairy sheep milked once-a-day during the 2021–2022 production season.

Trait[1]	Casein polymorphism (α_{s1}/α_{s2}/β-casein)	α-lactalbumin polymorphism	β-lactoglobulin polymorphism
Milk yield (L day^{-1})	0.79	0.94	0.18
Fat (%)	2.53	2.63	2.62
Protein (%)	2.43	3.38*	0.46
Lactose (%)	2.60	2.89	0.21
Casein (%)	1.35	4.58**	0.30
Casein:Protein	1.87	0.95	0.28
Calcium (mg 100 mL^{-1})	0.14	0.63	0.12
Urea (mg 100 mL^{-1})	2.03	1.44	1.80
SCS (Log_2SCC)	0.19	0.84	3.70
Protein composition (% TP)			
κ-casein	1.07	0.09	0.32
α_{s1}-casein	41.50***	1.22	1.45
α_{s2}-casein	60.40***	1.20	0.01
β-casein	4.00*	1.08	0.44
α-lactalbumin	0.02	15.60***	1.08
β-lactoglobulin	8.00**	0.67	0.44
Milk coagulation properties			
RCT (min)	0.26	0.43	1.70
K_{20} (min)	0.01	1.62	0.12
A_{30} (mm)	0.19	1.16	0.43
ILCY (%)	0.01	0.65	0.60
HCT (min)	3.26	0.08	5.02*
pH	2.96	0.70	3.50

Statistical significance is given as: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. [1] Casein:Protein = ratio of casein to protein, calculated as casein (%) divided by protein (%); SCS = somatic cell score (Log_2 SCC); SCC = somatic cell count; TP = total protein; RCT = rennet coagulation time; K_{20} = time to reach curd firmness of 20 mm; A_{30} = curd firmness at 30 min post rennet addition; ILCY = individual laboratory cheese yield; HCT = heat coagulation time.

3.4. Effects of animal factors

The F-values for the effects of animal factors on the milk traits investigated are presented in Supplementary Table S2. The least squares means of protein composition for different classes of ewe coat-colour, litter size, age, and different stages of lactation are presented in Supplementary Table S3. The detailed investigation of the effects of physiological and environmental factors on the protein composition was beyond the scope of the present study. These were included in the model to obtain an unbiased estimation of the effects of protein polymorphisms.

3.5. Effects of protein polymorphisms

The F-values for the effects of protein polymorphism are presented in Table 2. The least squares means of milk composition, processability, and protein composition, for each protein polymorphism, corrected for the fixed effects, are presented in Table 3, Table 4, and Table 5, respectively.

No effect of casein polymorphisms on milk production or milk composition was observed in the present study for this flock of dairy sheep. The only protein polymorphism with a significant effect ($p < 0.05$) on the gross milk composition of ewes was that of α-LA. The polymorphism named BB of α-LA was superior for contents of protein and casein in milk, followed by AB, and AA (BB > AB > AA). Polymorphism of β-LG almost had a significant effect on SCS, with polymorphism AB tending to have lower SCS.

The casein polymorphisms (combined α_{s1}-/α_{s2}-/β-CN) significantly affected the percentages of α_{s1}-, α_{s2}-, β-CN, and β-LG in TP. The polymorphism of α-LA significantly affected its concentration in TP. The polymorphism of β-LG did not significantly affect any of the protein concentrations. The concentration of κ-CN was not affected by any of the protein polymorphisms.

The only trait related to milk processability that was significantly affected by protein polymorphism was HCT. Polymorphism B of β-LG significantly increased milk heat stability, with AB milk coagulating 0.24 min (14 s) later than AA milk at 140 °C. Noteworthy, β-LG AB milk also tended to have a higher pH than AA milk, but this difference was not significant. The casein polymorphisms did not affect any traits relevant to cheese-making (RCT, A_{30}, K_{20}, and ILCY) (see Table 2).

Table 3
Least squares means (± standard errors) of milk composition (percentages of fat, protein, lactose, and casein), and SCS, for the different self-named protein polymorphisms.

Protein	Polymorphism[a]	Fat (%)	Protein (%)	Lactose (%)	Casein (%)	SCS (Log_2SCC)
Casein						
(α_{s1}/α_{s2}/β-CN)	AA/AA/AA	6.26 ± 0.09	5.50 ± 0.05	4.42 ± 0.02	4.17 ± 0.04	16.29 ± 0.17
	AB/AB/AB	6.06 ± 0.13	5.38 ± 0.07	4.77 ± 0.02	4.10 ± 0.05	16.39 ± 0.24
Whey fractions						
α-LA	AA	6.19 ± 0.11	5.37^{b} ± 0.06	4.77 ± 0.02	4.06^{b} ± 0.05	16.20 ± 0.21
	AB	6.09 ± 0.11	5.50^{ab} ± 0.06	4.72 ± 0.02	4.18^{a} ± 0.05	16.49 ± 0.21
	BB	6.44 ± 0.14	5.61^{a} ± 0.08	4.69 ± 0.03	4.27^{a} ± 0.06	16.25 ± 0.25
β-LG	AA	6.16 ± 0.09	5.46 ± 0.05	4.74 ± 0.02	4.14 ± 0.04	16.44 ± 0.17
	AB	6.38 ± 0.13	5.51 ± 0.07	4.72 ± 0.03	4.18 ± 0.06	15.97 ± 0.24

[a] Nomenclature was self-given to the protein polymorphisms in this population of dairy sheep, as they were being identified in the RP-HPLC chromatograms. Genomic studies and other laboratorial techniques for the description of protein structure will enable the confirmation of any protein genetic variants previously reported in the literature. α_{s1}/α_{s2}/β-CN = aggregate (haplotype) of α_{s1}-casein, α_{s2}-casein, and β-casein proteins; α-LA = α-lactalbumin; β-LG = β-lactoglobulin; SCS = somatic cell score calculated as Log_2SCC; SCC = somatic cell count. [a, b, c] Least squares means with different superscripts, within effect, are significantly different ($p < 0.05$).

Table 4
Least squares means (± standard errors) of milk coagulation properties, heat coagulation time, and milk pH, for the different self-named protein polymorphisms.

Protein	Polymorphism[a]	RCT (min)	K_{20} (min)	A_{30} (mm)	HCT (min)	pH
Casein						
(α_{s1}/α_{s2}/β-CN)	AA/AA/AA	14.63 ± 0.51	3.23 ± 0.25	52.00 ± 1.30	1.68 ± 0.09	6.59 ± 0.01
	AB/AB/AB	14.28 ± 0.71	3.20 ± 0.35	51.24 ± 1.82	1.86 ± 0.11	6.61 ± 0.01
Whey fractions						
α-LA	AA	14.41 ± 0.62	3.18 ± 0.30	51.69 ± 1.60	1.74 ± 0.10	6.60 ± 0.01
	AB	14.91 ± 0.63	3.53 ± 0.31	50.64 ± 1.61	1.70 ± 0.10	6.59 ± 0.01
	BB	14.22 ± 0.76	2.82 ± 0.37	53.85 ± 1.95	1.73 ± 0.12	6.60 ± 0.01
β-LG	AA	14.28 ± 0.52	3.19 ± 0.26	52.16 ± 1.34	1.67^{b} ± 0.09	6.59 ± 0.01
	AB	15.52 ± 0.72	3.31 ± 0.36	50.90 ± 1.86	1.91^{a} ± 0.11	6.61 ± 0.01

[a] Nomenclature was self-given to the protein polymorphisms in this population of dairy sheep, as they were being identified in the RP-HPLC chromatograms. Genomic studies and other laboratorial techniques for the description of protein structure will enable the confirmation of any protein genetic variants previously reported in the literature. RCT = rennet coagulation time; K_{20} = time to reach curd firmness of 20 mm; A_{30} = curd firmness at 30 min post rennet addition; HCT = heat coagulation time; α_{s1}/α_{s2}/β-CN = aggregate (haplotype) of α_{s1}-casein, α_{s2}-casein, and β-casein proteins; α-LA = α-lactalbumin; β-LG = β-lactoglobulin. [a, b, c] Least squares means with different superscripts, within effect, are significantly different ($p < 0.05$).

Table 5
Least squares means (± standard errors) of protein composition (percentages of κ-casein, α_{s1}-casein, α_{s2}-casein, β-casein, α-lactalbumin, and β-lactoglobulin, in total protein) for the different self-named protein polymorphisms.

Protein	Polymorphism[a]	κ-CN (% TP)	α_{s1}-CN (% TP)	α_{s2}-CN (% TP)	β-CN (% TP)	α-LA (% TP)	β-LG (% TP)
Casein							
(α_{s1}/α_{s2}/β-CN)	AA/AA/AA	7.73 ± 0.10	26.56^{b} ± 0.17	13.61^{a} ± 0.15	39.88^{a} ± 0.18	2.08 ± 0.05	10.12^{b} ± 0.11
	AB/AB/AB	7.88 ± 0.14	28.06^{a} ± 0.24	12.02^{b} ± 0.21	39.39^{b} ± 0.25	2.09 ± 0.08	10.55^{a} ± 0.15
Whey fractions							
α-LA	AA	7.74 ± 0.13	26.87 ± 0.24	13.16 ± 0.22	39.69 ± 0.22	2.29^{a} ± 0.06	10.23 ± 0.14
	AB	7.76 ± 0.13	27.16 ± 0.24	13.09 ± 0.22	39.66 ± 0.22	2.02^{b} ± 0.06	10.30 ± 0.14
	BB	7.82 ± 0.16	26.69 ± 0.29	13.53 ± 0.27	40.07 ± 0.27	1.80^{c} ± 0.07	10.09 ± 0.17
β-LG	AA	7.74 ± 0.11	26.84 ± 0.20	13.23 ± 0.18	39.81 ± 0.18	2.10 ± 0.05	10.25 ± 0.11
	AB	7.83 ± 0.15	27.18 ± 0.28	13.20 ± 0.25	39.64 ± 0.26	2.02 ± 0.08	10.14 ± 0.16

[a] Nomenclature was self-given to the protein polymorphisms in this population of dairy sheep, as they were being identified in the RP-HPLC chromatograms. Genomic studies and other laboratorial techniques for the description of protein structure will enable the confirmation of any protein genetic variants previously reported in the literature. α_{s1}/α_{s2}/β-CN = aggregate (haplotype) of α_{s1}-casein, α_{s2}-casein, and β-casein proteins; α-LA = α-lactalbumin; β-LG = β-lactoglobulin; κ-CN = κ-casein; α_{s1}-CN = α_{s1}-casein; α_{s2}-CN = α_{s2}-casein; β-CN = β-casein; TP = total protein. [a, b, c] Least squares means with different superscripts, within effect, are significantly different ($p < 0.05$).

3.6. Correlation between protein fractions and processability

The partial correlation coefficients between the content of protein fractions (% TP) and processability traits are presented in Table 6.

Most notable correlations were found between contents of κ-, α_{s1}-, β-CN, α-LA%, and ILCY. Increased κ- and α_{s1}-CN% were correlated with increased cheese yield, and the opposite was found for β-CN and α-LA%. Also, of notable importance, was the higher κ-CN% correlated with increased A_{30} and HCT. Additionally, higher α-LA% correlated with increased HCT, whereas higher β-LG% correlated with decreased HCT.

Table 6
Partial correlation coefficients between the content of protein fractions (percentages of κ-casein, α_{s1}-casein, α_{s2}-casein, β-casein, α-lactalbumin, and β-lactoglobulin, in total protein) and processability traits (RCT, K_{20}, A_{30}, ILCY, and HCT).

	κ-CN (% TP)	α_{s1}-CN (% TP)	α_{s2}-CN (% TP)	β-CN (% TP)	β-LG (% TP)	α-LA (% TP)
RCT (min)	−0.04	0.02	−0.04	0.08	−0.03	−0.12
	(0.46)	(0.69)	(0.47)	(0.18)	(0.66)	(0.05)
K_{20} (min)	−0.13	0.00	−0.11	0.13	0.02	−0.02
	(0.04)	(0.94)	(0.07)	(0.03)	(0.71)	(0.70)
A_{30} (mm)	0.17	−0.04	0.12	−0.10	−0.04	0.02
	(0.01)	(0.48)	(0.05)	(0.08)	(0.45)	(0.80)
ILCY (%)	0.39	0.29	−0.08	−0.36	0.07	−0.27
	(<0.001)	(<0.001)	(0.17)	(<0.001)	(0.27)	(<0.001)
HCT (min)	0.16	−0.12	0.00	0.07	−0.14	0.17
	(0.01)	(0.05)	(0.94)	(0.28)	(0.02)	(0.01)

RCT = rennet coagulation time; K_{20} = time to reach curd firmness of 20 mm; A_{30} = curd firmness at 30 min post rennet addition; ILCY = individual laboratory cheese yield; HCT = heat coagulation time; κ-CN = κ-casein; α_{s1}-CN = α_{s1}-casein; α_{s2}-CN = α_{s2}-casein; β-CN = β-casein; β-LG = β-lactoglobulin; α-LA = α-lactalbumin; TP = total protein.

4. Discussion

4.1. Average protein composition

To our knowledge, there are only a few reports available on the quantity of sheep milk casein fractions and they are contradictory, especially regarding the predominance of either α_{s1}-or α_{s2}-CN. Bramanti, Sortino, Onor, Beni, and Raspi (2003) also reported a higher concentration of α_{s1}-CN than of α_{s2}-CN fraction, in agreement with the present study. Protein composition was similar to the report of Moatsou et al. (2004) for Greek ewes, but different from the reports for Polish mountain (Kawęcka & Radkowska, 2023) and Romanian ewes (Pelmus et al., 2012). Differences might be largely attributed to the distinct sheep breeds and farming systems as feed is also reported to influence milk protein composition (Kawęcka & Radkowska, 2023).

4.2. Protein polymorphisms

Previous studies report κ-CN as monomorphic in dairy sheep, in agreement with the present study (Suárez-Vega et al., 2017). In East Friesian Dairy sheep, sequencing of CSN3 mRNA led to the identification of a new synonymous SNP (Giambra, Brandt, & Erhardt, 2014), which does not affect protein sequence. In Merino sheep, a microsatellite in intron 3 of ovine CSN3 was identified with five alleles (Corral, Padilla, & Izquierdo, 2010), and neutral amino acid exchange has also been reported (Ceriotti et al., 2004).

Others have also considered the polymorphisms of casein proteins (α_{s1}-, α_{s2}-, β-CN) as a haplotype that is inherited together (Gai, Uniacke-Lowe, O'Regan, Faulkner, & Kelly, 2021; Sacchi et al., 2005), in agreement with the findings in our study. This is due to the close location of the casein genes, which are organised in a cluster of less than 250 kb in length.

Also, in agreement with the present study, changes in the shape of double peaks were previously noted by Moatsou et al. (2004) in Greek sheep. This is suggested to be due to post-translational differences and to different genetic expressions of the level of phosphorylation. Other factors were also suggested to affect the level of protein phosphorylation such as the health and age of individuals, and the availability of phosphate (Amigo et al., 2000). In addition, seasonal variation of the level of glycosylation of κ-CN has been noted for grazing dairy cows (Li, Ye, & Singh, 2019), and these are worth investigating in dairy sheep.

The use of different HPLC methodologies in different studies (De Pascale et al., 2022; Picariello et al., 2009) impedes comparisons regarding the identification of genetic variants of α_{s1}-and α_{s2}-CNs. β-CN is also known to be highly polymorphic, though less phosphorylated than α_{s1}-CN and α_{s2}-CN (Chessa et al., 2010).

The polymorphisms of α-LA are the least studied among the milk proteins (Selvaggi, Laudadio, Dario, & Tufarelli, 2014). Less genetic variation is observed for this protein. Two ovine *α*-lactalbumin protein patterns (A and B) have been evidenced by starch gel electrophoresis, being the A variant the most common (Amigo et al., 2000; Schmidt & Ebner, 1972).

The most studied polymorphisms in sheep are those of β-LG, which have also been investigated by chromatography (El-Zahar et al., 2004; Picariello et al., 2012; Trujillo et al., 2000). Three variants of β-LG have been identified in the literature for dairy sheep (A, B, C), A and B being the most common. Thus, it is very likely that the β-LG variants identified in the present study are A and B.

4.3. Effect of protein polymorphisms on milk yield and composition

Protein genotypes of sheep have been previously associated with milk production and composition in other sheep populations (Corral et al., 2010; Dario, Carnicella, Dario, & Bufano, 2008; Noce et al., 2016; Pirisi et al., 1999; Pirisi, Fraghi, Piredda, & Leone, 1999; Sallam, 2023; Yousefi, Azari, Zerehdaran, Samiee, & Khataminejhad, 2013). However, contradictory results are reported, and this is attributed to breed differences, population sizes, frequency of distribution of genetic variants, and differences in methods used for statistical analysis. Furthermore, the effect of protein polymorphisms may change throughout lactation, due to differential expression of milk protein genes (Cardona et al., 2016).

There is limited information available in the literature regarding the effect of genetic variants of α-LA on quantitative milk traits (Sallam, 2023; Selvaggi et al., 2014). A strong association between α-LA genetic variants with milk protein and fat contents has been reported for Churra sheep (Garcia-Gamez et al., 2012), which aligns in part with the present study. It was suggested that an amino-acid substitution of α-LA would generate a decrease in lactose synthesis and milk osmotic pressure, affecting fat and protein concentrations and early studies have demonstrated the importance of α-LA as a regulator of lactose production and secretion of milk (Stacey et al., 1995). Another study found that heterozygote ewes of the LALBA gene tended to produce more milk with a greater total solid percentage (Sallam, 2023).

Although not significant in the present study, an association between β-LG genotype and SCC in ewes has been previously reported, also with lower SCC for heterozygous ewes (Triantaphyllopoulos et al., 2017). Effects of β-LG genotype on milk production and composition were found by others and the results reported were controversial (Corral et al., 2010; Dario et al., 2008; Ibrahim, Hassooni, & Alkhazraji, 2019; Ramos et al., 2009; Yousefi et al., 2013).

Although no significant effects of casein polymorphisms on milk production and composition were found in the present study, which could be a consequence of the small population size, others found casein genotypes to be strongly associated with ewe milk production and composition (Corral et al., 2010; Giambra et al., 2014; Pirisi, Piredda, et al., 1999; Ramos et al., 2009). In East Friesian and Lacaune ewes from

Switzerland and Germany, an α_{s1}-CN variant was associated with higher protein content (Giambra et al., 2014). Also, an interaction effect of CSN2 with CSN1S1 polymorphisms on milk production and composition was discovered in Awassi sheep (Al-Amareen & Jawasreh, 2022). In addition, genotypes of the CSN3 gene showed significant influences on the milk protein content in East Friesian sheep (Giambra et al., 2014).

No studies were found on the association between milk pH and ovine milk protein variants, however, for bovine milk, the pH of skim milk samples has been associated with α_{s1}-CN genetic variants (McLean, Graham, & Ponzoni, 1987). The polymorphisms of caseins and of β-LG almost had a significant effect on sheep milk pH in the present study.

4.4. Effect of protein polymorphisms on protein composition

While some studies have explored the association between protein genetic variants and differences in gross milk composition (Corral et al., 2010; Noce et al., 2016; Sallam, 2023), there remains a scarcity of research correlating protein genetic variants with protein composition in sheep milk (Nudda, Feligini, Battacone, Macciotta, & Pulina, 2003). It has been noted that variations in the coagulating properties of milk are predominantly linked to differences in the content of protein fractions rather than being solely influenced by the direct effect of protein genotypes (Bonfatti, Di Martino, Cecchinato, Degano, & Carnier, 2010; Cipolat-Gotet, Cecchinato, Malacarne, Bittante, & Summer 2018; Wedholm, 2008).

For instance, in cow milk, firmer curd was observed with lower proportions of α_{s2}-CN and β-CN or higher ratios of κ-CN (Jõudu, Henno, Kaart, Püssa, & Kärt, 2008). The concentration of κ-CN in the casein fraction is known to largely influence MPC because κ-CN is negatively correlated with casein micelle size. Low content of κ-CN increases the risk of non-coagulation because larger casein micelles are formed, which aggregate slower and form a softer curd (Ford & Grandison, 1986; Frederiksen et al., 2011). In agreement with the present study, Bonfatti et al. (2010) reported that RCT was favorably affected by high κ-CN content and by high percentage of κ-CN in total casein in bovine milk, and A_{30} increased with increased κ-CN content.

Additionally, milk protein composition is not only affected by the different protein polymorphisms, but can also vary due to factors related to the animal physiology such as lactation stage and parity number, which was noted in the present study, as well as with health status, feeding, and management practices (Bobe et al., 1998; Frederiksen et al., 2011).

Notably, protein composition has been extensively associated with milk protein genetic variants in dairy cows (Bonfatti et al., 2010; Jõudu et al., 2008; Ketto et al., 2017; McLean et al., 1987), in agreement with the present study where the effects of casein and α-LA polymorphisms on protein composition of sheep milk were significant. However, Nudda et al. (2003) found no significant effect of the ovine β-LG genotype on the contents (g L^{-1}) of β-LG or α-LA, which aligns with our findings.

In addition, in agreement with our study, McLean et al. (1987) noted that cow milk heat stability was positively correlated with κ-CN concentrations and negatively correlated with αs1-CN and β-LG concentrations. Also in agreement with our study, a previous study found that increased α-LA (increased α-LA:β-LG ratio) improved resistance to heat-induced coagulation, due to decreased protein-protein interactions, and that β-LG content had a destabilising effect (Crowley, Dowling, Caldeo, Kelly, & O'Mahony, 2016).

4.5. Effect of β-LG polymorphism on processability

In alignment with the present findings, it has been suggested that the Tyr of β-LG A has a role in the hydrophobic interactions, affecting the stability of micelles, and therefore B should show a higher denaturation onset temperature than ovine A (Amigo et al., 2000). For bovine milk, β-LG variant B was also considered more thermostable than variant A (Keppler, Sönnichsen, Lorenzen, & Schwarz, 2014).

It was found that during heat treatment of milk, a complex is formed between κ-CN and β-LG (Dalgleish, 1990). The destabilising effect of β-LG at a pH of minimum stability was linked to the ability to increase the hydrophobicity of casein micelles, sensitising casein micelles to heat-induced precipitation of calcium phosphate. However, at a pH of maximum stability, β-LG could chelate calcium (O'Connell & Fox, 2001). Hence, the relationship between β-LG variants and the heat stability of milk was found to be dependent on the pH range (Imafidon, Ng-Kwai-Hang, Harwalkar, & Ma, 1991), as well as milk concentration (McLean et al., 1987), and temperature applied (Jakob & Puhan, 1992). Consequently, conflicting results have emerged in the literature for bovine milk.

Although no significant effects of β-LG polymorphisms on the cheesemaking properties were found in the present study on ovine milk, significant effects of β-LG genotypes on MCP have been observed for bovine milk (Bonfatti et al., 2010; Marziali & Ng-Kwai-Hang, 1986).

4.6. Effect of casein polymorphisms on processability

Despite no direct significant effects of casein polymorphisms on processability being found in the present study, other studies reported significant associations with the renneting properties of milk (Noce et al., 2016; Pirisi, Piredda, et al., 1999). The lack of the negatively charged phosphate groups, which are the primary binding sites for Ca^{2+}, of an α_{s1}-CN variant was suggested to decrease the association of proteins in solution (Chianese et al., 1996)

Noce et al. (2016) found a particular SNP at the CSN1S1 gene to be associated with RCT, K_{20}, and A_{30}, with the shortest coagulation times and the highest values of curd firmness for heterozygous ewes. Those authors also reported significant associations between the CSN2 genetic variant and K_{20}, which had not been previously investigated in dairy sheep.

While limited studies have explored correlations between ovine casein polymorphisms and the technological properties of milk, extensive research has been conducted in bovine milk (Bonfatti et al., 2010; Ketto et al., 2019). The smaller net charge of a bovine α_{s1}-CN variant contributed to a stronger association (Schmidt, 1970), which resulted in a firmer curd, whereas a less hydrophobic variant contributed to a softer curd (Creamer, Zoerb, Olson, & Richardson, 1982). A bovine α_{s2}-CN variant was found to be less sensitive to Ca^{2+} due to the reduced number of anionic phosphoryl clusters (Swaisgood, 2003). In addition, variants of β-CN were also shown to have a direct effect on RCT, independent of the content of β-CN (Bonfatti et al., 2010).

Additionally, the level of casein phosphorylation is known to affect the buffering capacity of milk, with highly phosphorylated caseins associated with poor rennet and acid coagulation properties (Frederiksen et al., 2011; Ketto et al., 2017). Overall, glycosylation and phosphorylation of caseins can affect processability due to changes in the isoelectric point, molecular weight, hydrophobicity, and net charge of casein micelles (Huppertz, Fox, & Kelly, 2018), and further work is recommended to isolate and establish the effect of glycosylation and phosphorylation degree of the different casein polymorphisms on the coagulation properties of milk from individual sheep.

5. Conclusion

Protein polymorphisms were evidenced in the RP-HPLC chromatograms of individual sheep milk samples, despite the small flock size. There was quantitative variability in the protein fractions, especially for the contents of α-LA and κ-CN in total protein. The protein polymorphisms were associated with differences in the proportions of protein fractions, and these were correlated to the processability traits, especially the content of κ-CN, but also α-LA. Higher κ-CN was associated with overall better processability for cheese-making and heat-stability, and α-LA with better heat stability. There was a significant effect of β-LG polymorphism on milk heat stability, and this could be of

great interest to the manufacturers of sheep milk powder and beverages. Validation of the RP-HPLC method for the analysis of genetic variants of ovine milk proteins is necessary with the use of additional genomic studies, as the lack of sheep milk protein standards and the lack of studies using combined techniques hinder effective comparisons of chromatograms. Additionally, establishing a nomenclature committee for ovine milk protein identification would be beneficial.

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CRediT authorship contribution statement

Ana Carolina Marshall: Writing – review & editing, Writing – original draft, Visualization, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Vania Vigolo:** Methodology. **Massimo De Marchi:** Writing – review & editing, Methodology. **Nicolas Lopez-Villalobos:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Methodology, Conceptualization. **Simon M. Loveday:** Writing – review & editing, Supervision. **Mike Weeks:** Writing – review & editing, Supervision. **Warren McNabb:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

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