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Original article

Protein composition and polymorphism in the milk of Skopelos goats

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Abstract – Individual milk samples taken from goats of the Skopelos breed of Greece were analyzed by RP-HPLC using as standards the casein fraction of milks of known genotypes. Milk samples with particular characteristics with respect to the casein fraction were further analyzed by IEF and RP-HPLC/ESI-MS. The mean total protein content (36.7 ± 2.6 g·L^{-1}) and the mean casein content (29.7 ± 2.3 g·L^{-1}) were high. The same was true for the α_{s1}-cn fraction, which was 21.8% of the total cn fraction, whereas the β-cn percentage of total cn was 43.8%. The respective values for α_{s2}-cn and κ-cn were 13.7% and 13.8%. These quantitative characteristics were consistent with the predominance of the strong α_{s1}-cn variants B3, B4 and As/B1. In all samples analyzed by RP-HPLC/ESI-MS the deleted form differing from the respective complete form by Gln78 was found at different levels of phosphorylation, with the exception of variants F and D/G. The most frequent α_{s2}-cn variant was by far variant A followed by variants C and F, whereas compositional data implied the existence of a null allele. The detected β-cn forms were variants A and C in similar frequencies. Variant D (formerly B) of κ-cn predominated and the rare variant G was found. Different phosphorylation types of each casein variant and the characteristics of whey proteins were also determined.

goat milk protein / casein genotype / phosphorylation / protein quantification / polymorphism

摘要 – Skopelos 山羊奶蛋白质组成的蛋白质的多态性。以已知基因类型的乳酪蛋白为标准品，采用反相液相色谱分析了希腊 Skopelos 种山羊奶中蛋白质和酪蛋白的含量，采用等电聚焦和反相高效液相色谱－电喷雾质谱联用(RP-HPLC/ESI-MS)技术测定了酪蛋白的结构特征。乳中蛋白质的含量为 36.7 ± 2.6 g·L^{-1}，酪蛋白的含量为 29.7 ± 2.3 g·L^{-1}，α_{s1}- 酪蛋白占酪蛋白的 21.8%，上述测定值高于文献报导的最高值，而 β– 酪蛋白占酪蛋白 43.8% 低于文献报道的最高值。α_{s2}- 酪蛋白和 κ- 酪蛋白分别占酪蛋白的 13.7% 和 13.8%。在 α_{s1}- 酪蛋白变异体中，变异体 B3，B4 和 As/B1 占优势。所有样品经 RP-HPLC/ESI-MS 分析后证实，除了变异体 F 和 D/G 外，其他缺失型变异体与完整型的区别是在 Gln78 位上磷酸基的不同。α_{s2}-cn 变异体最大的基因频率是变异体 A，其次是变异体 C 和 F，试验证明其中可能存在无效的等位基因。β- 酪蛋白变异体缺失型是变异体 A 和 C，他们具有相似的基因频率。在 κ- 酪蛋白中变异体 D 占优势，而变异体 G 仅在个别品种羊奶中存在。本文还检测了每种酪蛋白变异体不同的磷酸化类型和乳清蛋白的特性。

关键词 山羊奶蛋白 / 山羊奶酪蛋白基因型 / 磷酸化作用 / 蛋白质定量分析 / 本地山羊 / 反相高效液相色谱 - 电喷雾质谱联用 / 多态性

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Résumé – Polymorphisme des protéines du lait de chèvre de la race Skopelos. Des laits individuels de chèvres de Skopelos (Grèce) ont été analysés par RP-HPLC et les profils protéiques ont été comparés avec ceux issus de lait de chèvres de génotypes connus au locus α_{s1}. Les échantillons qui présentaient des caractéristiques particulières de leur fraction caséique ont été analysés par IEF et RP-HPLC/ESI-MS. Les teneurs moyennes en protéines totales (36,7 ± 2,6 g·L^{-1}), et en caséines (29,7 ± 2,3 g·L^{-1}), étaient plus élevées que dans les laits des races hautement sélectionnées. Le pourcentage en α_{s1}-cn représentait 21,8 % des caséines totales, tandis que celui de la β-cn atteignait 43,8 %. Les pourcentages en α_{s2}-cn et κ-cn étaient respectivement 13,7 % et 13,8 %. Ces caractéristiques quantitatives étaient corrélées avec la prédominance des variants forts B3, B4 et As/B1 de la α_{s1}-cn. Dans tous les échantillons analysés par RP-HPLC/ESI-MS, la forme déplétée en Gln78 de la α_{s1}-cn présentait plusieurs niveaux de phosphorylation, à l'exception des variants F et D/G. Le variant le plus abondant de la α_{s2}-cn était le variant A suivi par les variants C et F. Les variants A et C de la β-cn ont été trouvés en fréquences similaires. Le variant D (antérieurement dénommé B) de la κ-cn était prédominant bien que le variant rare G était aussi trouvé. Plusieurs niveaux de phosphorylation de chacun de ces variants ont été aussi mis en évidence, ainsi que les principales caractéristiques des protéines du sérum.

polymorphisme / variant proteique / lait de chèvre / caséine / génotype / phosphorylation

1. INTRODUCTION

Skopelos goats are an autochthonous Greek breed [18] with a population of about 4500 animals raised in central Greece, mainly on Skopelos island, with a daily average milk production of 1.65 kg per animal and an average lactation period of 202 days. They are considered as an endangered dairy goat breed protected by the law.

It is well established that the complex qualitative and quantitative variability in goat caseins affects both the composition and technological behavior of milk. Seventeen α_{s1}-casein variants associated with high α_{s1}-casein content (3.6 g·L^{-1} per allele; A, B1, B2, B3, B4, C, L and M or 4.2 g·L^{-1} per allele, H) or medium (1.6 g·L^{-1} per allele; E and I) or low (0.6 g·L^{-1} per allele; D, F and G) or no α_{s1}-casein (O^1, O^2 and N) have been so far reported [4, 28, 42]. An additional source of α_{s1}-casein polymorphism is the simultaneous existence of deleted forms due to alternative splicing [16].

Five of the alleles of α_{s2}-casein (A, B, C, E and F) are associated with a production of 2.5 g·L^{-1} α_{s2}-casein per allele, one with decreased synthesis of α_{s2}-casein and one with zero α_{s2}-casein content [5, 23, 40, 41]. An additional variant (G) shown on isoelectric focusing gels has been reported by Erhardt et al. [15].

The four genetic variants of goat β-casein are associated with a normal β-casein content in milk, whereas there are two null alleles and one silent allele [12, 13, 17, 25, 35, 37]. There are 13 variants of goat κ-casein at the protein level and three silent alleles [20, 39, 50].

Many of the above-mentioned variants have been recently identified by using DNA typing of indigenous breeds from Southern Italy, West Africa or the Near East. It has been shown that goat casein variants differ among breeds and that they are related to region [6, 7, 20, 22, 39, 44, 47].

The aim of this research work was to study the protein fraction of the milk of the Skopelos goat breed of Greece. For this purpose, individual milk samples were taken from goats of the Skopelos, which were analyzed by RP-HPLC, using as standards the casein fraction of milks of known genotypes in the α_{s1} locus [35]. Their qualitative (number of peaks and retention time) and their quantitative characteristics (quantity of individual proteins) were studied. A number of samples with particular protein profiles were chosen to be further analyzed by IEF and RP-HPLC/ESI-MS, with the aim of characterizing them in detail.

2. MATERIALS AND METHODS

2.1. Milk samples

Sixty individual milks were randomly collected after the complete morning milking

of goats of the Skopelos breed. After the addition of sodium azide (0.4 g·L^{-1}), the milk samples were cooled down. A part of them was used for the compositional analyses and the other part was skimmed by centrifugation at 2000× *g* for 30 min at 4 °C. Total protein contents of milk samples were determined by means of an infrared spectroscopy apparatus (Milkoscan 133 A/S N, Foss Electric, Hillerod, Denmark) that was calibrated against the reference methods using goat's milks of different composition.

2.2. RP-HPLC of milk samples

The defatted milk samples were analyzed by RP-HPLC with the aim of visualizing and evaluating their protein profile. A Vydac C4 214 TP 5415 4.6 mm × 150 mm column (Separation group, Hesperia, CA, USA) was used. The HPLC system consisted of the Waters 600E pump (Waters, 34 Marple Street, Milford, MA, USA) and Millenium v. 3.05.01 software (1998, Waters). Solvent A was 1060 μL·L^{-1} trifluoroacetic acid in ultrapure water and Solvent B was 1 mL TFA, 800 mL acetonitrile and 200 mL ultrapure water. The flow rate was 1 mL·min^{-1}, the analyses were carried out at 40 °C and the eluent was monitored at 214 nm. A linear gradient from 350 to 620 mL·L^{-1} (from 35% to 62%) solvent B within 54 min was applied [21, 35]. Samples were prepared as follows: 0.5 mL of defatted milk was dissolved in 1 mL buffer, pH 7.0 (100 mmol·L^{-1} Tris, 8 mol·L^{-1} urea, 13 g·L^{-1} trisodium citrate and 20 mmol·L^{-1} dithiothreitol adjusted to pH 7.0 by HCl). After 1 h at 37 °C, 10 mL of Solvent A containing 6 mol·L^{-1} urea were added to the sample solution and the pH was adjusted to 2.1–2.2 with the addition of 0.5 mL of a TFA solution (100 mL·L^{-1}). After filtration through a 0.45-μm filter (Millipore Corporation, Bedford MA, USA), 50 μL of sample were injected. Two independent preparations of each sample were analyzed and the profiles were compared with those obtained from the analyses of whole casein standards containing known casein genotypes (κ-casein AA, α_{s2}-casein AA, α_{s1}-casein AA, EE and O^1O^1, and α_{s1}-casein CC).

2.3. RP-HPLC/ESI-MS of selected milk samples

After the assessment of the chromatographic profiles, 25 samples were selected for further analysis by RP-HPLC/ESI-MS. The selection was based on the retention time and on the quantification data obtained after the integration of their protein profiles (Sect. 2.2). A Vydac C4 214 TP 5215 2.1 mm × 150 mm column was used in a system coupling on-line RP-HPLC and ESI-MS. RP-HPLC was carried out on a Hewlett Packard 1100 system (Agilent Technologies, Massy, France) at a flow rate of 0.25 mL·min^{-1} at 40 °C, and the detection was by absorbance at 214 nm and by total ion current. Solvent A was 1060 μL·L^{-1} trifluroacetic acid (TFA) in ultrapure water and Solvent B was 1 mL TFA, 800 mL acetonitrile and 200 mL ultrapure water. The elution conditions were as follows: 370 mL·L^{-1} solvent B (37%) for 5 min, then a linear gradient from 370 to 570 mL·L^{-1} solvent B (from 37 to 57%) within 37.5 min was applied. The column was directly interfaced with a Sciex API III Plus mass spectrometer (Perkin-Elmer-Sciex, Thornhill, Ontario, Canada), through a post-flow splitter permitting the introduction of only 1/10 of the HPLC eluate into the mass spectrometer. The ion source voltage and the orifice voltage were set at 4–5 kV and 70/90 V, respectively. Positive ion mode was used and mass scans were acquired over a m/z range of 500–2400 with a step size of 0.3 g·mol^{-1} and a dwell time of 1 ms per step. The charge number of the multicharge ions, the deconvoluted mass spectra and the proteins Mr (mass) determination were obtained using the BioMultiView software 1.3.1. (PE-SCIEX). Fifty mg of lyophilized milk was dissolved in 800 mL buffer, pH 7.0 (100 mmol·L^{-1} Tris, 8 mol·L^{-1} urea, 13 g·L^{-1} trisodium citrate and 20 mmol·L^{-1} dithiothreiol adjusted to pH 7.0 by HCl). After 1 h at 37 °C, 3 mL of Solvent A containing 4 mol·L^{-1} urea were added to the sample solution and the pH was adjusted to 2.1–2.2 with the addition of 150 mL of a TFA solution (100 mL·L^{-1}). After filtration through a 0.45-μm filter (Millipore Corporation, Bedford MA, USA), 50 μL of sample were injected.

Table I. Quantitative characteristics of the protein fraction (expressed in $g \cdot L^{-1}$) of individual goat milk samples (n = 57) from the Skopelos breed.

	Total protein[a]	κ-cn[b]	α_{s2}-cn[b]	α_{s1}-cn[b]	γ-cn[b]	β-cn[b]	Total cn[b]	a-LA[b]	b-Lg[b]
mean	36.65	4.09	4.07	6.47	2.0	13.0	29.7	1.37	2.14
SD	2.63	0.56	0.69	1.96	0.54	1.15	2.28	0.22	0.45
min	31.3	3.12	2.62	1.31	1.15	10.3	25.0	0.94	1.37
max	42.3	5.61	5.95	10.1	3.41	15.5	34.6	2.01	3.89

[a] Estimated by Milkoscan (Sect. 2.1).
[b] Estimated by the area of the chromatographic peaks and by the compositional data (Sect. 3.1).

2.4. Isoelectric focusing (IEF) of whole casein

Whole casein prepared by acidification at pH 4.2 using 0.1 $mol \cdot L^{-1}$ HCl, of samples with characteristic protein profiles, was analyzed by isoelectric focusing on ultrathin polyacrylamide gels, as described by Moatsou et al. [31]. For comparison reasons, whole casein fractions of the standard milks of known genotypes were analyzed by the same method.

2.5. Statistical analysis

The software Statgraphics Plus for Windows 2.1 (Manugistics, Inc. Rockville, Maryland, USA) was used for the statistical analysis. Principal component analysis (PCA) was applied with the aim of grouping the milk samples regarding their α_{s1}-cn and α_{s2}-cn characteristics. The factors with eigenvalues >1 were retained.

3. RESULTS AND DISCUSSION

The elution times of α-la and β-lg were determined after the analysis of acid goat's wheys and individual whey proteins [30]. The elution time of γ-casein was determined after the analysis of a plasmin hydrolysate of goat β-cn [31]. The quantification of the individual protein fraction was based on the chromatographic areas of the peaks and on the total protein content of each sample. Three samples were not included in the study due to proteolysis.

The quantitative characteristics of the milk samples from the Skopelos breed are presented in Table I and some characteristic RP-HPLC profiles are presented in Figure 1. The total protein content, 36.7 ± 2.6 $g \cdot L^{-1}$, and the casein content, 29.7 ± 2.3 $g \cdot L^{-1}$, were higher than those of international highly selected breeds [33, 34, 46] and similar to those of the milk from indigenous Greek goats [1, 45]. The same was true for the α_{s1}-cn, which was 21.8% of the total cn fraction, compared with the 8–17% reported for highly selected breeds. On the contrary, the β-cn percentage of total cn of highly selected breeds, 48–60%, is higher compared with the 43.8% of the present milk.

The retention time of each peak was calculated relatively to β-cn to avoid variation from day to day, and it was symbolized as rRT. They were compared with the respective rRTs of goat casein of standard samples with known genotypes (Tab. II).

Twenty-five samples with profiles with particular characteristics or with marginal contents of total protein or individual caseins were chosen to be further analyzed by RP-HPLC/ESI-MS. For the assignment of the experimental masses of the casein peaks to the known variants, we took into consideration: (i) the known theoretical masses if the difference was <0.03%, and (ii) the peak area, since several α_{s1}-cn variants share very similar molecular masses and they have the same rRTs; but they have different levels of expression.

Table II. Retention times relative to β-cn (rRT) of individual goat caseins in the RP-HPLC profiles of standard samples with known genotypes.

	κ-cn A	κ-cn D[a]	α_{s2}-cn A/B	α_{s2}-cn C	α_{s1}-cn E	α_{s1}-cn A
n[b]	6	21	21	6	11	12
mean	18.13	17.82	13.94	12.16	5.11	4.19
SD	0.46	0.55	0.79	0.51	0.28	0.27

[a] Formerly B according to the nomenclature proposed by Jann et al. [20].
[b] Number of chromatographic runs.

3.1. α_{s1}-casein

The defective variants F, D and G could not be determined by ESI-MS or by IEF with certainty, especially in the heterozygous condition, due to their very low quantities (Fig. 1), because they are associated with 0.6 g·L^{-1} α_{s1}-cn production per allele. Furthermore, the D and G variants cannot be distinguished from each other, since they differ by 2.7 mass units. However, D/G variants were determined in the heterozygous condition in 5 out of the 25 samples analyzed by RP-HPLC/ESI-MS: in 2 samples with the strong α_{s1}-cn B3, in 2 with the strong α_{s1}-cn B4, and in one of them with the defective α_{s1}-cn F. Therefore, since the expression of weak variants could not be clearly distinguished from the expression of the null variants using the chromatographic profiles of the milks, the term defective variant was used to indicate their existence in some samples.

For the interpretation of the α_{s1}-cn results, the total of 57 milks were separated into five groups, taking into consideration the number of α_{s1}-cn peaks (1 or 2) and the rRT, as shown in Table III. Within each group, there were different levels of α_{s1}-cn quantities, which were pointed out with the aid of a Principal Component Analysis plot based on the α_{s1}-cn content, β-cn content and on the β-cn/α_{s1}-cn ratio of the samples. The genetic variants of α_{s1}-cn indicated in Table III were determined in the 25 samples that were further analyzed by RP-HPLC/ESI-MS.

The rRT of group A corresponds to the rRT of variant E (Tab. II). However, the α_{s1}-cn content of the samples of this group was not medium and experimental masses corresponding to strong variants B3 and B4 were also detected. Variants B3, B4 and E cannot be separated in the RP-HPLC profiles [3, 4, 9, 10, 21]. Furthermore, E and B4 have the same amino acid composition but different levels of expression (E is a medium and B4 is a strong variant). When only a strong variant was detected in the samples with medium α_{s1}-cn quantity, it was considered that it existed in the heterozygous condition along with a defective variant.

The same strategy was followed for the interpretation of the α_{s1}-cn fraction of milks of group B, bearing in mind that the strong As variant has the same amino acid composition as the medium I variant, and that variants As and B1 with 9 P co-elute each other and differ by one mass unit. However, in variant A there is a potential tenth phosphorylation site at Ser75 [29] that is eliminated in variant B1 due to Glu/Gln replacement at position 77. The quantification characteristics of Table III implied the existence of the strong variants As/B1 in the homozygous form in one sample with 8.5 g·L^{-1} α_{s1}-cn or the existence of the strong variant in the heterozygous condition with the medium variant I in the sample with 5.89 g·L^{-1} α_{s1}-cn. Similarly, it was evident that in the four samples with mean α_{s1}-cn content of 3.82 g·L^{-1}, the strong variant As/B1 existed with a defective variant or the medium variant I existed in the homozygous condition.

In group C, the existence of a strong variant along with a medium variant was also evident in the RP-HPLC profiles due to the great difference between the heights of the relevant peaks (Fig. 1). Twenty-eight milks out of 57 had a high α_{s1}-cn content, ranging from 7.5 to 8.5 g·L^{-1}, indicating the existence

Table III. Characteristics of α_{s1}-casein of individual goat milks from the Skopelos breed.

Groups	α_{s1}-cn peaks	rRT[a]	α_{s1}-cn content	n[b]	α_{s1}-cn, $g \cdot L^{-1}$ [c]	α_{s1}-cn/ total cn[c]	β-cn/ α_{s1}-cn[c]	Variants[d]	Samples analyzed by RP-HPLC/ ESI-MS[e]
A	1	5.18 ± 0.13	High	11	7.48 ± 0.73	0.25 ± 0.01	1.67 ± 0.18	B3 8P 9P 10P + B4 8P 9P 10P or B4 8P 9P or B3 7P 8P 9P	5
			Medium-high	5	6.25 ± 0.25	0.21 ± 0.01	2.16 ± 0.19	B3 8P 9P 10P or B4 8P 9P 10P + E 8P 9P 10P	2
			Medium	11	4.55 ± 0.64	0.16 ± 0.02	2.96 ± 0.19	E 8P 9P (10P) or B4 8P 9P (10P) + defective or B3 8P 9P 10P + defective	4
B	1	4.24 ± 0.14	High	1	8.50	0.26	1.58	As/B1 (6P) 7P 8P (9P)	1
			Medium-high	1	5.89	0.22	2.02	As/B1 (6P) 7P 8P (9P) + I (6P) 7P 8P (9P)	1
			Medium	4	3.82 ± 0.32	0.15 ± 0.01	3.37 ± 0.37	I 7P 8P 9P or As/B1 7P 8P 9P + defective	3
C	2	5.11 ± 0.15/ 4.11 ± 0.11	High	16	8.33 ± 0.91	0.27 ± 0.02	1.54 ± 0.24	As/B1 6P 7P 8P 9P + B4 (7P) 8P 9P 10P or As/B1 7P 8P (9P) + B3 8P 9P 10P	5
			Medium-high	6	6.12 ± 0.41	0.21 ± 0.02	2.16 ± 0.32	As/B1 7P 8P + E 8P 9P	2
D	2	5.97/7.22	Very low	1	1.31	0.05	10.6	F 2P 3P + D 2P/G 5P	1
E	1	7.3	Very low	1	1.75	0.06	8.08	D 2P/G 5P	1

[a] Retention time relative to that of β-cn.
[b] Number of samples in each group.
[c] Estimated as described in Section 3.1.
[d] Based on the experimental masses, rRT, level of expression of each variant and IEF profiles. Phosphorylations in parenthesis were rare and the most abundant of them are underlined.
[e] Samples of each group analyzed by RP-HPLC/ESI-MS.

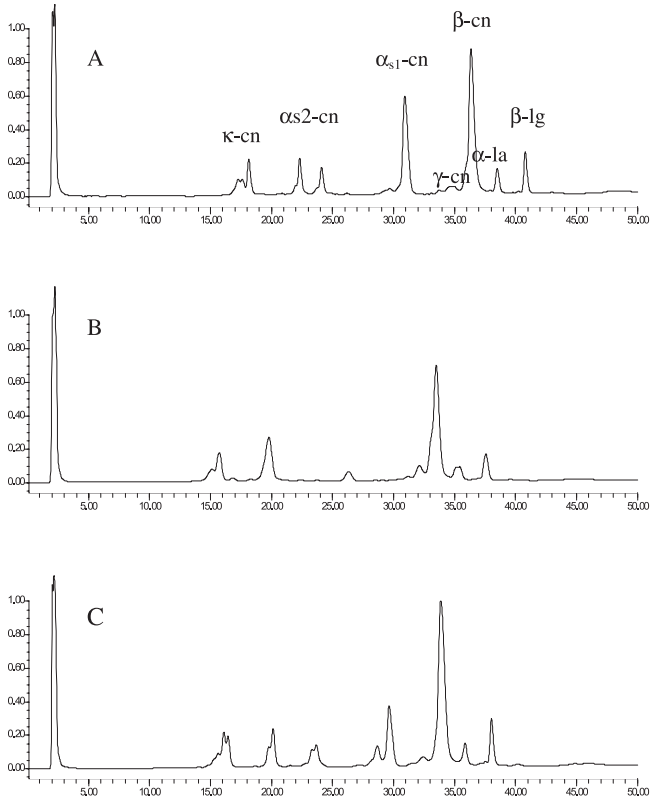

Figure 1. Some characteristic RP-HPLC profiles (A214) of individual goat milk samples from the Skopelos breed. Column: Vydac C4 214 TP 5415; elution conditions: as described in Section 2.2. The genotypes were determined by RP-HPLC/ESI-MS (Sect. 2.3). A: κ-cn DD, αs2-cn AC, α_{s1}-cn B3B4, β-cn AA; B: κ-cn DD, αs2-cn AA, α_{s1}-cn D/G, β-cn CC; C: κ-cn DG, αs2-cn AF, α_{s1}-cn As/B1E, β-cn AC.

of strong variants in the homozygous condition. Twelve milks had a medium–high content (from 5.9 to 6.3 g·L^{-1}), which implied the existence of a strong and a medium variant. Only two milks had a very low content, which corresponds to defective variants.

The strong variants determined by RP-HPLC/ESI-MS were mainly B3 (in 7 samples out of 25) and B4 (in 10 samples out of 25), followed by As or B1 (in 2 samples out of 25). The combination of rRT and α_{s1}-cn quantity showed that the medium variants were E and I. Therefore, the A and B variants were predominant, either in combinations with each other or with medium or defective variants, in accordance with the reports of Enne et al. [14], Martin et al. [28], Chianese et al. [8], Sacchi et al. [44] and Marletta et al. [27], on the abundance of strong α_{s1}-cn variants in the autochthonous dairy goat breeds of the Mediterranean region.

In all samples analyzed by RP-HPLC/ESI-MS the deleted form differing from the respective complete form by Gln78 was found at different levels of phosphorylation, with the exception of variants F and D/G. No other deleted forms were detected in the profiles, probably due to their low quantities.

Ferranti et al. [16] report that apart from genetic polymorphism, mature goat α_{s1}-cn exists as a mixture of at least seven molecular species with different peptide chain lengths, as a result of alternative skipping. The main component corresponds to the 199-residue-long form and the deleted proteins differ from the complete one by the absence of peptides 141-148, 110-117 or Gln78 or a combination of such deletions.

The main phosphorylation levels of α_{s1}-cn B3, B4 and E were three from 8P to 10P, the most abundant (based on intensity TIC) being the 9P form or the 8P form for the latter, whereas As/B1 were present in 4 phosphorylation levels from 6P to 9P, the most abundant being the 7P form. These results were in accordance with previous reports [35, 38, 48].

3.2. α_{s2}-casein

The characteristics of the α_{s2}-casein fraction of the individual milks are shown in Table IV. The presentation was based on the rRT of α_{s2}-cn peaks in RP-HPLC profiles, on their quantity ($g \cdot L^{-1}$) and on the ESI-MS analyses. Accordingly, samples were divided into four groups with regard to rRT of the number (1 or 2) of α_{s2}-cn peaks. Most of them, 34 out of 57, were homozygotes for variant A, whereas variant A was present in 55 samples. This finding was in accordance with several reports regarding the frequency of variant A in international [5] or native [15, 26, 27, 40, 41, 44] breeds.

A PCA plot based on α_{s2}-cn ($g \cdot L^{-1}$) content, on β-cn ($g \cdot L^{-1}$) and on the ratio of β-cn to α_{s2}-cn showed that there were two levels of α_{s2}-cn content within the total of samples with one α_{s2}-cn peak, that were at first considered as homozygotes for variant A or C. This plot implied the existence of a null α_{s2}-cn variant in the heterozygous condition in 9 samples out of 57.

The second most frequent variant was variant C in the heterozygous condition with null variant (group B) or with variant A (group C). Finally, the least frequent was the variant F, that was present only in the heterozygous condition (group D), similarly to the α_{s2}-cn of individual milks from the Indigenous Greek breed (data submitted for publication). Variant F is an abundant α_{s2}-cn variant in Italian goat breeds [26, 27, 40, 44].

The α_{s2}-cn is the most phosphorylated casein and in most cases it has been found with 7-11P, the most abundant type being 11P, as reported by Pierre et al. [38] and Neveu et al. [35]. In 4 samples out of 25 analyzed by RP-HPLC/ESI-MS, types with 12 P and 13 P were observed, as reported by Trujillo et al. [48]. The differentiation of variant B of α_{s2}-cn was not possible, because it co-elutes with variant A and has almost the same mass. However, it has 1P lower than A, due to amino acid substitution, Glu64 → Lys64, that affects phosphorylation site Ser62 [35], but no α_{s2}-cn with such a phosphorylation pattern was found by ESI-MS analysis. Moreover, some randomly selected samples were analyzed by IEF since α_{s2}-cn A and B have different profiles. No sample was assigned to variant B, similarly to individual milks from the Indigenous Greek breed (data submitted for publication).

3.3. β-casein

The mean β-cn content of the samples was 13 ± 1.15 $g \cdot L^{-1}$, and in 43 out of a total of 57, it ranged from 11.6 to 14.2 $g \cdot L^{-1}$.

Goat β-cn variants could not be distinguished from each other because they co-elute each other in the RP-HPLC profiles, have similar IEF profiles and they are associated with a normal β-cn content. Therefore, in Table V, only the 25 samples analyzed by ESI-MS are presented. Variants A [43] and C [35] were present in both homozygous and heterozygous forms in 3 phosphorylation levels, the most abundant being 5P and 6P in almost similar quantities. Furthermore, the quantification data did not imply the existence of a null allele [13] in the heterozygous condition. The β-Cn variants A and C were almost equally present.

In one sample, an unknown mass of 23 876 was found in the β-cn peak along with variant C, in two levels of phosphorylation, which has been also found in the milk of the Indigenous Greek breed and is under investigation.

Table IV. Characteristics of α_{s2}-casein of individual goat milks from the Skopelos breed.

Groups	α_{s2}-cn peaks	rRT[a]	α_{s2}-cn content	n[b]	α_{s2}-cn, g·L^{-1} [c]	α_{s2}-cn/ total cn[c]	β-cn/ α_{s2}-cn[c]	Variants[d]	Samples analyzed by RP-HPLC/ ESI-MS[e]
A	1	13.77 ± 0.29	High	34	4.25 ± 0.58	0.15 ± 0.02	3.14 ± 0.38	A 6-11P or A 6-11P 12P 13P	16
			Low	7	3.19 ± 0.34	0.11 ± 0.01	4.23 ± 0.31	A 10-11P	1
B	1	12.0	Low	2	3.09	0.10	4.21		0
C	2	13.72 ± 0.18/ 12.07 ± 0.15	High	11	4.01 ± 0.62	0.13 ± 0.02	3.05 ± 0.40	A 7-11P/C 8-11P or A 9-11P 12P/C 8-11P	5
D	2	13.72 ± 0.12/ 10.16 ± 0.08	High	3	4.83 ± 0.66	0.16 ± 0.02	2.75 ± 0.18	A 7-11P/F 7-11P	

[a] Retention time relative to that of β-cn.
[b] Number of samples in each group.
[c] Estimated as described in Section 3.1.
[d] Based on the experimental masses, rRT, level of expression of each variant and IEF profiles. Phosphorylations in parenthesis were rare and the most abundant of them are underlined.
[e] Samples of each group analyzed by RP-HPLC/ESI-MS.

Table V. Types of β-cn in individual goat milks from the Skopelos breed determined by RP-HPLC/ESI-MS.

Variant[a]	n[b]	β-cn, g·L^{-1} [c]	β-cn/total cn [c]
A 4P 5P 6P	7	12.9 ± 1.3	0.44 ± 0.06
C 4P 5P 6P	8	12.9 ± 0.7	0.46 ± 0.05
A 4P 5P 6P + C 4P 5P 6P	9	12.9 ± 1.5	0.42 ± 0.06
C 5P 6P + unknown	1	12.23	0.44

[a] Based on the experimental masses, retention times relative to β-cn (rRTs) and IEF profiles. Phosphorylations in parenthesis were rare and the most abundant of them are underlined.
[b] Number of samples with this type of β-cn.
[c] Estimated as described in Section 3.1.

Table VI. Types of κ-cn in individual goat milks from the Skopelos breed.

Variant[a]	n[b]	κ-cn, g·L^{-1} [d]	κ-cn/total cn [d]
A (1P) 2P	1	3.8	0.12
D^3 1P 2P	18	4.0 ± 0.5	0.15 ± 0.02
A (1P) 2P + D^3 1P 2P	4	3.9 ± 0.1	0.14 ± 0.005
D^3 (1P) 2P + G (1P) 2P	2	3.8	0.17

[a] Based on the experimental masses, retention times relative to β-cn (rRTs) and IEF profiles. Phosphorylations in parenthesis were rare and the most abundant of them are underlined.
[b] Number of samples with this type of κ-cn.
[c] Formerly B variant, according to nomenclature proposed by Jann et al. [20].
[d] Estimated as described in Section 3.1.

3.4. κ-casein

The glycosylated forms are eluted just before the main peak [32]; therefore, only the main peak (Fig.1) was taken into consideration for the interpretation of the ESI-MS results. The presentation in Table VI is according to the most recent nomenclature, proposed by Jann et al. [20], in which the former B variant [50] has been renamed as D and vice versa. The goat κ-cn variants were not separated on the C4 column and on the IEF gels. In fact, Prinzenberg et al. [39] report that the κ-Cn variants are grouped into 2 visible IEF patterns, according to their isoelectric points: A^{IEF} (variants A, B, C, F, G, H, I, J and L) and B^{IEF} (variants D, E, K and M).

The mean κ-cn content of the samples was 4.09 ± 0.56 g·L^{-1}, and in 48 out of a total of 57, it ranged from 3.1 to 4.8 g·L^{-1}. The different κ-cn alleles have not been associated with differences in their level of expression. However, Chianese et al. [8] report that B^{IEF} is present in milks with higher casein content than A^{IEF}.

The variant D (formerly B) of κ-cn predominated in the samples analyzed by ESI-MS, as happens with the majority of the European goat breeds [6, 44, 50]. In 2 samples out of 25, there was a mass attributed to κ-cn G found in Italian and Turkish breeds [39, 50]. The major form of κ-Cn was the 2P form, although the 1P form was also evident, as previously reported [35, 38, 48].

3.5. Whey proteins

The ratio β-Lg/α-La was 1.58 ± 0.33 and in the majority of samples (49 out of 57) it

ranged from 1.1 to 1.9. The α-La mass detected by RP-HPLC/ESI-MS analyses was 14 198 ± 1, which corresponds to the theoretical mass of goat α-La (14 192.2). The mass 18 197 ± 1 detected in the β-Lg peaks of the milks of the present study corresponds to the theoretical mass of this goat protein (18 191.3). In some samples a mass of 18 523 ± 1 was detected in the front part of the β-Lg peak that is consistent with a covalent linkage of lactosyl residue to the protein [24, 48]. No protein variants were observed, in accordance with the previous works that report only silent alleles for goat α-La [11] and β-Lg loci [2, 19, 36, 49].

4. CONCLUSION

The goat's milk of the autochthonous Skopelos breed had a higher total protein content and a higher α_{s1}-cn content compared with the milk of international breeds. This characteristic was consistent with predominance of the strong α_{s1}-cn variants B3, B4 and As/B1. The most frequent α_{s2}-cn variant was by far variant A followed by variants C and F, whereas compositional data showed the existence of a null allele. The detected β-cn forms were variants A and C in similar frequencies. Variant D (formerly B) of κ-cn predominated and the rare variant G was found.

Therefore, according to the findings of the present study, the milk of the Skopelos breed is a "strong" milk suitable for cheesemaking, although further studies with respect to micelle characteristics are needed. Furthermore, it can be concluded that the RP-HPLC analysis of defatted milk can be a simple and fast way to study with reasonable certainty the complex characteristics of the goat protein fraction by taking into consideration the RT and the quantity of the peaks.

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