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## EFFECT OF $\beta$ -LACTOGLOBULIN GENE POLYMORPHISM, LACTATION STAGE AND BREED ON MILK TRAITS IN CHIOS AND KARAGOUNIKO SHEEP BREEDS

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### Abstract

The animal selection with favourable phenotypes of the past has been, currently, replaced by the genotype selection on quantitative traits, assisted by the expanding molecular techniques in the context of livestock improvement. In this study, the c.112T>C polymorphism in exon II of  $\beta$ -lactoglobulin ( $\beta$ -LG) gene was investigated in Karagouniko and Chios sheep breeds by using polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP), and possible associations with milk traits were examined. In total, 125 blood DNA samples were isolated for PCR-RFLP analysis and the respective 217 milk samples' composition profile was obtained. The goodness of fit test to Hardy-Weinberg equilibrium (HWE) for  $\beta$ -LG genotypes was estimated and associations found between  $\beta$ -LG genotypes and raw milk composition. Two alleles and three genotypes were observed (AA, AB and BB) in both breeds, and Chios breed significantly deviated ( $P \leq 0.05$ ) from Hardy-Weinberg equilibrium (HWE). Conclusively, linear mixed model analysis on samples, from both breeds collectively, showed significant effects of  $\beta$ -LG genotype on lactose percentage and somatic cell count (SCC), lactation stage on daily milk yield and protein, while the breed effect was significant only on daily milk yield.

**Key words:** sheep,  $\beta$ -lactoglobulin, polymorphism, lactation, breed

The milk industry has strong interest in the raw milk quality, which is a pre-requisite for dairy products, especially from sheep that are almost exclusively used

in yogurt- and cheese-making. Genotype selection on quantitative traits, under livestock improvement, has advantaged sheep breeders in animal selection plans. Genome-wide association studies in animal breeding, due to its polygenic nature has attracted a lot of attention in the recent years, greatly improving the phenotype predictability and becoming the landmark in animal science research (Wu et al., 2014).

$\beta$ -lactoglobulin is one of the most important whey proteins in sheep milk which, in the latter, it features a unique protein fraction profile (Moatsou et al., 2005). Moreover,  $\beta$ -lactoglobulin protein content depends on breed and lactation stage, in sheep and goat (Hejtmánková et al., 2012). Polymorphic genes, such as  $\beta$ -LG, were proven to be associated with traits of interest in sheep milk, therefore, similar genes can comprise entities of detectable molecular markers in a current selection method for milk traits. The genetic polymorphism of  $\beta$ -LG gene was initially found at the protein level in cattle (Aschaffenburg and Drewry, 1955), and later was examined in sheep breeds. The examined polymorphic site has shown a DNA transition (X12817.1:c.112T>C) and three polymorphic genetic variants (A, B, and C) have been described, while C allele has been shown to be rare, originated and mainly detected in Spanish Merinos (Erhardt, 1989; Recio et al., 1997). This transition comprises of a Tyr/His substitution in position +20 of the protein (Kolde and Braunitzer, 1983), where the  $\beta$ -LG variant A has Tyr (codon TAC) and variant B has His (codon CAC) (Gaye et al., 1986). After the detection of polymorphism in sheep, subsequent studies have demonstrated the  $\beta$ -LG gene polymorphic effect on milk yield, protein, fat, lactose content etc. (Kusza et al., 2015) as well as the impact of protein genetic variants on technological properties of milk (Selvaggi et al., 2014). The association of genetic polymorphisms with milk traits is especially important for rare indigenous sheep breeds. There is a lack of genetic information for important indigenous sheep breeds around the world, i.e. allele and genotype frequencies, the goodness of fit to HWE, and importantly, and there are unexplored associations between genetic polymorphisms in sheep genome with the milk traits.

In our study, Karagouniko and Chios sheep breeds were employed, as they are considered two of the most popular indigenous sheep breeds in Greece. Karagouniko breed is multinumbered in many areas, especially in North-Western Greece. Also Chios breed is considered as one of the highest milk producers, although animal numbers of Chios breed have declined recently and the breed is currently under protection (Natura project; <http://www.agrobiodiversity.net/greece/>). Recently, Chios sheep breed has been recruited as an important breed from the Mediterranean basin, for sheep genomic representation in the International Sheep Genomics Consortium (ISGC), which is committed to sequence the sheep genome, improve and annotate SNP haplotype and detailed sheep physical maps etc. (Heaton et al., 2014; Mastrangelo et al., 2014).

Thus, the purposes of our study were, to focus on Chios and Karagouniko dairy sheep breeds, estimate allele and genotype frequencies as well as the goodness of fit to HWE. Furthermore, the present study investigates possible associations related to the above polymorphism, in conjunction with the milk traits, which will improve our knowledge in relation to the genetic potential of indigenous sheep breeds, around the

world. Furthermore, it was also our immediate concern to investigate the breed as well as the lactation stage effect in these sheep breeds, in order to assess the significance of either of these effects on milk yield and quality traits.

## **Material and methods**

### **Blood DNA and milk samples**

For this study, blood samples were collected from 125 ewes of Chios ( $n=59$ ) and Karagouniko ( $n=66$ ) breeds at their third or fourth lactation, from the farm of the experimental station of the Agricultural University of Athens. The animals were fed under a system of indoor nutrition consisting of a standard concentrate and forage. Blood was taken from jugular vein and genomic DNA was extracted from leucocytes according to an in-house adapted protocol given by Miller et al. (1988). The genomic DNA was also checked for purity, integrity and concentration under a spectrophotometer and by agarose gel electrophoresis.

The analysis for the milk traits was based on 217 milk samples in total from Chios and Karagouniko breeds. Milking was performed twice daily with a milking machine, under hygienic conditions and procedures attached to the animal welfare by the ethical committee of our Institution and the guidelines of the Research Ethics Committee of the Department of Animal Science and Aquaculture of the Agricultural University of Athens. The milk samples were taken for the analysis of chemical composition and rheological values, by combining the afternoon sampling of the previous day (04:00 pm) with the morning sampling (06:00 am) in one pool. For the analysis of chemical composition and rheological properties, the two milk aliquots were cooled and transported to the dairy laboratory; the first was accounted for the rheological properties and the second for the chemical composition. Each animal provided at least a milk sample and all the combined samples, as above, were divided in two aliquots for further analysis. There were 2–7 consecutive samplings for each animal during the lactation period from both breeds, except two of the ewes that have contributed with one sample, and twelve ewes contributed with 7 samples, during lactation. The number of samples from all animals were, 21, 16, 26, 32, 40, 43 and 39 corresponding to the 1st, 2nd, 3rd, 4th, 5th, 6th and 7th sampling, respectively, whose sum equals to 217 total milk samples.

Project procedures attached to the animal welfare were approved by the ethical committee of our Institution and the guidelines of the Research Ethics Committee of the Faculty of Animal Science and Aquaculture of the Agricultural University of Athens.

### **Primer design and PCR-RFLP procedure**

$\beta$ -LG gene genotypes were identified as previously reported (Feligini et al., 1998) by PCR. The PCR amplification of the exon II of  $\beta$ -LG gene in sheep (GenBank Acc. No: X12817.1), i.e. DNA fragment of 120 bp was amplified by the primer set: forward primer, 5'-CAACTCAAGGTCCCTCTCCA-3' and reverse primer,

5'-CTTCAGCTCCTCCACGTACA-3', on an ABI Thermal Cycler (Life Technologies, Cambridge, England). The PCR program routine was: denaturation at 95°C for 5 min at start, followed by 36 cycles subroutine at 95°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min. The RFLP included an enzyme cut, of the PCR products (20 µL) using 10 units of *RsaI* restriction enzyme in a 25 µL total reaction volume, for 2½ h at 37°C. The restriction fragments were directly analysed by 3% agarose gel electrophoresis, and visualized under UV light. Two alleles were found, i.e. the A allele yielded three bands of 64, 39, and 18 bp, the B allele yielded two bands of 102 and 18 bp, while the heterozygote group AB, had all the four fragments (i.e. 103, 64, 39, and 18 bp).

### Determination of chemical composition, SCC and rheological traits

Milk samples were analysed for fat, protein, lactose, solids-not-fat (SNF) and total solids (TS) by the infrared method using a Milkoscan 133 (Foss Electric, Hillerød, Denmark) calibrated against the Mojonniér method for fat analysis, Kjeldahl method for protein, and the polarimetric method for lactose according to Association of Official Analytical Chemists (AOAC) official methods (AOAC, 1990). SCC were determined with a Fossomatic cell counter (Foss Electric). Milk rheological properties were expressed by three parameters, i.e.:  $R_{CT}$  (rennet coagulation time in min),  $K_{20}$  (curd firming time in min) and  $A_{30}$  (curd firmness in mm), while the coagulation process run in 30 min for each milk sample. In more detail, each milk sample (10 ml) was maintained at 35°C for 30 min before mixing with 200 µl of rennet enzyme 1.6% (vol/vol) in H<sub>2</sub>O, and upon enzyme addition, was immediately analysed at 35°C.  $R_{CT}$ ,  $K_{20}$  and  $A_{30}$  were obtained by the Formagraph's (Foss Electric, Hillerød, Denmark) graphic representation using FormaWin32 software.

### Statistical analysis

The genotype and allele frequencies were counted directly in each sheep population. The chi-square test was performed to test the goodness of fit to HWE, for the  $\beta$ -LG genotypes. In order to evaluate the main effects of breed, genotype and lactation stage (fixed effect factors) and their interactions on the milk traits, we used restricted maximum likelihood (REML) method in a linear mixed model (SPSS Statistics for Windows, Version 13.0) for repeated measurements per subject (ewe) with the autoregressive order one [AR(1)] to modelling the covariance structure of the residuals and individual ewe sampling as the repeat measure factor. The animals after genotyping, were categorized in 3 genotype groups for both breeds, i.e. AA (n=30), AB (n=66) and BB (n=29) genotypes, two lactation stage groups, i.e. the early lactation A (27–100 d), n=112, and late lactation B (101–200 d), n=105 and breed. Also, the collected total milk samples corresponding to the 3 genotypes, were 92 in Karagouniko breed and 125 in Chios breed. Overall total milk samples for both breeds = 217.

The animals were pooled from both breeds and statistical analysis of the animal effect as one population was applied. All groups tested for normality (D'Agostino and Pearson) and data transformed when necessary (e.g. log<sub>10</sub> cells) to meet model assumptions.

## Results

### Genotype and allele frequencies

Two alleles (A and B) of  $\beta$ -LG gene (Figure 1) were detected by PCR-RFLP, in the two breeds (Table 1). Nevertheless, C allele was not detected in the present or in a previous study for these breeds (Kranis, unpublished data).

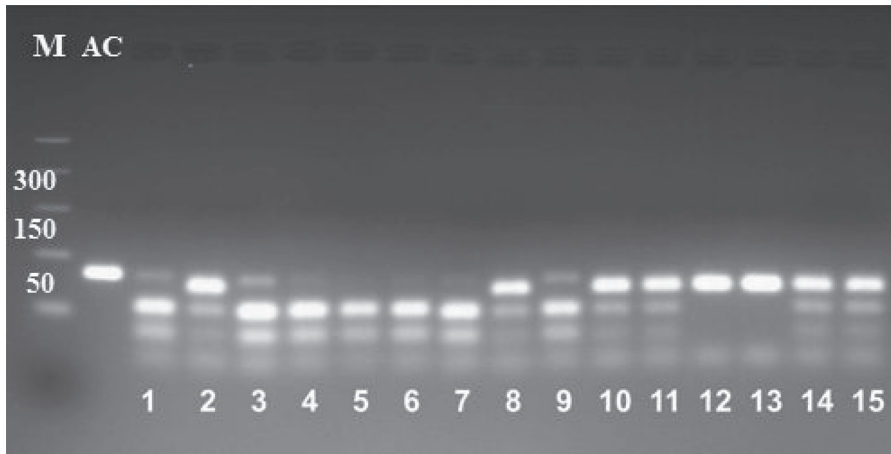


Figure 1. PCR-RFLP analysis results from the amplified PCR products of the 120 bp exonic II fragment of the ovine BLG gene, digested with *RsaI* enzyme and run on a 3% agarose gel. M=DNA markers, numbers indicates size in base pairs, AC= uncut PCR product. BLG genotypes in lanes 1, 3, 4, 5, 6, 7 and 9: genotype AA, lanes 2, 8, 10, 11, 14 and 15: genotype AB, lanes 12 and 13: genotype BB

Table 1. Distribution of  $\beta$ -LG genotype numbers, observed genotype frequencies and allele frequencies in Karagouniko and Chios sheep breeds

Sheep breed	N	Genotype numbers			Observed genotype frequencies			Allele frequencies		$\chi^2$
		AA	AB	BB	AA	AB	BB	A	B	
Karagouniko	59	22	24	13	0.373	0.407	0.220	0.576	0.424	1.640ns
Chios	66	8	42	16	0.121	0.636	0.243	0.439	0.561	5.590 *

$\chi^2$  – goodness to fit test to HWE. ns – non significant, \* – significant ( $P \leq 0.05$ ), significant differences between observed and expected allele frequencies.

In the present study, B and A alleles were detected with greater frequencies in Chios and Karagouniko breeds, respectively. The  $X^2$  analysis for the goodness of fit to HWE for the  $\beta$ -LG genotypes showed a significant deviation ( $P \leq 0.05$ ) in Chios breed, while that was not the case for Karagouniko breed (Table 1). The genotype frequencies for heterozygotes were greater in both breeds, while homozygotes varied

between the breeds, i.e.  $AB > AA > BB$  in Karagouniko, and  $AB > BB > AA$  in Chios breed. As a plausible inference we can conclude that deviation from HWE in Chios breed was caused by the large number of heterozygotes ( $AB$ ), among the other genotype groups (Table 1). Importantly, heterozygote frequency for Karagouniko breed, did not deviate significantly from HWE ( $P > 0.05$ ), while allele frequencies for the alleles A and B, had opposite trend in these breeds.

### Effect of $\beta$ -LG genotype on milk traits, composition and rheological properties

In this study the associations between  $\beta$ -LG genotype and daily milk yield, composition and quality traits were examined as shown in Table 2. From the mixed effect model analysis in the pooled animals from both breeds, no significant differences were found between genotypes in milk composition and rheological properties, but only in lactose percentage and SSC number. Particularly, we observed greater lactose percentage for genotype  $AB$  compared to genotype  $AA$ , but not to genotype  $BB$ . We also found a greater SCC number for genotype  $AA$  compared to genotype  $AB$ , but not to genotype  $BB$ . The other milk traits remained unaffected and no significant differences were observed. Interestingly,  $\beta$ -LG genotype  $AB$  was associated with the highest milk yield, although no significant differences were observed between the genotype groups (Table 2). Also,  $\beta$ -LG genotype  $AA$  was associated with the highest fat, protein percentage and renneting properties ( $r_{ct}$ ,  $A_{30}$ ), although no significant differences were observed between the genotype groups (Table 2). Conclusively, associations were found between  $\beta$ -LG polymorphism and lactose percentage and SSC number and no associations with milk yield or other composition and renneting properties of milk.

Table 2. Genotype effect of  $\beta$ -lactoglobulin on milk traits in both Karagouniko and Chios sheep breeds

Milk trait	Genotypes of $\beta$ -lactoglobulin <sup>1</sup>							Level of significance
	$\beta$ -LG	AA	$\beta$ -LG	AB	$\beta$ -LG	BB	P-value	
	mean	SEM	mean	SEM	mean	SEM		
Daily milk yield (mL)	904.90	201.65	1,058.34	84.26	770.11	115.81	0.139	ns
SCC (log10 cells.mL <sup>-1</sup> )	3.02 a	0.24	2.32 b	0.10	2.36 ab	0.13	0.03	*
Fat (%)	6.21	0.34	6.04	0.16	5.69	0.19	0.236	ns
Protein (%)	5.71	0.26	5.63	0.10	5.48	0.14	0.633	ns
Fat/Protein ratio	1.09	0.05	1.08	0.02	1.05	0.03	0.552	ns
Lactose (%)	4.96 a	0.17	5.42 b	0.07	5.32 ab	0.10	0.053	*
Solids non-fat (%)	11.77	0.97	12.29	0.33	11.69	0.48	0.567	ns
Total solids (%)	17.79	0.53	17.93	0.21	17.27	0.29	0.20	ns
R <sub>CT</sub> (min)	13.10	1.50	12.46	0.56	13.17	0.82	0.752	ns
K <sub>20</sub> (min)	2.69	0.71	2.26	0.27	2.23	0.39	0.836	ns
A <sub>30</sub> (mm)	36.8	2.46	38.68	0.83	38.06	1.23	0.742	ns

The least squares means  $\pm$  SEM are shown for both Karagouniko (N=92) and Chios breeds (N=125).

a, b – means within a row with different letters differ at  $P \leq 0.05$ .

<sup>1</sup>Milk samples of genotyped AA animals, N = 17, AB animals, N = 134 and BB animals, N = 66 (see Material and methods), ns – non significant.

### Effect of lactation stage on milk traits

From the results of the statistical analysis that were accounted for the lactation stage, significant differences emerged between lactation stage A and lactation stage B. In more detail, there was a prominent effect of the stage of lactation, in the pooled animals from both breeds, on milk yield and protein percentage, while the other milk composition and quality traits remained unaffected with no significant differences, as shown in Table 3. Notably, as shown from the analysis, there were no significant differences in protein and fat percentage or other quality traits ( $P>0.05$ ) (Table 3). Nevertheless, it was a trend for higher levels in solids-not-fat, total solids and renneting properties ( $R_{CT}$ ,  $A_{30}$ ) at lactation stage B compared to lactation stage A, although without significant differences (Table 3).

Table 3. Effect of lactation stage on milk traits in both Karagouniko and Chios sheep breeds

Milk trait	Stage of lactation <sup>1</sup>				P-value	Level of significance
	A		B			
	N = 112	SEM	N = 105	SEM		
	mean		mean			
Daily milk yield (mL)	1,148.29 a	83.86	673.95 b	88.50	0.000	***
SCC (log10 cells.mL <sup>-1</sup> )	2.56	0.10	2.58	0.11	0.802	ns
Fat (%)	6.00	0.14	5.96	0.16	0.743	ns
Protein (%)	5.52 a	0.11	5.69 b	0.12	0.044	*
Fat/Protein ratio	1.09	0.02	1.06	0.02	0.076	ns
Lactose (%)	5.26	0.07	5.20	0.08	0.153	ns
Solids non-fat (%)	11.49	0.42	12.35	0.49	0.101	ns
Total solids (%)	17.60	0.22	17.72	0.25	0.511	ns
R <sub>CTP</sub> (min)	12.63	0.64	13.20	0.75	0.415	ns
K <sub>20</sub> (min)	2.44	0.30	2.34	0.34	0.726	ns
A <sub>30</sub> (mm)	37.46	1.06	38.24	1.23	0.538	ns

The least squares means  $\pm$  SEM are shown for both, Karagouniko and Chios breed.

a-d – means within a row with different small letter differ. \* – significant ( $P\leq0.05$ ).

\*\*\* – significant ( $P\leq0.001$ ). <sup>1</sup>Stage of Lactation: A – (27 to 100 days post partum).

B – (101 to 200 days post partum), ns – non significant.

### Breed effect on milk traits

The mean differences between Karagouniko and Chios breeds on milk yield and composition traits are shown in Table 4. The breed effect on daily milk yield, was significant ( $P<0.05$ ), while fat, protein, fat:protein ratio, total solids  $R_{CT}$ ,  $K_{20}$  and  $A_{30}$ , were not significantly different between Karagouniko and Chios breeds (Table 4). In more detail, the significant difference mainly affected the daily milk yield between Chios and Karagouniko breeds, i.e.  $1,065.51\pm108.92$  vs.  $803.35\pm98.69$  mL, respectively. The latter difference attributes superiority to Chios breed compared to Karagouniko breed, in relation to daily milk yield.

Also, significant interaction was found between breed and genotype or lactation stage, under the mixed model analysis. Specifically, two-way breed\*genotype interactions were found significant in fat percentage; for example between Karagouniko breed of genotype *BB* and Chios breed genotype *AA*, at lactation stage A ( $P\leq0.05$ ),



as well as lactation stage B ( $P \leq 0.05$ ) (data not shown). Nevertheless, the breed effect alone, was not found significant in fat percentage (Table 4). Furthermore, two additional two-way breed interactions were also found significant ( $P \leq 0.05$ ), i.e. breed\* $\beta$ -LG genotype *AA* and breed\*lactation stage A in protein percentage. In more detail breed\*genotype interactions were found significant for Karagouniko breed of genotype *BB* ( $P=0.008$ ) and Karagouniko breed at lactation stage A ( $P=0.005$ ) in protein percentage, with lower values compared to Chios breed, while no differences were found in protein percentage at the stage of lactation B ( $P > 0.05$ ) (data not shown). Unimportant differences were observed in any other source of variance.

Table 4. Breed effect between Karagouniko and Chios breeds on milk traits

Milk trait	Breed			SEM	P-value	Level of significance
	Karagouniko	SEM	Chios			
	mean		mean			
Daily milk yield (mL)	803.35	98.69	1,065.51	108.92	0.049	*
SCC (log10 cells.mL <sup>-1</sup> )	2.65	0.12	2.50	0.13	0.340	ns
Fat (%)	6.06	0.16	5.99	0.17	0.725	ns
Protein (%)	5.69	0.13	5.46	0.13	0.163	ns
Fat/Protein ratio	1.05	0.02	1.09	0.02	0.222	ns
Lactose (%)	5.19	0.08	5.26	0.09	0.517	ns
Solids non-fat (%)	11.81	0.46	11.97	0.49	0.779	ns
Total solids (%)	17.88	0.25	17.48	0.28	0.232	ns
R <sub>CT</sub> (min)	13.603	0.74	12.23	0.80	0.154	ns
K <sub>20</sub> (min)	2.47	0.35	2.13	0.38	0.466	ns
A <sub>30</sub> (mm)	36.92	1.15	38.84	1.24	0.172	ns

The least squares means  $\pm$  SEM are shown for Karagouniko (N=92) and Chios breeds (N=125). Means within a row differ. \* – significant ( $P \leq 0.05$ ) or ns – non significant.

Conclusively, the breed effect results show that Chios breed has higher daily milk yield compared to Karagouniko breed during the 6-month lactation period of the study, while the remaining milk quality traits were not significantly different between the two breeds.

## Discussion

Indigenous sheep breeds are of great economic importance in dairy industry contributing to a high percentage in cheese making and a large variety of dairy products, especially those named as “protected designation of origin” (PDO). The work hypothesis that was put forward in this investigation regarding the genotype effect of  $\beta$ -LG gene, lactation stage and breed on the milk traits has been challenged by the limited sample size, although the profound effect of some of the factors examined here, was enhanced by the mixed model analysis of pooled animals’ data from both breeds. As a prominent example of the latter is the lactation stage and its profound effects on milk traits, supported by previous reports (Amigo et al., 2000; Dario et al.,



2008; Kawecka and Radko, 2011; Rozbicka-Wieczorek et al., 2015). Moreover, our results for the allele and genotype structure of the studied populations, as reported in the literature, show high variability in allele and genotype frequencies of  $\beta$ -LG gene (Selvaggi et al., 2015). This high allele variability has been studied around the world in various breeds, such as the monomorphic population of breeds from Saudi Arabia for either of the alleles as well as intermediate frequencies, such as the Friesian breed, although the list is not exhaustive (Kawecka and Radko, 2011; Selvaggi et al., 2015). Similar monomorphic populations for  $\beta$ -LG gene have been also observed in a previous study from a Synthetic breed created from indigenous North-Western Greek breeds in our lab using an upgrade breeding scheme (crossbred 50% Boutsiko breed  $\times$  25% Chios breed  $\times$  25% Arta breed) (data not shown).

In the present study the estimated allele and genotype frequencies were different between Chios and Karagouniko breeds. In more detail, heterozygotes were the highest frequencies from both breeds, while homozygote allele frequencies showed the opposite in the frequencies between these two breeds. The highest frequency in allele A was detected in Karagouniko breed, which has been also shown for another breed (Boutsiko breed), in our experimental farm (data not shown). Interestingly, in a previous study in Chios breed (Kranis, unpublished data) as well as the current study, we have observed high allele B and genotype *AB* frequency and deviation from HWE (see results section and Table 1). Moreover, the greater frequency of genotype *AB* has been reported in breeds such as Lacaune and Tsigaja, which we have also observed in Karagouniko and Chios breeds. According to Kawecka and Radko (2011) and Rozbicka-Wieczorek et al. (2015), the high frequency of genotype *AB* has been also observed in British dairy sheep, Hungarian Merinos, Racka and Polish Lowland breeds, whereas the genotype *AB* and allele B were predominant. On the contrary, the frequency of A allele is dominant in different sheep breeds (Amigo et al., 2000; Dario et al., 2008) and greater allele A frequency was observed in Karagouniko breed, which has been also reported in Racka and East Friesian sheep (Kawecka and Radko, 2011), and greater B allele frequencies, similar to our observations in Chios breed, have been reported in Awassi and other breeds (Arora et al., 2010; Kusza et al., 2015). The latter prevalence of B allele is mainly encountered in sheep breeds from India, with its ancestral origin (Selvaggi et al., 2015). No evidence was found of C allele in our study, which is considered a rare variant detected only in few breeds such as Merinoland, Lacha, Carranzana, Spanish Merino, Serra da Estrela, White and Black Merino, originated from the Spanish Merinos (Selvaggi et al., 2015).

In a population that is evolving over time, the effect of selection, favouring one allele could be the cause of allele frequency changes, apart from the random effects, genetic drift or migration. Previous studies (Dario et al., 2008) have examined possible associations between genetic polymorphism and milk properties, for breeding purposes. Although the number of animals in the current study was not that large, our results were consistent with previous genotyping study for the same polymorphism in Chios breed and larger sample size ( $n=160$ ) (Kranis, unpublished data), which has shown significant deviation from HWE ( $P \leq 0.05$ ). Also consistent was our finding for the association between genotype and milk traits, and particularly genotype *AB* with higher lactose percentage (Yousefi et al., 2013), although no genotype associa-

tion was found on daily milk yield or other milk traits and rheological properties, in contrast to other reports (Amigo et al., 2000; Corral et al., 2010). We have also observed a different trend where  $\beta$ -LG genotype *AB* was associated with the highest milk yield than the reported genotype *BB*, although not significantly different between the genotype groups. The  $\beta$ -LG genotype factor did not affect daily milk yield in the pooled animals from both breeds (Table 2) or from the analysis within breed (data not shown), although differences were found for lactose percentage and SCC between  $\beta$ -LG genotype *AA* and genotype *AB* but not genotype *BB* (refer to Table 2). The latter result concerning lactose percentage was consistent with the finding of Yousefi et al. (2013) and the predominance of genotype *AB*. Also, the observed trend in daily milk yield favoured by genotype *AA* or *AB*, which was in agreement with the findings in Sarda breed or East Friesian sheep (Selvaggi et al., 2015).

Furthermore, Dario et al. (2008), found strong associations between genotype *AB* with fat and protein content while other researchers failed to find significant associations (Kawecka and Radko, 2011). Nevertheless, it was a downward trend in milk quality values towards the genotype groups: *AA*, *AB* and *BB*, for the percentage of fat, protein, fat:protein ratio and  $K_{20}$  (min). The latter results mean a superiority of genotype *AA*, compared to *AB*, compared to *BB*, for most of the quality traits from both breeds, apart from the curd firming time ( $K_{20}$ ), which is negatively influencing cheese-making properties. These results could also be explained by the influence of environmental factors, the breed or possibly the small sample size of the examined populations.

From our analysis data within breed, we have also found that homozygotes *AA* in Chios breed have higher milk yield compared to heterozygotes *AB* or homozygotes *BB*, for fat, protein and total solids (TS) percentage of milk; also TS percentage was found significantly greater in genotype *AB* of Karagouniko breed (data not shown), which is consistent with the finding of Recio et al. (1997) but inconsistent with the results of Amigo et al. (2000). This putative relationship could be possibly explained by the high protein percentage, which is associated with the genotype *AA*, mainly because of the high casein content and the relative amounts of kappa- and beta-casein in genotype *AA* animals (Amigo et al., 2000). This is not observed in our results and leads to the hypothesis that casein content in genotype *AA* animals of Karagouniko and Chios breeds may not be as high as in other breeds (Amigo et al., 2000), to positively affecting rheological properties ( $R_{CT}$ ,  $A_{30}$ ), despite the high protein percentage as described in the results' section. Furthermore, the above hypothesis predicts a superiority of such a type of milk, with significantly high protein percentage from Chios breed from genotype *AA* ewes, for cheese processing, because of its clotting and curd firming time (Selvaggi et al., 2014).

Due to the small number of animals in such a pilot study, further validation of the results is necessary with larger sample size to confirm these associations and their significance on the milk traits under such analysis. The latter will restrict higher confounding risk and false positive results, although strong and robust trends were shown in these results by the multiple statistical analysis. Therefore, we could assume that there is yet no clear association between the *A* or *B* allele presence with milk yield or certain milk traits in Karagouniko and Chios sheep breeds.

As far as the prominent effect of the stage of lactation is concerned, we found significantly greater levels in late lactation (stage B), for protein and total solids percentage in Chios breed, which has been also observed in East Friesian ewes (Kuchtik et al., 2008). The observed significant protein percentage rise towards the lactation stage B, with a concurrent decline in fat percentage, however insignificant could be, is explained by the action of known loci in cattle affecting milk fat production, such as *agpat6* gene (Bionaz and Loor, 2008). These loci expressed in the mammary gland, have shown a tendency to increase over the first 60 days of lactation followed by a decrease afterwards (Bionaz and Loor, 2008). Although the latter tendency was noticeable in our results from the effect of the stage of lactation, fat percentage decrease was not significant in both Chios and Karagouniko breeds, unlike the significant protein increase mentioned earlier. Notably, as shown from the analysis of pooled animals from both breeds for the lactation stage effect, there were no significant differences in other traits apart from the daily milk yield and protein percentage, with the latter being consistent with the finding of Kuchtik et al. (2008), who also found significant effect on the contents of all milk components. The latter limited similarity could be due to the low numbers of studied individuals of Karagouniko and Chios breeds, as statistical analysis of our data exposes only striking differences among the traits, the breed influence on lactation stage pertinent to milk components (Kuchtik et al., 2008), environmental factors and finally the number of days post partum consisting of the lactation stage groups in the analysis.

In the study, we assume that the observed differences, however insignificant in fat and protein content between the two breeds, are caused by the combinatorial effect between breed and environment, and remain stable within breed during lactation, as reported recently for Tsigai and Improved Valachian ewes (Orancova et al., 2015). Also, the breed effect did not affect the studied milk traits, considering the pooled animals from both breeds, but only the daily milk yield. Similar observations for the genotype effect of  $\beta$ -LG variants have been reported previously in non-dairy sheep breeds, which did not affect the principal milk ingredients but only the total protein level and the components of protein fraction of milk (Rozbicka-Wieczorek et al., 2015). In comparison with previously reported values from within breed analysis (data not shown), the fat percentage mean in Karagouniko breed was lower, and Chios percentage mean was higher than in Spanca or Turcana breeds (Mierlita, 2011), respectively, while fat percentage in Chios breed was close to the Awassi breed, and Karagouniko breed was close to East-Friesian sheep breed (McKusick et al., 2001). An interpretation of the breed effect results makes likely the fact that inherent breed differences could be intensified by the combined effect of  $\beta$ -LG genotype and breed, favouring a trait (e.g. milk yield, fat etc.). This is an important observation, which is in agreement with previous reports that  $\beta$ -LG genotype *AA* is associated with a higher content in TS, fat, protein, casein and basic components of milk, in Massesse, Manchega and Merino breeds (Amigo et al., 2000; Corral et al., 2010).

Summarising the work, the higher genotype frequency for genotype *AB* was determined in both breeds, while Chios breed significantly deviated from HWE. The  $X^2$  analysis revealed opposing allele frequencies in these breeds, with higher allele A frequency in Karagouniko and higher allele B frequency in Chios breed, showing

different selective pressure. The  $\beta$ -LG genotypes seem to have insignificant effect on most of the milk traits and yield, when the statistical analysis was applied on the pooled animals from both breeds. Thus, our data highlight the genotype influence on milk composition that is only limited on lactose percentage and SCC, but not significantly affecting other milk components or daily milk yield. Judging from the above open routes of selection for the breeder, at least for milk yield, it could be mainly the choice of early lactation stage and breed. The latter fixed effects were emphasized by the important interactions of either genotype or lactation stage effect with the breed, which have to be examined with caution as further analysis with higher number of individuals is necessary. Furthermore, statistical analysis within breed has shown that, for the studied breeds the genotype does not affect milk yield within breed as much as the lactation stage, however it was important for the milk composition traits, such as fat and protein percentage, which also affects cheese-making properties. It becomes obvious that early stages of lactation are more important than late, as shown for daily milk yield and other traits (fat, protein etc.), in both of our analyses in these breeds, but is also important for the technological properties of milk for the farmers and breeders (Johnson et al., 2001); although, many of the milk component values are stabilized at the end of lactation.

Further experimental work is due to include larger sample size for the above sheep breeds as well as detailed lipid profile and protein analysis that will decipher the marker potential of  $\beta$ -LG variants to a detailed screening of important milk ingredients. Considering that milk yield and basic milk components' information, such as fat, protein, renneting properties etc. especially for indigenous breeds is very scarce and in high demand, similar pilot studies, like the current one, can highlight the genetic potential of unknown sheep breeds for further investigation. Because of the significance of these components on technological properties of milk and cheese (Johnson et al., 2001), the effect of the breed and genotype information for selection purposes may be useful to improving milk yield and quality. For the above investigation goal, last but not least PCR-RFLP albeit not new technique, it has offered a great deal in genotype screening, especially for small scale genotyping efforts, predicting the genetic potential of sheep breeds. This will be beneficial not only for sheep breeders but will be also supporting incomplete gene mapping projects across species, which are under way, such as in goat, focusing on production traits and gene function.

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