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EFFECT OF ABCG2 GENE POLYMORPHISM ON MILK PRODUCTIVITY IN AWASSI EWES

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Abstract

The ABCG2 gene is responsible for transporting many molecules across cell membranes and is expressed in various tissues, including the mammary gland. In this regard, the ABCG2 gene has been considered as a candidate gene related to the quantity, composition and quality of milk yield in sheep. The purpuse of present study was to establish the genetic diversity in this gene in connection with the study of possible dependencies of genotypes in ABCG2 with milk production in the ewes of the Awassi breed reared in Bulgaria. A highly polymorphic state of the ABCG2 gene was found, with the presence of two alleles - wild "+" and mutant "-" with frequencies of 0.49 and 0.51, respectively, and three genotypes - homozygous +/+ (0.31), heterozygous +/- (0.36) and homozygous -/- (0.33). Regarding milk productivity, no statistically significant differences were found between the different genotypes of the ABCG2 gene.

Key words*: Awassi sheep, ABCG2 gene, PCR-RFLP, genetic polymorphism, milk productivity.*

INTRODUCTION

Sheep farming is a potentially profitable agricultural activity in areas unsuitable for other agricultural production. Sheep milk, which is more suitable for processing than cow and goat milk, is obtained predominantly from low- to medium-yielding local breeds raised under extensive conditions for meat, milk, wool and hides (Gutiérrez-Gil et al., 2014), but also from specialized dairy breeds. Globally, the demand for sheep milk and its products, such as cheese and yogurt, is influenced by consumer preferences for traditional and specialty dairy products. As the world population continues to grow, the consumption of a variety of dairy products is expected to increase, highlighting the importance of sustainable development in the sheep milk sector. To meet growing consumer demands, it is necessary to increase sheep milk yield and improve the efficiency of sheep farming practices (Garzón et al., 2023; Ceyhan et al., 2022).

The Food and Agriculture Organization of the United Nations (FAO) forecasts that world milk production will reach approximately 979 million tons in 2024, reflecting an increase of 1.4% compared to 2023. Milk production takes place in all EU countries and represents a significant share of the value of EU agricultural output. Total EU milk production is estimated at around 155 million tons per year according to Eurostat. While cow's milk dominates the market, accounting for around 81% of total production, sheep's milk, together with goat's and camel's milk, contributes around 4%. This suggests that although sheep's milk represents a smaller share of the global dairy sector, it remains an important and specialised component of the industry. Most sheep's milk production is concentrated in certain regions, particularly in the Mediterranean countries and the Balkans, where sheep farming has deep-rooted cultural and economic importance. In the European Union, countries such as Spain, Greece, France and Italy are notable producers of sheep milk, with Spain producing approximately 1.1 million tons and Greece 0.9 million tons from sheep and goats combined in 2023 (Mioč et al., 2024; FAO, 2024).

In Bulgaria, sheep milk production has remained relatively stable in recent years. According to data from the Ministry of Agriculture and Food, in 2022, sheep milk production in Bulgaria amounted to 54.742 million litres, which represents a significant decrease of 19.5% compared to 2021. The dairy industry in Bulgaria is experiencing structural changes and market consolidation, which contributed to increasing

efficiency in dairy farming and milk processing. In 2023, a total of 50,255 thousand litres of raw milk were produced in Bulgaria from 901,200 ewes. As of November 1, 2023, the total number of sheep in the country was 1,072,768 - 2.2% below the level of a year ago (Agricultural Report, 2024).

Genomic markers for genetic variation allow selective intervention in breeding and early assessment with high accuracy, so that good production traits in sheep can be better transmitted to their offspring (Hayes et al., 2013; Wang et al., 2023). In recent years, various candidate genes related to yield and milk production qualities have been tested, such as DGAT1, β-lactoglobulin, prolactin, ATP-binding cassette subfamily G member 2 (ABCG2) (Gutiérrez-Gil et al., 2014; Xu & Li, 2017; Singh et al., 2019).

ABCG2 is an ABC transporter, a member of the ATP-binding cassette (ABC) superfamily, which is thought to be important for protection against xenobiotics (chemical compounds that do not belong to the natural composition of living organisms) (Robey et al., 2009). ABCG2 also has an important function in the development and differentiation of the mammary gland (Alim et al., 2013). The gene encoding the ABCG2 protein is located on chromosome 6 of the ovine genome, is composed of 20 exons separated by introns, and is expressed in several tissues, including the mammary gland (Al-Mamun et al., 2015).

ABCG2 is thought to be associated with milk production traits in sheep (Árnyasi et al., 2013), for example in the Manech Tête Rousse, a dairy breed used for cheese production in the Pyrenees region (Rochus et al., 2018). According to Gutiérrez-Gil et al. (2014) the ABCG2 gene strongly influences the amount of milk produced in sheep. Studies have shown the presence of a polymorphism in the ABCG2 gene. A mutation representing an insertion/deletion of 35 bp was found in the ABCG2 gene of sheep (Árnyasi et al., 2013). Oner et al. (2014) reported the "-" allele as the predominant allele and its frequency was in the range of 0.50-0.65. In a study by Árnyasi et al. (2013) the "+" allele was observed with a higher frequency, establishing a relationship between the "-" allele and the higher SCC in the milk of the studied sheep.

Hofmannová et al. (2018) identified a polymorphism in intron 5 of the ABCG2 gene in the Lacaune and East Friesian breeds raised in the Czech Republic. This mutation is located in the non-coding region and does not cause changes in the amino acid sequence, but the introns may carry transcriptional regulatory elements (Árnyasi et al., 2013; Hofmannová et al., 2018). In Lacaune animals, the deletion allele is predominantly found, while in East Friesian animals the non-deletion allele predominates. This mutation of the ABCG2 gene had an effect on the somatic cell count in milk. The I allele may be associated mainly with the lower limit of the somatic cell count (SCC) in the milk of healthy animals, for which increased resistance to mastitis has not been demonstrated (Hofmannová et al., 2018).

Climate change both globally and in our latitudes is one of the reasons for the search for more resistant sheep breeds. Awassi is the most numerous and widespread sheep breed in southwest Asia, with variation observed between individual flocks in terms of milk productivity, size and wool quality (Epstein, 1985). The milk yield of Awassi sheep varies widely (from 73 kg to 506 kg) (Galal et al., 2008; Al-Samarai & Al-Anbari, 2009; Gootwine, 2011; Meydan et al., 2024) due to various reasons: level of selection, duration of lactation, climatic influences, birth order, milking frequency, production year and last but not least genetic diversity (Ali et al., 2020; Meydan et al., 2024). The interest in the Awassi breed is also related to healthy eating - the milk fat of the Awassi breed has a better ratio of omega-3 and omega-6 compared to the milk fat of other breeds (Ayadi et al., 2024), and in general we can say that animals of the Awassi breed have higher quality meat and milk (Merzah et al., 2023).

The present study aims to determine the genetic diversity in intron 5 of the ABCG2 gene in connection with the study of possible dependencies of genotypes in ABCG2 with milk production in the ewes of the Awasi breed reared in Bulgaria.

MATERIALS AND METHODS

Animals. The study was conducted with ewes of the Awassi breed, raised in a private flock in the village of Sredkovets, Shumen district, Bulgaria (Figure 1). The flock was also the subject of our previous study (Dimitrova et al., 2024) with the consent of the owner. Blood samples were collected from the jugular vein of 61 clinically healthy animals in vacuum tubes containing EDTA as an anticoagulant by the state veterinarian in charge of the farm.

Figure 1. Awassi ewes from a private herd - the village of Sredkovets, Shumen district, Bulgaria (own source)

The animals are raised in a semi-intensive, pasture-manure system. The lambing campaign on the farm usually starts in the second half of December and ends by the end of March, according to the insemination campaign in the respective year. Milking is mechanized and is carried out twice, after the lambs are weaned at about 2-3 months of age.

The control of the productive qualities of the Awassi sheep breed in Bulgaria is carried out by the "Breeding Association for Dairy Sheep" (RAMO). According to the regulatory provisions, control of milk productivity was carried out by measuring the amount of milk in liters milked during the milking period of the animals (1st and 2nd lactation) using the AC method, specified in the nomenclature of the International Committee for Animal Control (ICAR). For the studied herd, the provided data on the individual milk productivity of the controlled sheep were equated for a 120-day milking period (TMM120).

DNA isolation Genomic DNA was extracted from whole blood using a manual commercial DNA purification kit (Illustra Blood Genomic Prep DNA Purification Kit, GE Healthcare) following the manufacturer's protocol. The quality and quantity of the DNA were assessed via spectrophotometric analysis (Biodrop spectrophotometer) and agarose gel electrophoresis using a 1% agarose gel (Bioline) with 1× TAE buffer (Thermo Scientific).

PCR procedure PCR reactions were conducted in a total volume of 10 µL, containing 40 ng of DNA template, 0.2 µL ddH_2O, 20 pM of each primer, and 5 µL of 2× (1.5 mM $MgCl_2$) MyTaq™ HS Red Mix (Bioline).

The primer set used for amplification (F:5'-GCCTCTTCTCCCATACGTC-3' and R:5'-AAACCAGTTGTGGGCTCATC-3') was based on the study by Árnyasi et al. (2013), generating PCR products of 232 bp (with deletion) or 267 bp (no deletion).

PCR amplifications were performed using a erityPro 96-Well (Applied Biosystems by Thermo Fisher Scientific) according to the following parameters: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation (94°C for 30 s), annealing (52.6°C for 65 s), and elongation (72°C for 1 min), with a final extension step at 72°C for 10 min.

The fragment sizes were determined by agarose gel electrophoresis, using a Ready-to-Use 50 bp DNA Ladder (Thermo Scientific) on a 2.4% agarose gel (Bioline), stained with RedGel™ Nucleic Acid Stain (Biotium), and analyzed under UV exposure.

Statistical analysis. The data on milk yield for a 120-day milking period (TMM120) were processed using the Statistica software product. To determine the effect of the ABCG2 gene polymorphism on milk yield for a 120-day milking period, the one-way ANOVA analysis of variance model was used.

RESULTS AND DISCUSSIONS

Genotyping of the ABCG2 gene was performed by polymerase chain reaction (PCR) itself, capable of distinguishing between fragments based on the presence or absence of the mutation (deletion/insertion) and subsequent electrophoresis. As a result, fragments of intron 5 of the ABCG2 locus with two different lengths were amplified - 232 bp resulting from the deletion and 267 bp in the absence of the deletion (Figure 2).

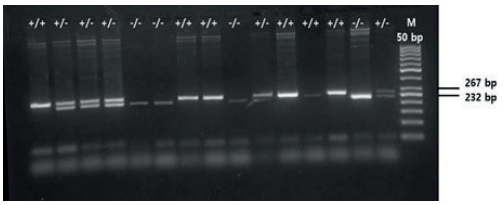

Figure 2. Agarose gel electrophoresis of PCR fragments of ABCG2 gene and DNA ladder visualized on 2.4 % agarose gel under UV light (own source)

The fragment representing the wild-type allele "+" is 267 bp in size, and the fragment with a 35 bp deletion representing the mutant allele "-" is 232 bp in size. A highly polymorphic state of the ABCG2 gene was found in Awassi sheep, the wild-type allele "+" has a frequency of 0.49, and the mutant allele "-" - 0.51. Three genotypes were identified with the following frequencies - homozygous wild type +/+ - with 0.31, heterozygous +/- with 0.36 and homozygous mutant -/- - with 0.33. In this herd, however, the expected heterozygosity is higher than the established one, and the inbreeding coefficient is positive, which also indicates the presence of inbreeding, and the results obtained are statistically reliable (Table 1).

Table 1. Allele and genotype frequencies, observed and expected heterozygosity, coefficient of inbreeding in studied Awassi herd

Breed	n	Allele frequency		Genotype frequency			Heterozygosity		F_{is}	χ^2	p
		+	-	+/+	+/-	-/-	Ho	He			
Awassi ABCG2	61	0.49	0.51	0.31	0.36	0.33	0.361	0.500	0.278	5.39	0.02*

*statistically significant difference ($p<0.05$)

In our previous study of the ABCG2 gene in Bulgarian sheep breeds, we tested a total of 90 animals from three Merino breeds - Askanian Merino, Caucasian Merino and Karnobat Merino, and polymorphism was found in all three breeds with the presence of two alleles and three genotypes, with the mutant allele frequencies being higher. The frequency of allele "-" was highest in Askanian Merino sheep breeds - 0.76 and the frequency of genotype -/- is also highest in animals of the same breed - 0.68 (Dimitrova et al., 2019).

A predominant allele "–" was reported by Oner et al. (2014) in Turkish sheep breed Kıvırcık and frequency was in the range of 0.60. On the contrary, the "+" allele was found with a higher frequency in another study conducted in Hungary in Gyimesi Racka and Awassi sheep (Árnyasi et al., 2013). The genotype frequencies for the Awassi breed were: 0.39 for +/+, 0.46 for +/- and 0.15 for -/-, which differ from the results obtained by us for the same breed.

Hofmannová et al. (2018) identified all three genotypes in a study of Lacaune and East Frisian sheep. In the Lacaune breed, the deletion allele was predominantly found (0.694), while in the East Frisian the non-deletion allele was primarily found (0.784).

Bozhilova-Sakova et al. (2022) studied two flocks of the Bulgarian dairy synthetic population - the first from the Agricultural Institute in Shumen - 116 ewes with frequencies of the wild-type allele "+" - 0.53 and of the mutant allele "-" 0.47; the second from the Institute of Animal Husbandry - Kostinbrod - 68 ewes with frequencies of the wild-type allele "+" - 0.71 and of the mutant allele "-" - 0.29. All three possible genotypes were identified in both flocks. In the sheep from Shumen, the heterozygous genotype had a frequency of 0.63, and in those from Kostinbrod - 0.32; both flocks were not in Hardy-Weinberg equilibrium. The results obtained for both herds differ significantly from our results.

In our previous study, with sheep from the same flock, we found that the total milk productivity of the sheep was 126.317 (l) per 120-day milking period (Dimitrova et al., 2024). It is known that the milk productivity of the Awassi breed sheep varies in different countries of its distribution (Galal et al., 2008). For example, Üstüner & Mustafa (2013) found that the milk yield of the Awassi breed sheep in the Central Anatolian Region of Turkey was 196.5 kg for a 184.3-day lactation period, and for sheep of the same breed in Iraq, Al-Samarai et al., (2014) reported a milk yield of 103.57 kg for a 107.44-day lactation period. The milk productivity of the genotypes identified in the present study is shown in Table 2.

Table 2. Effect of the genotype of ABCG2 gene on the milk yield for a standard 120-day milking period

Traits	Genotype +/+ (n=19)			Genotype +/- (n=22)			Genotype -/- (n=20)			p-value
	Number of milk records	Average	CV%	Number of milk records	Average	CV%	Number of milk records	Average	CV%	
TMM120, total	28	126.123	45.90	35	126.645	38.15	38	126.158	46.89	0.94

The carriers of the three genotypes are almost evenly distributed, their milk yield is close to the average for the studied sheep, and is in very close limits for the homozygous genotypes "+/+" (126.123 l) and "-/-" (126.158 l). The milk yield is slightly higher for the heterozygous genotype "+/-" (126.645 l), but no relationship between the ABCG2 gene polymorphism and milk yield in the different genotypes was established.

In a study conducted on 30 sheep from the Bulgarian Dairy Synthetic Population, two genotypes were identified in intron 5 of the ABCG2 gene: homozygous (-/-) and heterozygous (+/-). The analysis revealed no statistically significant difference in milk production between heterozygous individuals (+/-) and those with the homozygous (-/-) genotype at a 5% significance level, corroborating the findings of the present study (Bozhilova-Sakova et al., 2022a).

CONCLUSIONS

A genetic polymorphism in intron 5 of the ABCG2 gene was identified in the studied Awassi ewes with the presence of two alleles with similar frequencies. Three genotypes were identified with the following distribution - homozygous wild type +/+ - with 31%, heterozygous +/- - with 36% and homozygous mutant -/- - with 33%. The expected heterozygosity is higher than the established one, and the inbreeding coefficient is positive, which indicates the presence of inbreeding in the studied herd of the Awassi breed.

The milk yield of ewes carrying the three genotypes is nearly identical, closely aligning with the average yield of the studied sheep. The homozygous genotypes "+/+" (126.123 l) and "-/-" (126.158 l) exhibit very similar yields, while the heterozygous genotype "+/-" shows a slight increase (126.645 l). However, no significant relationship was established between the polymorphism in intron 5 of the ABCG2 gene and milk yield across the different genotypes.

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