RELATIONSHIP OF SECRETED PHOSPHOPROTEIN 1 PROMOTER /EXON 3 GENE POLYMORPHISM/ G>A/1207 & A>G /1212 IN SOME ECONOIC VALUES AND LIVER ENZYME OF AWASSI SHEEP

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ABSTRACT

This study was aimed to identify the genetic polymorphism of the Secreted phosphoprotein 1 (SPP1) gene in a sample of Awassi sheep consisting of 48 ewes and its 62 newborns. This study lasted for six months, The results revealed that there were two variations (2 SNPs) in the Promoter Exon 3 of gene SPP1, the first variance SNP1 the position was (SNP1 A > G: P/1212/(84 bp), AG. The second variance Snp2, G > A: P/1207/ (92 bp), There were two genetic components of this gene, GG and GA, at a percentage rate of 60.42 and 39.58%, respectively, and there were high-morale (P≤0.01) differences in the birth weight of the ewe, The GA hybrid ewe outperformed the G-pure terrestrial genetic structure. This study concludes that the mutant GG genetic structure does not appear in the SNP1 and AA does not appear in the SNP2 of the gene's studied region. There is a correlation between the heterogeneity obtained from the study of SPP1 gene and the production of daily milk and some liver enzymes in passive sheep that can be exploited in election and improvement programmes.

Keywords: Secreted phosphoprotein1(SPP1) -Awassi sheep-milk production - AST.
***Part of Ph.D.dissertattion of the 1st author.**

التراكيب الوراثية لجين SPP1/ برموتر الاكسون 3 G>A/1207 و A>G /1212 وعلاقتها بعدد من الصفات الانتاجية وانزيمات الكبد لدى الاغنام العواسي في العراق

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المستخلص

هذه الدراسة هدفت الى تحديد المظاهر الوراثية لجين Secreted phosphoprotein 1 (SPP1) في عينة من الأغنام العواسي مكونة من 48 نعجة ومواليدها البالغ عددها 62 , استغرقت هذه الدراسة مدة ستة اشهر, إظهرت النتائج وجود تغايرين (2 SNPs) في منطقة الحفاز للاكسون الثالث جين SPP1، التغاير الأول SNP1 كانت الموضع (SNP1 P/1212 :A>G/ 84 bp)، AG. اما التغاير الثاني Snp2, P/1207 :G>A/ 92 bp)، إذ كان هنالك تركيبن وراثيين لهذا الجين هما GG و GA بنسبة مئوية بلغت 60.42 و 39.58 % على التوالي، وان هنالك فروق عالية المعنوية (P≤0.01) في وزن النعاج عند الولادة، إذ تفوقت النعاج ذات التركيب الوراثي الهجين GA على مثيلاتها ذات التركيب الوراثي البري النقيG. يستنتج من هذه الدراسة عدم ظهرر التركيب الوراثي الطافر GG في SNP1 و AA في SNP2 للمنطقة المدروسة من الجين، وان هنالك علاقة بين التغاير الذي تم الحصول عليه من دراسة جين SPP1 وانتاج الحليب اليومي وبعض انزيمات الكبد في الاغنام العواسي بالامكان استغلالها في برامج الانتخاب والتحسين .

الكلمات المفتاحية: جين SPP1, منطقة المحفز, أغنام العواسي, إنتاج الحليب, إنزيمات الكبد.
***البحث مستل من اطروحة الدكتوراه للباحث الاول.**

Received:13 /7/2023, Accepted:5/11/2023, Published:December 2025

INTRODUCTION

Prominent sheep breeds raised in Iraq, and they are among the most widespread. They are distinguished by their high adaptability to harsh environmental conditions (6, 1, 21). Studies have focused on the development of methods to enhance the genetic composition through an emphasis on functional blood traits, molecular genetics, quantitative genetics, and breeding strategies and programs (4, 9,5 , 17). There are many genetic markers closely associated with the variation and phenotypic pattern of important traits, including milk and meat production (12,9, 8,18). It is possible to predict the phenotypic variation of the desired traits in an early stage (13), which represents functional mutations in influential genes. These techniques in the field of genetic sequence analysis provide numerous benefits in determining the genetic structure of genes. Through them, it becomes possible to identify the primary enzymes produced by the genetic material, which play a key role in important physiological processes and nutritional pathways associated with the phenotypic characteristics of the organism. Following this, genetic improvement programs can be developed based on this knowledge (22). such as identifying genetic markers associated with milk production traits and enhancing them (10,23). By utilizing these advanced techniques, researchers are able to improve the genetic performance of local sheep breeds and increase their productivity. One of these genes is the SPP1 gene (Secreted Phosphoprotein 1), which is located in the first half of sheep chromosome 6, within the region spanning from position dp 36645289 to 36658205. This gene has a length of 6.55 kilobases and consists of 12,916 nucleotide bases. Its ID is 443058 in *Ovis aries* (OAR6). Interestingly, this gene is found in a region that overlaps with quantitative trait loci (QTLs) associated with milk traits in cattle (Bos Taurus) on BTA6 (Bus Taurus), as reported by Gutierrez (7). The coding region NC_056059 on cDNA has a size of 539 base pairs. The SPP1 gene significantly influences the increase in sheep's weight. This is attributed to the gene's impact on the metabolic process and its optimal utilization of available nutrients, ultimately leading to increased production. Additionally, it has an effect on resistance to certain diseases by enhancing the immune system of animals, thereby protecting them from various infectious diseases (24). The SPP1 gene plays a crucial role in the differentiation of the mammary gland and the branching of mammary ducts (14). It also regulates the expression of quantitative traits responsible for milk production and its components (24). There is encoding for a protein called Osteopontin, abbreviated as OPN. This protein is essential for the activities of fibroblasts, B cells, and bone cells. It is responsible for bone tissue growth and improving economic traits in sheep through its role in reproduction (13). The SPP1 gene has an impact on liver enzymes, including ALP (Alkaline Phosphatase), AST (Aspartate Aminotransferase), and ALT (Alanine Aminotransferase), which play a fundamental role in amino acid metabolism at their normal levels. These enzymes are involved in the transfer of amino groups. ALP enzyme is responsible for transferring phosphate groups across cell membranes. Since the enzymes are present in both blood and tissues, they play a crucial role in all metabolic activities in the body. This increase in enzymatic activity, along with an increase in amino group-carrying enzymes like AST(Aspartate Aminotransferase), is associated with increased metabolic activity in the body. This aligns with the interpretation provided (3), which explained that an increase in overall bodily metabolic activity, including high sexual activity, leads to increased activity of amino group-carrying enzymes, including AST. Studies have shown that the enzyme ALP is found in high concentrations in the liver and on the secretory surfaces of cells (2). The aim of the research was to identify the genetic variations in the SPP1 gene, specifically the G>A/1207 and A>G/1212 variants, in a sample of Awassi sheep. This included studying the polymorphism distribution and allele frequency of SPP1 gene/ Exon 3 , and additionally, the research sought to understand the relationship between these genetic variations and various production traits as well as liver enzymes.

MATERIALS AND METHODS

The research was conducted at the animal field site affiliated with the Babylon Station in the Jabla district of the Musayyib Sheep Farming Project, located 50 kilometers away from the Baghdad Governorate. The study duration spanned from January 1, 2022, to June 1, 2023. It involved a sample of local sheep, consisting of 48 ewes and 62 of their offspring. The genetic material of the Secreted phosphoprotein 1 (SPP1) gene was extracted from the studied samples for the purpose of determining the genotype of the studied segments, including the distribution ratios of genetic features and allelic frequencies. This was done after collecting blood samples and conducting field measurements of the studied traits. The weights and dimensions of the lambs at birth and weaning were recorded, and the concentrations of liver enzymes ALT, AST, and ALP were measured, along with the analysis of milk components. The laboratory work was conducted at the Laboratory of Graduate Studies in Biotechnology and Molecular Genetics, University of Baghdad, College of Agricultural Engineering Sciences, Department of Animal Production, using a sample of Awassi sheep consisting of 48 ewes. The results showed the presence of two genetic variations (2 SNPs) in the promoter region of the third exon (Exon3) of the SPP1 gene.

Blood collection: A 5 ml blood sample was collected from the Jugular vein of each sheep under study into a test tube containing K2 anticoagulant to prevent clotting, and it was stored frozen until the extraction process . The identification numbers of the animals were recorded for the purpose of monitoring all the required measurements.

DNA Extraction: DNA was extracted from the blood using the Geneaid Kit following the method described by Russel and Samrook (19). The nucleic acid was isolated from the blood sample and loaded onto a GD Column from the kit. The tubes were then stored at a temperature of -20°C until further work was completed, as shown in Figure 1.

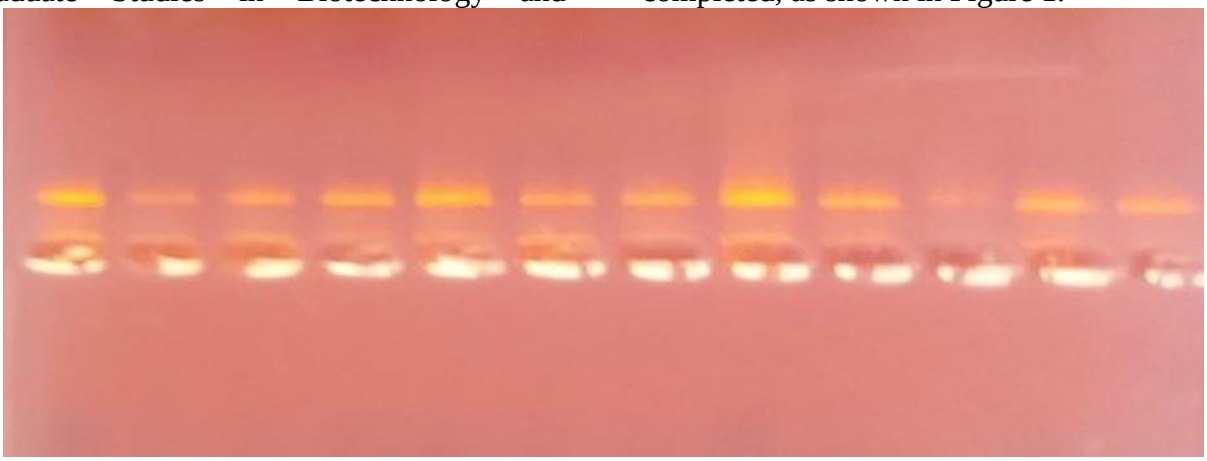

Figure 1. Genomic DNA Migration of Awassi Sheep

Molecular analysis of the SPP1 gene

For the purpose of performing molecular analysis of the studied gene on the blood samples collected from the Awassi sheep and stored at freezing temperatures (-20°C), they were thawed for laboratory testing. The kits were prepared, and primers (primers) and tools were designed in the laboratory.

The primer design for the SPP1 gene

An area of the SPP1 gene was identified, a length of 539 base pairs of nitrogenous bases, as shown in Table 1. This was done for the purpose of molecular detection and determining the allelic diversity of the genes and mutations present in the SPP1 gene (13, 15, 16).

Table 1. Designed Primers for the SPP1 gene

Gene	Position	Sequence	Size (bp)
SPP1	intron 2 -- exon 3	F: 5'-GAGATGGAAAATAGAGGTGGC-3'	539
Primer	4 intron	R: 5'-AGCAGGCACCCAATAAATACT-3'	C 58

To assess the quality of the sample, DNA concentration was measured using the Quantus Fluorometer device provided by Promega, an American company specializing in DNA

analysis. After designing, the primers were prepared by MACROGEN, in the form of dried powder, and they were dissolved by adding 300 microliters of deionized water, free from ions. The size of the studied fragment of the SPP1 gene was confirmed to be 539 base pairs (bp) through gel electrophoresis. The primer strength was measured using a PCR gradient to determine the appropriate annealing temperature for primer binding was 58°C. In the gel electrophoresis process, 3 microliters of DNA ladder marker (ranging from 100 to 1000 bp) were placed in the first well of the gel. Then, 5 microliters of the PCR product were loaded into the remaining wells. The sample is relayed on the Acarose gel at a concentration of 1.5% and the power of the voltage is stabilized on 70 volts and at a current of 60 mV for 60 minutes, The Ethidium Bromide formula is placed in the installation, as shown in Figure 2. The results, after amplifying the targeted gene fragment using PCR, were sent to the Scientific Pulse Laboratory for Molecular Biology and Genetic Analysis.

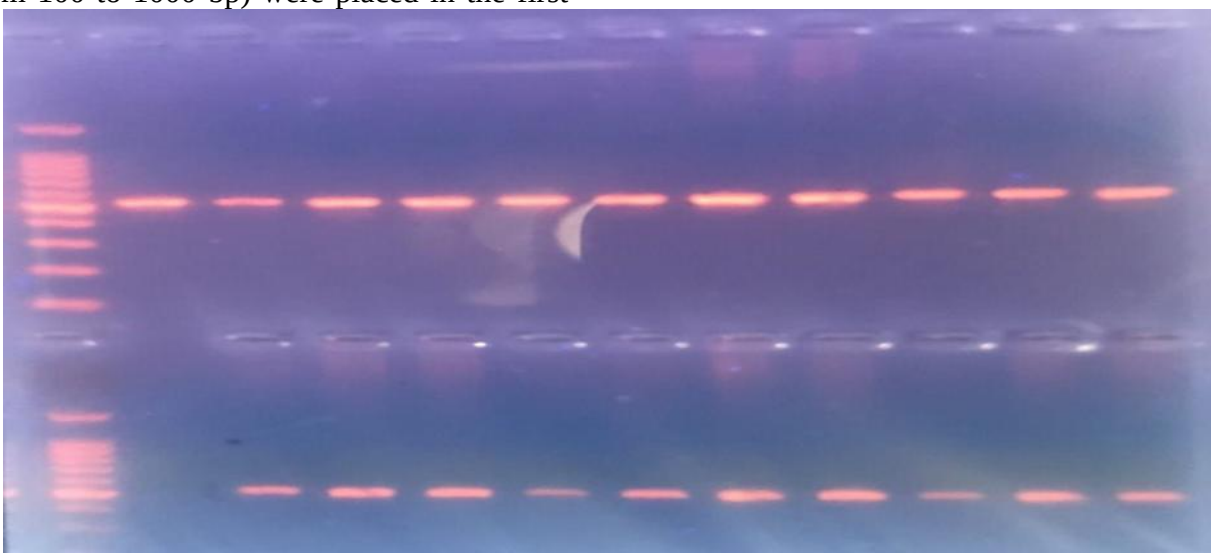

Figure 2. Gel electrophoresis for SPP1 gene (539bp) base pairs in Awassi sheep

Statistical analysis: The Statistical Analysis System- SAS (15) program was used for statistical data analysis to study the impact of genetic variations in the SPP1 gene on the studied traits. The Duncan multiple range test was applied to compare the means of significant differences by using the Least Square Means method. Additionally, the Chi-square ($\chi 2$) test was employed to compare the percentage distribution of genetic structures.

RESULTS AND DISCUSSION

Genotype and allele frequency: The genotype and allelic frequency for the first variation in the SPP1 gene, located in the promoter region of exon3, SNP1/A>G: P/1212in Awassi Sheep (Table 2). The genotypes were divided into AA, AG, and GG, with percentages ranging from 41.67 to 56.25 and 2.08%, respectively. The allelic frequencies for A and G were 0.70 and 0.30, respectively, as indicated by this. As shown in Figure (3), there were statistically significant differences (P 0.01) in the genotype distribution within the sample. It's important to note that the distribution of genotype and allelic frequencies was vary based on factors such as the gene, the studied region, sample size, breed, environmental conditions, and chance.

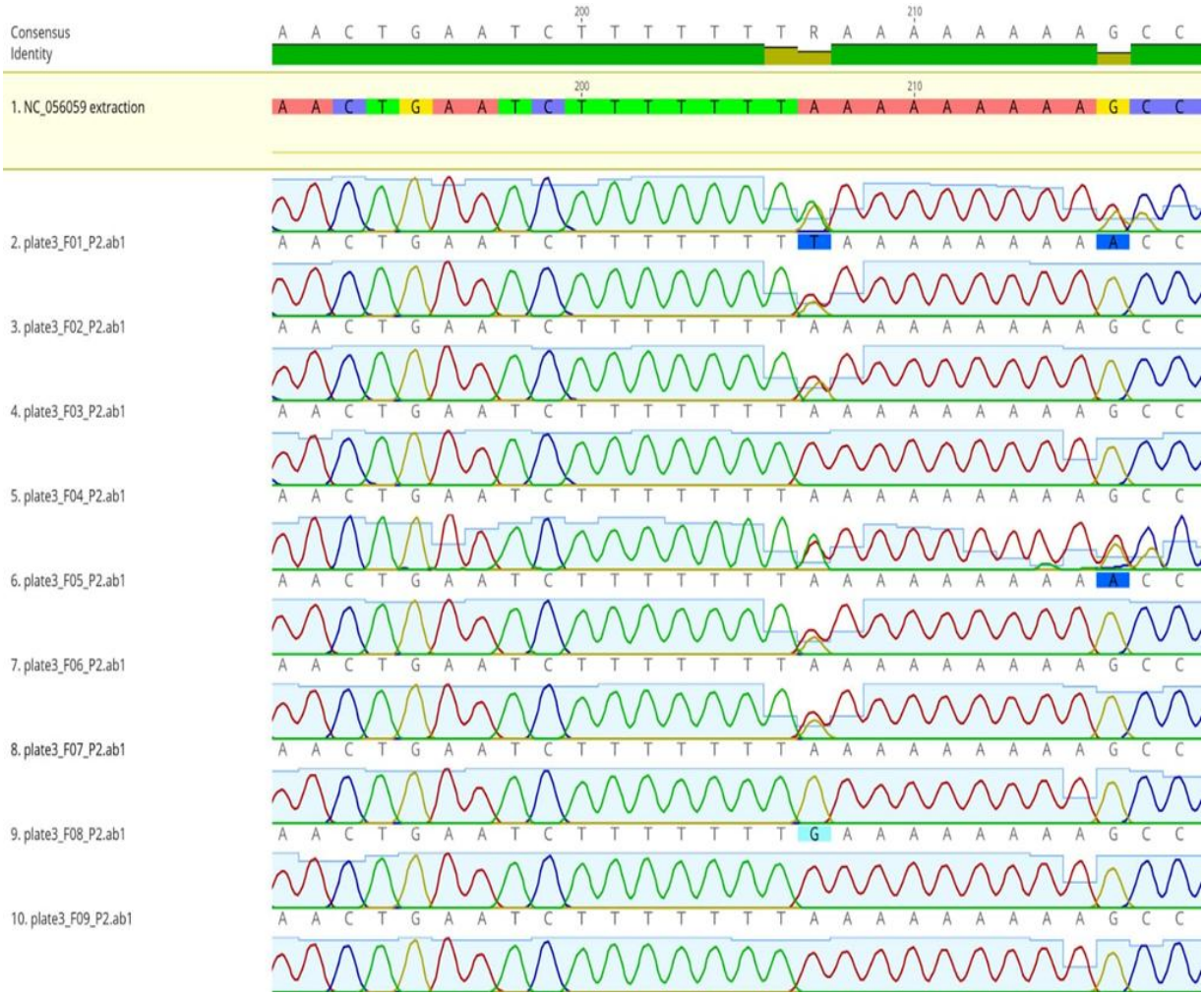

Figure 3. Displays the analysis of the first SNP1(1) and the second SNP2(11) mutations in the promoter region of exon 3 of the SPP1 gene

Table 2. Polymorphism distribution and allele frequency of SPP1 Gene Promoter Exon 3 Position SNP1 (A>G: P/1212)

Polymorphism	Number	(%) Percentage
AA	20	41.67
AG	27	56.25
GG	1	2.08
Total	48	100 %
Chi-Square ($\chi 2$)	----	** 15.791
Allele		Frequency
A		0.70
G		0.30
	.(P≤0.01)**	

Genotype and allele frequencies of the SPP1 gene's second variation in exon 3promoter SNP2, G>A: P/1207 / 92- bp: Genotype and allele frequencies of the SPP1 gene in the exon 3 promoter region SNP2, G>A: P/1207 (92-bp) for the studied sheep sample are shown in Table (3). The genotypes observed were GG and GA, with no individuals carrying the mutated genotype AA within the studied sample. The numbers of individuals carrying these genotypes were 29 and 19, with percentages of 60.42% and 39.58%, respectively. This indicates that the allele frequencies for G and A were 0.80 and 0.20, respectively. There were statistically significant differences (P≤0.01) in the distribution of genotype within the sample. Figure3. provides an analysis of G>A:P/1207/SNP2 in the SPP1 gene in the exon 3 promoter region.

Table 3. Polymorphism distribution and allele frequency of SPP1 Gene Promoter Exon 3 Position SNP2 (G>A: P/1207).

Polymorphism	Number	Percentage(%)
GG	29	60.42
GA	19	39.58
AA	0	0.00
Total	48	100%
Chi-Square ($\chi 2$)	----	37.125**
Allele	Frequency	
G	0.80	
A	0.20	
.(P≤0.01)**		

Relationship of the SPP1 gene with the exon 3 promoter SNP1/ A>G:

P/1212 and its impact on daily milk production and lactation oeriod: The results from Table (4) showed a significant difference (P≤0.05) in daily milk production between ewes with different genetic compositions based on the analysis of the SPP1 gene SNP1 (A>G: P/1212). Ewes with the heterozygous genotype AG (335.96 20.57 kg) outperformed their counterparts with the homozygous genotype AA (316.76 23.51 kg).However, there were no significant differences between the two polymorphism AA and AG in terms of lactation period, as their rates were very close. The differences in milk quantity produced in current results may be due to increas gene expression in individuals carrying the AG genotype.

Table 4. Relationship between SPP1 Gene (A>G: P/1212) polymorphism with Daily Milk Production and lactation period of Awassi Sheep

SPP1 polymorphism	Number of ewes	Mean ± Standard error (cm) Daily milk production (kg)	Lactation period (day)
AA	20	316.76 ±23.51	111.15 ±8.94
AG	27	335.96 ±20.57	112.01 ±8.33
Significant		*	NS
* (P≤0.05), NS: Non-Significant.			

Relationship of SPP1/SNP1 Gene (A>G: P/1212) polymorphism and Liver enzyme level in Awassi: The results in Table 5 indicate that the levels of ALT and ALP enzymes were not significantly affected by the SPP1 gene SNP1 (A>G:) polymorphism in Awassi sheep. However, there were significant differences in the AST enzyme, with a concentration of 128.76 ± 12.07 international units per liter in ewes with the AA genotype, while it was lower in their counterparts with the heterozygous AG composition (84.19 ± 6.34 international units per liter). The variation in AST enzyme levels with different genotype in the studied region of the SPP1 gene may be attributed to differences in gene expression. This could be related to increased gene expression associated with higher metabolic activity in the body, leading to increased enzymatic activity, including AST. These findings align with the interpretation provided by, who explained that increased overall metabolic activity, including sexual activity, leads to enhanced activity of amino acid-carrying enzymes, including AST.

Table 5. Relationship SNP1 Gene (A>G: P/1212) polymorphism and Liver enzyme level in Awassi

Genotype	Number of ewes	Mean ± Standard error (U/L)		
		ALT	AST	ALP
AA	20 (40 sample)	32.91 ±2.63	128.76 ±12.07	161.82 ±10.70
AG	27 (54 sample)	33.06 ±3.51	84.19 ±6.34	156.74 ±8.22
Significant		NS	*	NS
* (P≤0.05), NS: Non-Significant.				
ALT= Alanine aminotransferase , AST= Aspartate aminotransferase , ALP= Alkaline phosphatase.				

Relationship of the SPP1, G>A: P/1207 gene variant with growth traits in Awassi sheep: Table (6) revealed the association of the genetic variations of the SNP2 (G>A:1207) gene with growth traits in Awassi sheep. There were highly significant differences (P≤0.01) in

the birth weight of the ewes, with ewes carrying the heterozygous GA genotype (67.51 ± 3.83 kg) outperformed their counterparts with the pure GG genetic (56.98 ± 1.90 kg). This difference can be attributed to the higher gene expression of the GA genotype compared to the GG. Although there were no significant differences in birth weight between the GG and GA genotypes, lambs born to ewes with the GA genotype showed significant improvements (P≤0.05) in both weaning weight (23.34 ± 1.13 vs 19.02 ± 1.40 kg) and average weight gain from birth to weaning (18.52 ± 0.37 vs 14.53 ± 0.44 kg) compared to their counterparts with the GG genotype. These results align with the superior birth weights of ewes, as the weight of the mother at birth is correlated with the weights of the births.

Table 6. Relationship between SPP1 Gene polymorphism (G>A: P/1207) and growth traits in Awassi Sheep

Genotype	Number of ewes	Mean ± Standard error			
		Dam weight	Birth weight	Weaning weight	Gain (between birth and weaning)
GG	29	56.98 ±1.90	4.49 ±0.37	19.02 ±1.40	14.53 ±0.44
GA	19	67.51 ±3.83	4.82 ±0.46	23.34 ±1.13	18.52 ±0.37
Significant		**	NS	*	*

* (P≤0.05), ** (P≤0.01), NS: Non-Significant.

Relationship of the SPP1, G>A: P/1207gene variant with daily milk production and milk season length in Awassi sheep: Table (7) revealed a significant difference (P≤0.05) in daily milk production between ewes with different genetic variations according to the SNP2 (G>A:1207) gene analysis. Ewes with the hybrid GA genotype (341.63 ± 22.64 kg) outperformed their counterparts with the purebred GG genotype (305.19 ± 21.88 kg) in terms of daily milk production. However, there were no significant differences between the GG and GA genotypes in lactation period, with respective averages of 109.64 ± 9.07 and 115.01 ± 8.91 days. This suggests that the variation in milk quantity produced may be attributed to an increased gene expression in individuals carrying the GA genetic variant.

Table 7. Relationship between SPP1 Gene (G>A: P/1207) polymorphism and Daily Milk Production and lactation period of Awassi Sheep

SPP1 polymorphism	Number of ewes	Mean ± Standard error (cm)	
		Daily milk production (kg)	Lactation period (day)
GG	29	305.19 ±21.88	109.64 ±9.07
AA	19	342.63 ±22.64	115.01 ±8.91
Significant		*	NS

* (P≤0.05), NS: Non-Significant.

Conclusion

SPP1 gene has established a significant relationship between polymorphism / variations identified within the daily milk production, weaning weight and AST enzyme level in Iraqi awassi sheep. Furthermore, it posits that the influence exerted by this gene on milk composition and growth appears to be confined to particular attributes.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

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