



Research Article

The Role of the Annexin Gene in Milk Composition and Udder Dimensions Traits in Sheep

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ZATA: Conceptualization and design of the study, animal sampling, laboratory and molecular analyses, statistical analysis, interpretation of results, and manuscript writing. AHS: Contribution to data analysis, interpretation of genetic and phenotypic results, and critical revision of the manuscript. GRA: Assistance in sample collection, laboratory work, and manuscript review.

Keywords

ANXA9 gene, SNPs, Udder dimensions, Milk compositions, Awassi sheep



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Abstract | This study was conducted at the sheep and goat breeding station in Shatrah, Thi-Qar Governorate, southern Iraq, and included 39 Awassi sheep aged 2-4 years. The study aimed to investigate the effects of ANXA9 gene polymorphisms on certain udder size and milk composition traits in Awassi sheep to use these genetic markers for early breeding and to test whether three intronic SNPs in ANXA9 (C172G, G385T, G393C) are associated with udder dimensions and milk composition in Awassi ewes from southern Iraq. Blood samples were collected from the jugular vein of each sheep, and genomic DNA was extracted. The target fragment of the ANXA9 gene was amplified using the PCR, and the amplicons were analyzed through gel electrophoresis to verify the size of the targeted segment. All molecular procedures were performed at the Molecular Genetics Laboratory, University of Thi-Qar. The results revealed three single-nucleotide polymorphisms (SNPs) in intron 4 of chromosome 1 at positions 172 C/G, 385 G/T, and 393 G/C. Statistical analysis using the Chi-square test showed no significant differences in genotype distribution for these SNPs. Milk composition and udder morphology are economically important in dairy sheep, yet the contribution of candidate genes such as ANXA9 (Annexin A9) remains incompletely resolved. Annexins are Ca²⁺-regulated, membrane-binding proteins that organize membrane domains and participate in lipid/lipoprotein trafficking in secretory epithelia, including the mammary gland. Recent ovine work has linked polymorphisms in ANXA9/FASN/SCD1 with variation in the milk fatty-acid profile. However, a significant effect ($P \leq 0.05$) of the genotypes resulting from the genetic variation at the (C172G) SNP which heterozygous genotype (CG) showed superiority in udder length, with a mean of 20.10 cm, while the wild-type genotype (CC) was superior in teat circumference, with a mean of 5.18 cm. Though there was no significant effect of the three genotypes on the other udder dimension traits (udder circumference, udder width, and teat length), there was a single significant effect ($P \leq 0.05$) of the genotypes resulting from the first SNP (C172G) in the *ANXA9* gene on milk density. The CG genotype had a significantly higher mean value (39.58). On the other hand, no significant differences were observed among animals carrying the three genotypes of the G385T and G393C SNPs in any of the milk composition traits. The C172G locus showed genotype-specific differences in udder length and milk density, whereas G385T and G393C showed no detectable effects; ANXA9 remains a plausible marker to explore in selection programs for local Awassi populations.

Novelty Statement | This study is the first to comprehensively assess intronic SNPs of the ANXA9 gene (C172G, G385T, and G393C) in relation to both udder morphological traits and milk composition in local Awassi sheep from southern Iraq. The identification of a genotype-specific association between the C172G SNP and udder length as well as milk density provides novel molecular evidence supporting ANXA9 as a promising candidate gene for marker-assisted selection in dairy sheep under local production systems.

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Introduction

In recent years, there has been a growing interest in the relationship between diet and health due to the established links between the two. Although dairy products, especially milk products, are generally considered healthy and widely consumed around the world, their composition still requires further research. Most existing studies focus on yield and often overlook the quality factors of milk (Jameel and Sahib, 2016; Al-Jaryan *et al.*, 2022). In Iraq, sheep are an important part of the livestock industry and make an important contribution to the supply of animal products, including milk. However, the productivity of local sheep breeds remains relatively low. Milk is an important nutritional source of protein, calcium, potassium, and antioxidants (Barlowska *et al.*, 2011; Al-Jaryan *et al.*, 2023). The concentrations of these compounds are affected by multiple genetic factors (e.g., breed and gene variation) as well as non-genetic factors (e.g., age, lactation period, farrowing period, pregnancy status, milking method, and feeding system) (Capistrak *et al.*, 2002). Traits that require more scientific attention include udder morphological characteristics, which play a key role in milk production sustainability, lactation capacity, and milk flow. These traits are also considered crucial in the selection of dairy animals (Al-Hered, 2008). Milk yield traits in Iraqi Awassi sheep are well documented, but gene-level determinants of milk quality and udder morphology remain underexplored. The *ANXA9* gene, a member of the annexin family whose membrane-bound proteins modulate vital cellular processes such as signaling, growth regulation, and stability of membranes, has been implicated in the metabolism of adipose tissue as well as expression in the mammary glands during lactation, particularly in sheep breeds indigenous to southern Iraq, like the Awassi. Annexin A9 is an atypical annexin expressed in mammary tissue and implicated in lipid handling; annexins scaffold membrane domains in a Ca²⁺-dependent manner (Gerke *et al.*, 2005). Across ruminants, ANXA9 variants have been associated with milk-fat yield in dairy cattle (Martínez-Royo *et al.*, 2010) and milk fatty-acid profile in sheep when considered with lipid genes (Pecka-Kielb *et al.*, 2021). However, no study has examined whether ovine ANXA9 intronic SNPs are related specifically to udder dimensions together with milk composition in local Awassi ewes. Therefore, this study examined whether genetic variations in the *ANXA9* gene correlated with the composition of milk or morphological attributes of the udder within this population, with implications for milk production efficiency and animal welfare. A thorough investigation of the gene's involvement may help optimize pastoral management strategies for the sustenance of this culturally significant but vulnerable rural enterprise. Therefore, this study aimed to detect the SNPs in the ANXA9 gene and its relationship to milk production traits in Awassi sheep in Iraq.

Materials and Methods

Animals and management

Thirty-nine Awassi ewes (2–4 years) kept at the Al-Shatrah station (Thi-Qar, Iraq); morning milking was used for sampling. Units for udder traits are reported in centimeters (cm).

Milk analysis

Composite milk (~50 mL/ewe) analyzed on a LACTOSCAN ultrasonic analyzer; density reported in kg/m³ per manufacturer documentation (typical range 1015–1040 kg/m³) Lactoscan Manual (2019). Milk fat, protein, lactose, and non-fat solids are given in percentage (%).

DNA extraction and quality control

Genomic DNA was extracted at the University of Thi-Qar using the Geneaid kit; integrity verified by agarose gel electrophoresis.

PCR amplification and sequencing

Primers were used as described by Almaamory and Al-Anbari (2023); The thermal cycling protocol included an initial denaturation step at 95 °C for 4 minutes. This began with 35 cycles, each one consisting of 30 seconds denaturation at 95°C, 40 seconds annealing at 55.5°C, and 45 seconds extension at 72°C. A final extension was executed for 7 minutes at 72°C to completely cultivate the target fragment. The cycling conditions were as described, and the resulting amplicons were Sanger-sequenced, with SNPs verified using BLAST.

Statistical analysis

A General Linear Model (GLM) was applied using SAS 9.4 (SAS Institute, 2018), with genotype as a fixed effect and age as a covariate (model unchanged). Means are reported as mean ± SE. Exact χ^2 p-values for Hardy-Weinberg equilibrium tests (Table 1) and 95% confidence intervals (CIs) for key significant genotype means were provided.

Statistically significant differences among means were compared using Duncan's Multiple Range Test (Duncan, 1955), according to the following statistical model:

$$Y_{ijk} = \mu + X_i + A_j + e_{ijk}$$

Y_{ijk} : The observed value, μ : The overall mean of the trait, X_i : The effect of gene polymorphism, A_j : The adjusted age effect, e_{ijk} : The random error. The Chi-square test was used to compare the percentages of the genetic genotypes.

Results and Discussion

The gene fragment of the ANXA9 gene (675 bp) to be tested was amplified using specific primers and the DNA samples. Subsequently, the PCR product samples

were electrophoresed on a 1.5% agarose gel and visualized using a UV gel imaging system (Figure 1).

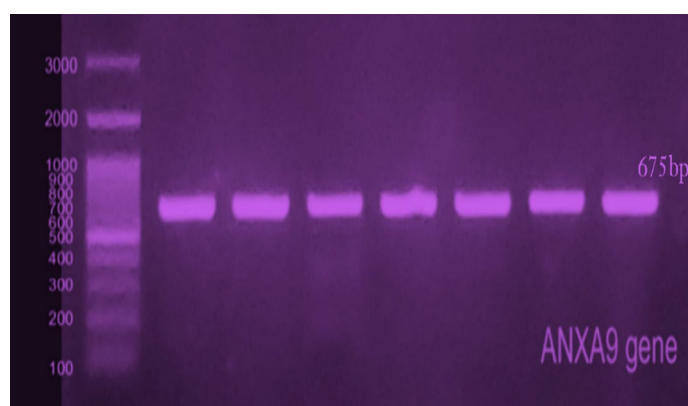


Figure 1: PCR product of the *ANXA9* gene in sheep.

The results of the sequence alignment analysis of the *ANXA9* gene in Awassi sheep showed that the studied region is located on chromosome 1 of the gene and constitutes part of intron 4, the entire exon 5, and part of intron 5. The presence of three SNPs at sites 172 C>G, 385 G>T and 393 G>C has been observed; all mutations occurred in intron 4. The first of them appeared in three genotypes, which are CC, CG and GG, and the second were GG, GT, and TT, while the third were CC, CG and GG genotypes (Figure 2).

In Table 1, the genotype frequency values indicate that the Chi-square values were non-significant for all three SNPs. This suggests that the population is in equilibrium at all three SNP loci, according to the Chi-square test, as no significant deviation from the expected distribution under Hardy-Weinberg equilibrium was observed. Exact χ^2 p-values for genotype distributions were C172G p= 0.405, G385T p= 0.509, G393C p= 0.631, indicating no deviation from HWE in this sample.

Table 1: Allelic frequency and genotypes of the *ANXA9* gene in Awassi sheep.

Chi-square	Frequen- cy	Al- leles	Frequen- cy	No	Geno- type	SNP
0.6923 NS	0.54	C	0.31	12	CC	1
			0.46	18	CG	C> G 172
	0.46	G	0.23	9	GG	
	1.00		1.00	39		
0.4359 NS	0.47	G	0.205	8	GG	2
			0.540	21	GT	G> T 385
	0.53	T	0.255	10	TT	
	1.00		1.00	39		
0.2308 NS	0.47	G	0.23	9	GG	3
			0.49	19	GC	G> C 393
	0.53	C	0.28	11	CC	
	1.00		1.00	39		

NS: non-significant

Udder dimensions traits

The statistical analysis results demonstrated a significant effect ($P \leq 0.05$) of the genotypes resulting from the genetic variation at the first SNP (C172G) on two udder dimension traits. The heterozygous genotype (CG) showed superiority in udder length, with a mean of 20.10 cm, compared to the wild-type (CC, 18.30 cm) and mutant-type (GG, 13.12 cm). On the other hand, the wild-type genotype (CC) was superior in teat circumference, with a mean of 5.18 cm, compared to both the heterozygous and mutant genotypes, while there was no significant effect of the three genotypes on the other udder dimension traits (udder circumference, udder width, and teat length) in Awassi sheep (Table 2). The significant C172G effects confined to udder length and density suggest locus- or LD-specific regulation rather than

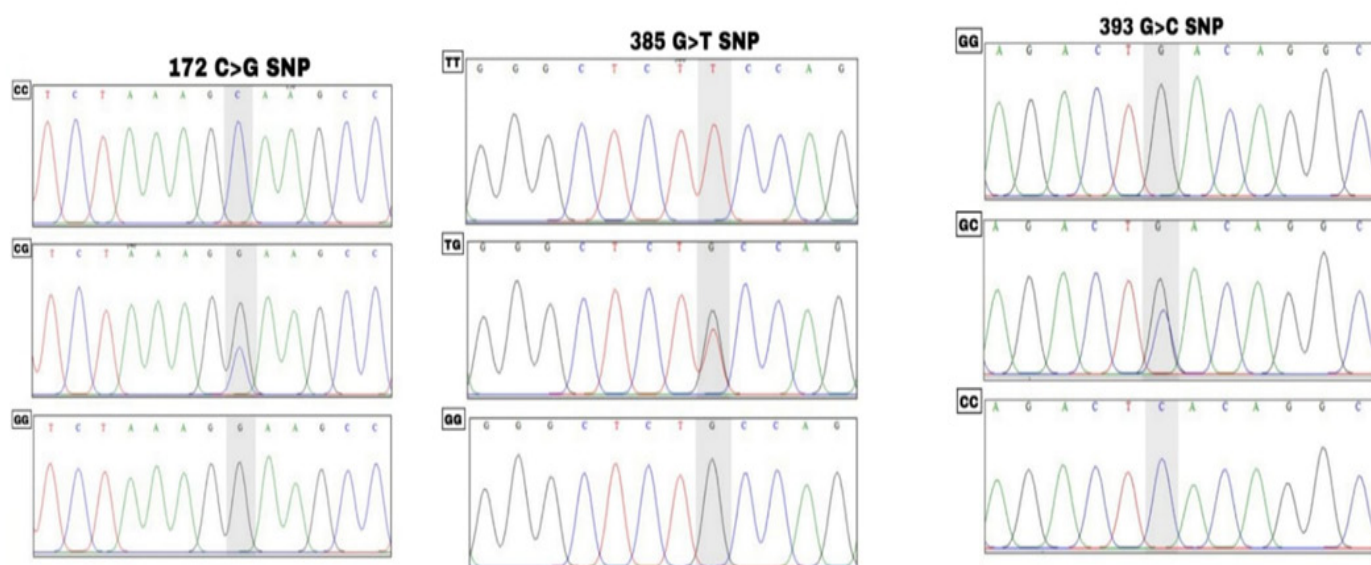


Figure 2: The three SNPs in intron 4 of chromosome 1 in the *ANXA9* gene in sheep.

a genome-wide shift in genotype frequencies. Additionally, our results indicated that no significant effect was observed for the second (G385T) and third (G393C) SNPs on any of the studied udder dimension traits. This lack of effect may be due to their location within non-functional regions of the gene, whereas the first SNP (C172G) is located in regulatory regions that might influence gene expression, protein structure, or may be in linkage disequilibrium with causative mutations, thus affecting some of the studied traits. Moreover, the differences observed between the three SNPs can also be attributed to the complex nature of quantitative traits, such as udder dimensions, which are influenced by multiple genes and environmental factors (Visscher *et al.*, 2012; Wang *et al.*, 2020).

Milk composition traits

As shown in Table 3, 4 and 5, there was a single significant effect ($P \leq 0.05$) of the genotypes resulting from the first SNP (C172G) in the *ANXA9* gene on milk density. The heterozygous genotype (CG) had a significantly higher mean value (39.58) compared to the mutant genotype (GG), while no significant difference was observed between the wild-type (CC) and the heterozygous (CG) genotypes. On the other hand, no significant differences

were observed among animals carrying the three genotypes of the second (G385T) and third (G393C) SNPs in any of the milk composition traits in Awassi sheep (Tables 6, 7). In cattle, *ANXA9* polymorphism associates with milk-fat yield (Martínez-Royo *et al.*, 2010); in sheep, studies of *ANXA9* alongside *FASN/SCD1* link genotypes to fatty-acid profiles (Pecka-Kielb *et al.*, 2021).

Table 2: The effect of the genotypes of the CG>172 SNP in the *ANXA9* gene on udder dimension traits in Awassi sheep.

Teat circumference	Teat length	Mean $\pm$ S.E			No	Genotypes
		Udder width	Udder length	Udder circumference		
5.185 $\pm$ 0.594a	4.445 $\pm$ 0.374	11.535 $\pm$ 0.653	18.309 $\pm$ 1.694a	36.618 $\pm$ 1.788	12	CC
b 4.127 $\pm$ 0.349	4.303 $\pm$ 0.318	11.712 $\pm$ 0.430	20.108 $\pm$ 1.207a	38.100 $\pm$ 1.893	18	CG
0.383ab $\pm$ 4.727	3.810 $\pm$ 0.351	11.006 $\pm$ 0.423	13.123 $\pm$ 1.197b	34.431 $\pm$ 1.277	9	GG
*	NS	NS	*	NS	39	Sign.

NS: non-significant, *: $P \leq 0.05$

Table 3: The effect of the genotypes of the GT>385 SNP in the *ANXA9* gene on udder dimension traits in Awassi sheep.

Teat circumference	Teat length	Mean $\pm$ S.E			NO	Genotypes
		Udder width	Udder length	Udder circumference		
5.556 $\pm$ 0.793	4.286 $\pm$ 0.572	11.747 $\pm$ 0.822	15.875 $\pm$ 1.949	37.306 $\pm$ 2.263	8	GG
4.233 $\pm$ 0.322	4.203 $\pm$ 0.292	11.490 $\pm$ 0.386	18.687 $\pm$ 1.291	37.616 $\pm$ 1.602	21	GT
4.572 $\pm$ 0.387	4.254 $\pm$ 0.268	11.303 $\pm$ 0.550	18.034 $\pm$ 1.712	34.671 $\pm$ 1.796	10	TT
NS	NS	NS	NS	NS	39	Sign.

NS: non-significant.

Table 4: The effect of the genotypes of the GC>393 SNP in the *ANXA9* gene on udder dimensions traits in Awassi sheep.

Teat circumference	Teat length	Mean $\pm$ S.E			NO	Genotypes
		Udder width	Udder length	Udder circumference		
4.797 $\pm$ 0.519	4.374 $\pm$ 0.388	11.99 $\pm$ 0.705	19.332 $\pm$ 1.768	37.958 $\pm$ 2.204	9	GG
4.511 $\pm$ 0.414	4.110 $\pm$ 0.316	11.162 $\pm$ 0.418	18.047 $\pm$ 1.344	36.295 $\pm$ 1.457	19	GC
4.560 $\pm$ 0.467	4.329 $\pm$ 0.371	11.660 $\pm$ 0.508	16.625 $\pm$ 1.766	36.714 $\pm$ 2.381	11	CC
NS	NS	NS	NS	NS	39	Sign.

NS: non-significant.

Table 5: The effect of the genotypes of the CG>172 SNP in the *ANXA9* gene on milk composition traits in Awassi sheep.

Density	NFS	Mean $\pm$ S.E			NO	Genotypes
		Lactose	Protein	Fat		
38.678a $\pm$ 1.598	10.453 $\pm$ 0.416	4.778 $\pm$ 0.195	4.765 $\pm$ 0.269	5.715 $\pm$ 0.731	12	CC
39.585 $\pm$ 2.043 a	10.071 $\pm$ 0.471	4.565 $\pm$ 0.164	4.629 $\pm$ 0.271	5.093 $\pm$ 0.510	18	CG
34.032 $\pm$ 3.077b	10.500 $\pm$ 0.355	4.562 $\pm$ 0.188	4.802 $\pm$ 0.196	5.444 $\pm$ 0.609	9	GG
*	NS	NS	NS	NS	39	Sign.

NS: non-significant, *: $P \leq 0.05$, NFS: Non-fat solids

Table 6: The effect of the genotypes of the GT>385 SNP in the ANXA9 gene on milk composition traits in Awassi sheep.

Mean ± S.E					NO	Genotypes
Density	NFS	Lactose	Protein	Fat		
36.5780000 3.877	10.3660000 0.363	4.6300000 0.220	4.7600000 0.260	5.1740000 0.701	8	GG
39.2578571 1.920	10.1378571 0.441	4.5607143 0.152	4.6342857 0.251	5.1585714 0.476	21	GT
37.3857143 2.792	10.4714286 0.469	4.7700000 0.214	4.8042857 0.287	5.8671429 0.790	10	TT
NS	NS	NS	NS	NS	39	Sign.

NS: non-significant, *:P≤0.05, NFS: Non-fat solids

Table 7: The effect of the genotypes of the GC>393 SNP in the ANXA9 gene on milk composition traits in Awassi sheep.

Mean ± S.E					NO	Genotypes
Density	NFS	Lactose	Protein	Fat		
37.486± 3.791	10.386 ±0.618	4.680 ±0.289	4.934± 0.302	6.044 ± 1.212	9	GG
39.644 ±1.611	10.440± 0.428	4.743± 0.134	4.646± 0.275	5.260± 0.383	19	GC
36.423 ±3.233	9.926± 0.428	4.415± 0.201	4.655± 0.210	5.070±0.662	11	CC
NS	NS	NS	NS	NS	39	Sign.

NS: non-significant, *:P≤0.05, NFS: Non-fat solids

In a similar study by [Almaamory and Al-Anbari \(2023\)](#) on the effect of genetic polymorphism in the *ANXA9* gene on some milk production and composition traits in Awassi sheep, the researchers found no significant effect of this gene on these traits except for a single significant effect on milk fat percentage, in this case, the mutant genotype (GG) showed superiority over the heterozygous genotype (TG), while no significant difference was observed between the wild-type and mutant genotypes. Annexins, including ANXA9, reversibly bind anionic phospholipids in a Ca²⁺-regulated manner and organize membrane microdomains ([Gerke et al., 2005](#)). In mammary epithelium, such scaffolding may influence lipid droplet trafficking and apocrine secretion, offering a plausible mechanism whereby intronic variation that affects ANXA9 expression/splicing could modulate milk traits. Although introns are not translated into proteins, single-nucleotide polymorphisms (SNPs) within these regions can significantly influence gene expression by modifying gene splicing or affecting gene regulatory elements ([Rigau et al., 2019](#)). Therefore, it is important to study SNPs in introns when analyzing gene expression and functional differences between individuals. Despite not being translated into proteins, introns can harbor SNPs that significantly impact gene expression ([Jari and Salim, 2023](#)). These effects may occur through alterations in gene splicing or through the modulation of gene regulatory elements ([Rigau et al., 2019](#)). Therefore, investigating SNPs within intronic regions is crucial for understanding gene expression patterns and the functional variability observed among individuals.

Conclusions

This work identifies an intronic ANXA9 variant (C172G) associated with udder length and milk density in Awassi ewes, while two nearby SNPs showed no detectable effects. Given annexins roles in membrane organization and lipid handling, ANXA9 remains a biologically credible candidate for marker-assisted selection. Future work should quantify ANXA9 expression/splicing across genotypes and validate associations in larger, multi-flock cohorts with standardized phenotyping.

Declarations

Acknowledgment

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IRB approval

The experimental procedures were reviewed and approved by the Scientific Committee of the Department

of Animal Production, Faculty of Agriculture and Marshlands, University of Thi-Qar, Iraq.

Ethical statement

All animal handling and experimental procedures were conducted in accordance with institutional ethical guidelines for the care and use of animals approved by the University of Thi-Qar. All efforts were made to minimize animal stress and discomfort.

Generative AI and AI-assisted technology statement

The authors declare that no generative artificial intelligence or AI-assisted technologies were used in the design of the study, data collection, data analysis, or writing of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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