Fatty Acid Synthase Polymorphism of Awassi Sheep and its Impact on Fatty Acid Composition

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ABSTRAK

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Kandungan lemak intramuskular ternak ditentukan dengan sintase asam lemak (FASN). Profil asam lemak lemak berhubungan dengan kesehatan manusia. Penelitian ini melakukan eksplorasi hubungan antara polimorfisme gen FASN pada domba Awassi dan pengaruhnya terhadap komposisi asam lemak. Penelitian ini menggunakan 100 ekor domba jantan Awassi yang berumur antara 1 sampai 2,5 tahun. DNA molekuler diisolasi dari setiap sampel darah; genotipe, reaksi pengurutan, dan alat in silico kemudian digunakan untuk mengonfirmasi varian dalam fragmen yang diamplifikasi. Terdapat dua genotipe (GG dan GA) dari gen FASN ovine (exon 2). Novel missense c.186 G>A teridentfikasi dalam genotipe GA. Genotipe GA secara signifikan (P<0,05) meningkatkan lemak intramuskular, kandungan asam lemak tak jenuh yang lebih tinggi, dan kandungan asam lemak jenuh yang lebih rendah dibandingkan dengan genotipe GG. Kumulatif dalam analisis silico menunjukkan efek merugikan dari SNP c.186 G>A pada aktivitas FASN. Genotipe GA intramuskular domba Awassi memiliki kandungan lemak jenuh relatif rendah terhadap lemak tak jenuh. Hasilnya menegaskan bahwa c.186 G>A SNP dalam variasi gen FASN ovine berpotensi berguna untuk menilai sifat-sifat karkas. Di masa mendatang, hal ini dapat mengarah pada pemilihan hewan yang lebih efisien dengan profil asam lemak yang lebih sehat, menghasilkan daging berkualitas lebih tinggi.

Kata Kunci: Domba Awassi, Gen FASN, Komposisi Asam Lemak

ABSTRACT

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Livestock intramuscular fat content is determined by fatty acid synthase (FASN). The fatty acid profile of the fat is of relevance to human health. Thus, this study explores the relationship between the polymorphism of the *FASN* gene in Awassi sheep and its impact on fatty acid composition. The study used 100 Awassi rams, ranging in age from 1 to 2.5 years. Molecular DNA was isolated from each blood sample; genotyping, sequencing reactions, and *in silico* tools were subsequently used to confirm the variants in amplified fragments. The results revealed two genotypes (GG and GA) of the ovine *FASN* gene (exon 2). The novel missense c.186 G>A was identified in the genotype GA. The GA genotype had significantly (P<0.05) increased intramuscular fat, higher unsaturated fatty acid content, and lower saturated fatty acid content than the GG genotype. Cumulative *in silico* analysis indicated a detrimental effect of the SNP c.186 G>A on FASN activity. The intramuscular GA genotype of Awassi sheep had a low saturated content relative to unsaturated fats. The result confirmed that the c.186 G>A SNP in ovine *FASN* gene variation is potentially helpful in assessing carcass traits, and this might lead to a more efficient selection of animals with healthier fatty acid profiles, resulting in higher-quality meat.

Key Words: Awassi Sheep, *FASN* Gene, Fatty Acids Composition

INTRODUCTION

The fatty acid synthase (FASN) gene is a potential candidate for regulating livestock meat fat composition (Kaplanová et al. 2013; Al-Thuwaini & Kareem 2022). The fatty acid composition of livestock meat impacts consumer health (Grzes et al. 2016; Dervishi et al. 2019). High consumption of saturated fatty acids (SFAs) causes high blood cholesterol levels and cardiovascular diseases (Hudson et al., 2020). The FASN gene mapped on chromosome 11 in sheep and on chromosome 19 in cattle that encodes the fatty acid synthase (FASN) enzyme (Kaplanová et al. 2013; Pećina & Ivanković 2021), which organizes de novo biosynthesis of long-chain SFAs (Shi et al. 2019; Otto et al. 2022). Moreover, most fatty acids require the FASN enzyme for animal body fat deposition (Chu et al. 2015). Recent studies revealed that genetic variants in the FASN gene are associated with animal fatty acid composition (Crespo-Piazuelo et al. 2020; Mwangi et al. 2022). A partial explanation for the variation in meat fatty acid composition between animals could be due to these polymorphisms (Esteves et

al. 2019), in which DNA variants in the FASN gene have been found to affect the animal fatty acids content in subcutaneous and intermuscular fat (Zhang et al. 2021). Genetic variation in the FASN gene has been shown to affect the fatty acid composition of livestock fat (Mwangi et al., 2022). These SNPs include SNP g.257C>T within exon 32 in the Czech sheep (Sztankoova et al. 2018), the g.17924G>A SNP in the Hanwoo cattle, the g.17924A>G SNP in Nellore cattle (de Souza et al. 2012), and SNP g.16930T>A in yaks (Chu et al. 2015). Furthermore, the significant influence of FASN g.17924A>G SNP has been confirmed with decreased SFA in commercial crossbred cattle (Kaplanová et al. 2013). Meanwhile, these variations differ between homozygous and heterozygous, which causes the variation in unsaturated fatty acids in homozygous compared with the heterozygous (Chu et al. 2015). The Awassi sheep produce milk and meat, and they are a valuable resource for many resource-poor farmers in the Middle East (Haile et al. 2019). Awassi sheep showed superior meat quality characteristics than other sheep breeds (Suliman et al. 2021). This breed has desirable carcass characteristics and meat quality, mainly due to their fat tail allowing them to have leaner carcasses and trimming fat more efficiently. Compared to more traditional sheep breeds, the fat stored in the tailed may contribute to a lower fat level in various cuts of meat (Oramari et al. 2014; Farah et al. 2019). Due to the above considerations, no research has been conducted on the relationship between the FASN gene and intramuscular fat content in Awassi sheep. Thus, this study investigated the effects of single nucleotide polymorphisms (SNPs) of the FASN gene in Awassi sheep and their influence on fat composition.

MATERIALS AND METHODS

Animal populations, determination of the fatty acid profile, and DNA isolation

The study was conducted following the international recommendations on animal care and use under Al-Qasim Green University's approval (Agri, No. 015,3,12), between October 2017 and June 2018, at the College of Agriculture / Department of Animal Production. This study included 100 Awassi sheep (aged 1-2.5 years and weighing 40 to 60 kg). A selection of animals was made based on three herds in Iraq's middle Euphrates region. Each flock studied included 10-12 rams mated randomly with about 20-25 ewes per ram. Animals were raised on seasonal grass-fed with concentrated foods (2.5% of their body weight, 59% barely, 40% bran, and 1% salt) and freshwater until slaughter. All samples were slaughtered at abattoirs of Babylon. From each animal, the longissimus dorsi (LD) muscle samples (~100 g) were taken at 45 min postmortem between the 12 and 13th ribs, collected, and processed to determine the fatty acid profile. Fatty acid content was analyzed by the high-performance liquid chromatography (HPLC) method, according to Salimon et al. (2017). Then, the values of saturated fatty acids (SFAs) (Caprylic C8:0, Capric C10:0, Lauric C12:0, Myristic C14:0, Palmitic C16:0, Stearic C18:0, and Arachidic acid C20:0), monounsaturated fatty acids (MUFAs) (Ricinoleic C18:1cis, Ricinelaidic C18:1trans, Oleic C18:1n-9, Petroselinic C18:1n-9, Elaidic C18:1n-9, and Vaccenic C18:1n-7), and polyunsaturated fatty acids (PUFAs) (α-linoleic C18:2n6, α-linolenic C18:3n-3, β-linolenic C18:3n-3) were calculated. Blood was collected (5 ml) from the jugular vein to extract genomic DNA by the high salt method of Al-Shuhaib (2017).

PCR amplification and SNP genotyping

Primers were constructed using the Primer-BLAST online server, according to the sequence of the FASN gene for ovine (GenBank accession numbers NC_019468.2). The primer sequences with a product length of 325 bp used to be as follows: FASN,exo2-F: 5′ CAGATGGAGGAGGGGGATCA 3′ and FASN,exo2-R: 5′ GTCCACAGAAGCGGTGA GAA 3′. This PCR experiment consisted of the following steps: 5 min to initial denaturation, 30 cycles for each 30 sec of 95°C denaturation, 62.9°C annealing, and 72°C elongation, followed by 5 min of 72°C final extension. PCR amplicons were verified for specificity using agarose gel electrophoresis before being subjected to Single-Strand Conformation Polymorphism (SSCP) experiments. Genotyping was conducted according to the protocol described by Imran et al. (2020). The SSCP patterns on the gels were visualized by Byun et al. (2009) using silver staining.

DNA sequencing

All genotypes were sequenced downstream using a BioNeer sequencing machine, in Daejeon, South Korea, with forward and reverse primers. The received chromatograms were edited and aligned using DNA Star, EditSeq. / ClustalW, with the sequences published in the GenBank database taken as a reference. The SnapGene Viewer, ver. 4.0.4 (GSL. Biotech. LLC), and ensemble genome browser 96 (https://asia.ensembl.org/index.html), were used to visualize and check the novelty of the observed mutations of the FASN gene.

In silico analysis

Several computational prediction tools were used to assess the implications of the observed missense variants for mutant protein structure, function, and stability. SIFT, a tool that determines whether substitutions affect the functionality of proteins using

sequence homology and amino acid properties; SNAP2, a tool that predicts mutated protein functionality; and I-Mutant2.0, designed to predict protein stability changes based on single point mutations from protein sequences and structures (Imran et al. 2020). The detected nsSNP was virtually visualized using Phere2, ver 2.0, and PyMol-v1, https://www.schrodinger.com/pymol, predicting FASN's 3D structure prior to and subsequent mutation.

Data analyses

The genetic diversity indices of Awassi sheep, including allele frequency, genotype frequency, observed heterozygosity (H*o*), expected heterozygosity (H*e*), the effective number of alleles (N*e*), and Hardy-Weinberg equilibrium, were analyzed using PopGen32 software, v. 1.31. Statistical analysis was conducted using SPSS v23.0 (IBM, NY, USA). Student's t-tests were used with the following model to examine the effects of genotype on various phenotypic traits:

$$Y_{ij} = \mu + \alpha i + e_{ij}$$

where Y_{ij} is the phenotypic trait, μ is the overall mean, αi is the effect of genotype, i^{th} genotype (i = GG, GA), and e_{ij} is random error assumed to be NID (0, $\sigma^2 e$).

Normality was tested using the Kolmogorov–Smirnov test. Preliminary statistical analyses indicated that age, season, and nutrition were not found to affect the fat traits being investigated, and thus, they were not included in the model.

RESULTS AND DISCUSSION

Genotyping and genetic diversity of the *FASN* gene

The ovine FASN gene's genotyping analysis was conducted using PCR-SSCP and DNA sequencing techniques. The SSCP analysis revealed two GG and GA genotype variations within the DNA samples amplified by the ovine FASN (exon 2) (Figure 1). The genetic analysis revealed that the predominant genotype was GA, with a genotype frequency of 79%. Based on the $\chi 2$ tests (Table 1), FASN gene (exon 2) polymorphism in Awassi sheep was not at Hardy-Weinberg equilibrium at this locus. Higher heterozygosity than expected was observed. For the c.186 G>A SNP, the genetic diversity analysis found higher values for Ne and Higher; this reflected a very high genetic diversity for the FASN gene in ovine populations.

The current genotyping results revealed two genotypes (GG and GA) of the ovine FASN gene (exon 2). Sheep with the GA genotype had lower saturated fatty acids, higher IMF (%), and unsaturated fatty acids. Sequencing reactions confirmed that these two SSCP patterns were positioned within GA genotypes. The FASN gene polymorphism exerts a crucial role in lipogenesis and saturated fatty acid synthesis (Inostroza et al. 2013; Renaville et al. 2018; Malgwi et al. 2022). Several researchers have reported the genetic variations of the FASN gene associated with fatty acid content in livestock. Sztankoova et al. (2018) revealed that the SNP g.257C>T in exon 32 of the FASN gene influences fatty acid composition, in which the genotype TT has higher values for MUFAs and polyunsaturated fatty acids compared to the genotype CC in the Czech sheep population. Besides, the SNP g.17924A>G affects fatty acid content in Nellore cattle (de Souza et al. 2012). Furthermore, the SNP g.16930T>A is associated with higher fatty acid content in yaks (Chu et al. 2015).

Sequence and *in silico* analysis of *FASN* gene

Post-PCR genotyping analysis revealed two different genotypes of Awassi sheep. Sequence analysis of the ovine FASN locus identified seven novel SNPs between the two resolved genotypes and the FASN (exon 2) NCBI reference sequences (GenBank accession numbers NC_019468.2). The pattern of each SNP discovered by sequencing is listed in (Supplementary Table 1). Several SNPs were discovered in FASN (exon 2) reference compared to GG and GA genotypes. Four novel SNPs were identified in genotype GA (G 103 C, G 186 A, C 224 G, T 290 C), compared with two novel SNPs in genotype GG (A 109 C, G 111 C). Both genotypes shared one common SNP (G 287 C) (Supplementary Table 1).

Concerning in silico analysis, all the utilized in silico prediction tools were given deleterious signals for c.186 G>A. The I-Mutant 2 tool additionally confirmed the deleterious effects of c.186 G>A, which predicted a reduction in stability of the FASN protein upon mutation with this SNP (Figure 2 and Supplementary Table 2). This amino acid substitution caused a protein with reduced stability and may have had a detrimental effect on the function of the FASN enzyme, which organizes de novo the synthesis of saturated fatty acids.

To verify whether these SNPs cause the deleterious effect on the encoded FASN protein. Various in silico methods were employed to assess the structural and functional consequences of the novel SNPs. Most in silico analyses referred to the detrimental effects of c.186 G>A SNP on the FASN enzyme. Several nsSNPs can modify enzyme function, alter protein structure, or cause protein interaction disruptions (Patel et al. 2015). As the FASN enzyme is responsible for the biosynthesis of SFA (Otto et al. 2022), it is possible to speculate that the g. 50787138 A>G SNP is responsible for the decreased protein capability to undertake its scheduled task of the FASN enzyme. These cumulative deleterious consequences of c.186 G>A were detected only in the

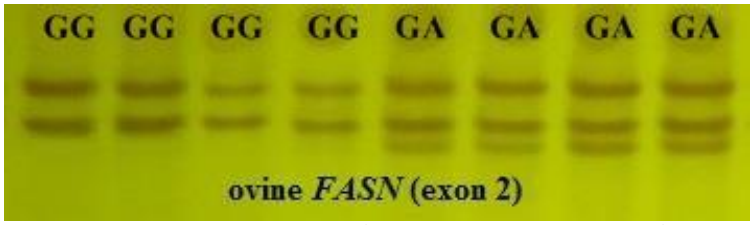

Figure 1. SSCP non-denaturing polyacrylamide gel electrophoresis of ovine *FASN* gene (exon 2) PCR fragments exhibited two SSCP banding patterns in Awassi sheep. SSCP experiment was performed in 10% polyacrylamide gels (37.5:1) at 200 V for 4 h

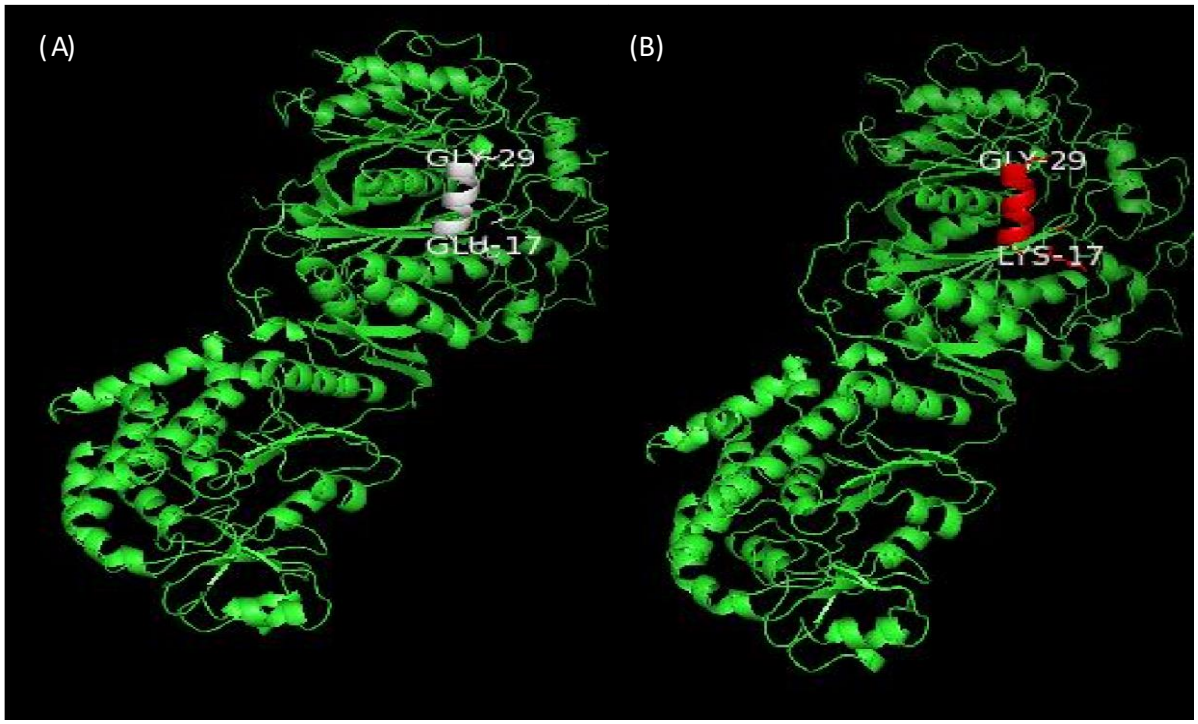

Figure 2. Virtual 3-D structure of ovine FASN. A) Reference type protein (Before mutation), B) mutant protein (in GA genotype)

GA genotype, making them more favored in unsaturated fatty acids content than the GG genotype. This result concord with the study by Bartoň et al. (2021) revealed the association between the FASN gene variant and unsaturated fatty acid content in diverse cattle breeds. Moreover, Otto et al. (2022) demonstrated that g. 50787138 A>G SNP in the FASN gene were associated with a healthier longissimus dorsi fatty acid composition.

FASN (exon 2) gene polymorphism with intramuscular fat content and fatty acid composition

Association analysis for the fatty acid composition in the longissimus dorsi muscle and FASN (exon 2) genotypes is shown in Table (2). A significant association of the FASN (GA) genotypes was observed for the highest content of IMF (%), and lowest content of myristic acid of SFA, total SFA, and with unsaturated fatty acid in which the GA genotype had the highest content of ricinoleic, oleic, total MUFA, PUFA including α-linoleic, and α-linolenic compare to the GG genotypes.

Concerning association analysis, the FASN gene was related to fatty acid content. Compared to the GG genotype, the GA genotype had significantly ($P \leq 0.05$) higher quantities of unsaturated fatty acids and less saturated fatty acids. Fatty acid synthase enzyme participated in de novo lipogenesis in livestock (Bartoň et al. 2021; Otto et al. 2022). A similar study also showed a significant association between FASN levels and fatty acid content, primarily due to FASN polymorphism, resulting in higher mitochondrial oxidation of fatty acids (Mahmoud et al. 2016). Add to that, g. 17924A>G SNP of the FASN gene in Friesian cows is revealed to be associated with the fatty acids composition (Inostroza et al. 2013). Another study showed that three SNPs in the FASN gene are associated with cattle growth and carcass characteristics (de Souza et al. 2012). As a result of the present study, the longissimus dorsi (LD) muscle fatty acid profile is determined concerning the polymorphisms studied, and the results could be used for sheep flock selection: the GA genotype is highly promising for human consumption due to the polymorphism of the FASN gene.

Table 1. Genetic diversity parameters for the *FASN* gene in Awassi breeds.

Genotype frequencies (n)		Allele frequencies		Ho	He	Ne	PIC	$\chi 2$
GA	GG	A	G	0.79	0.48	1.91	0.36	42.01
0.79 (79)	0.21 (21)	0.40	0.60					

Abbreviations= (n) samples number, χ^2 – chi-square, *Ho*= observed heterozygosity, *He*= Expected heterozygosity, *Ne*= effective allele number. All Chi-square tests have one degree of freedom and are within the significance level of P<0.05

Table 2. Relationship between *FASN* (exon 2) gene polymorphism with animal age, carcass weight, IMF (%), and fatty acids composition (% of total FA) in Awassi sheep

Parameters	LSM ± SE		*P* value
	GG	GA	
Animal age at slaughter (year)	2.18 ± 0.07	2.01 ± 0.08	0.53
Carcass weight (kg)	23.32 ± 1.63	22.91 ± 1.04	0.41
IMF (%)	3.06 ± 0.15	5.22 ± 0.35	**0.01**
SFA (% of total FA)			
C8:0	4.09 ± 0.40	3.84 ± 0.12	0.20
C10:0	4.11 ± 0.54	3.88 ± 0.21	0.43
C12:0	1.34 ± 0.16	1.49 ± 0.24	0.16
C14:0	3.73 ± 0.36	2.12 ± 0.19	**0.03**
C16:0	1.90 ± 0.15	1.78 ± 0.27	0.29
C18:0	0.01 ± 0.003	0.03 ± 0.001	0.48
C20:0	1.47 ± 0.11	1.38 ± 0.26	0.13
Total SFA	46.79 ± 2.08	37.41 ± 1.92	**0.03**
MUFA (% of total FA)			
C18:1cis	0.87 ± 0.01	1.18 ± 0.09	**0.01**
C18:1trans	1.52 ± 0.33	1.68 ± 0.26	0.44
C18:1n-9	3.55 ± 0.12	5.74 ± 0.65	**0.01**
C18:1n-9	1.69 ± 0.24	1.52 ± 0.33	0.55
C18:1n-9	0.37 ± 0.08	0.39 ± 0.03	0.13
C18:1n-7	0.11 ± 0.001	0.09 ± 0.002	0.30
Total MUFA	22.20 ± 2.37	29.14 ± 1.83	**0.03**
PUFA (% of total FA)			
C18:2n6	0.65 ± 0.07	1.15 ± 0.23	**0.01**
C18:3n-3	5.01 ± 0.70	4.99 ± 0.64	0.15
C18:3n3	0.41 ± 0.09	0.89 ± 0.02	**0.01**
Total PUFA	12.84 ± 1.75	13.03 ± 2.53	0.20

LSM= Least square mean ±SE standard error, SFA= saturated fatty acid, MUFA= monounsaturated fatty acid, PUFA= polyunsaturated fatty acid. The P-value with statistical significance is indicated in bold numbers

CONCLUSION

The result confirmed that a low SFA content and a high level of MUFA and PUFA characterize the intramuscular fat composition of the GA genotype of Awassi sheep. The c.186 G>A SNP has a considerable negative impact on meat fatty acid composition in Awassi sheep. Therefore, the c.186 G>A SNP in ovine *FASN* gene variation could be useful for assessing carcass traits. Moreover, it may be effective for improving the future direct selection of animals with a healthier fatty acid content, thus improving meat quality.

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