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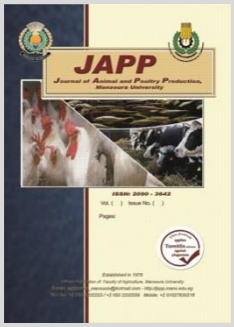
Genetic Polymorphisms of *Fabp4* Gene and Their Association with Milk Traits in Barki Ewes

Sallam, A. M.* and A. H. Ibrahim

Animal and Poultry Breeding Department, Desert Research Center. 1st Mathaf El-Mataryia st., 11753, Mataryia, Cairo, Egypt



ABSTRACT

The fatty acid binding proteins (*FABPs*) were recognized in cattle as promising candidate genes for various economic traits in general and particularly milk. Nevertheless, limited information is available about the contribution of these genes in sheep. Alongside, there is an increasing interest for sheep dairy production worldwide. Single strand conformation polymorphism technique was used to investigate the polymorphisms in *FABP4* gene in Barki sheep breed. Three genetic variants (named *A*, *B* and *C*) were identified with allelic frequencies 34.55%, 31.33%, and 34.12% for the corresponding alleles, respectively. Significant associations were found between the *FABP4* genotypes and fat percentage (*FP*; *P*=0.027), lactose percentage (*LP*, *P*=0.015) and total solid percentage (*TSP*; *P*=0.001) in milk. However, no significant association (*P*>0.05) was reported for milk yield per day (*DMY*) or percentage of the protein (*PP*). Absence of allele A significantly decreased *FP* (-1.28%; *P*=0.04), LP (-1.67%; *P*=0.04), *PP* (-1.36; *P*=0.01), and TSP (-6.66%; *P*=0.01), while allele C tended to increase TSP (+4.82%; *P*=0.06). Worthwhile, ewes with the BC genotype produced more *DMY* (363.48±27.74g/day; *P*=0.26) with higher *TSP* (22.99±1.27%) and *LP* (7.88±0.33%) compared to ewes with other genotypes. Likewise, ewes that carry AC genotypes tended to produce milk with higher *FP* (5.69±0.40%; *P*=0.025) and *PP* (6.70±0.40%; *P*=0.27). Results showed that the *DMY*, *FP*, and *TSP* significantly influenced by the age and parity of ewe. This study presented a valuable information concerning the *FABP4* gene polymorphisms as potential candidate gene underlying genetic markers for milk production in Barki sheep.

Keywords: Single Nucleotide Polymorphism (*SNP*), Fat percentage, Candidate gene, association

INTRODUCTION

Globally, milk production is a trait of interest that was under intensive selection for many years in livestock. Alongside to the unique dairy products of sheep for the human consumption, another concern of milk production as the main source to sufficient feed of the newborn lambs (Abousoliman *et al*., 2020). This is eventually lead to producing healthy lambs until weaning, which is dependent mainly on their dam's milk production and composition (Amel *et al*., 2019). Therefore, including this trait in genetic improvement programs of livestock was maximized during the recent years.

Sheep were the one of the first animals to be domesticated worldwide because they are easy to be managed by humans. This may be because of their smaller size, higher adaptation, behavior, and higher feed efficiency, compared with other livestock (Balthazar *et al*., 2017). Generally, sheep are bred double-purposely in Egypt for mutton and milk production. Barki is highly adaptabted to desert conditions and predominantly located in the north-western coastal region of Egypt (Sallam *et al*., 2019a). It is considered as a main source for milk and meat for a large proportion of the Egyptian community. The breed has been included in several crossbreeding programs with high-productive exotic breeds with little progress (Sallam *et al*., 2019b). Imposing the advanced molecular genetic techniques in selection schemes of Barki sheep may achieve a considerable improvement of the breed characteristics.

Over the last few years, candidate gene approach has been extensively used as an effective tool to identify genetic markers for economic traits in livestock. Single nucleotide polymorphisms (*SNP*s) were used as the most preferable genetic markers in the genome for the trait of interest (Altshuler *et al*., 2010; Bush and Moore, 2012). These *SNP*s may be identified and typed within specific genes of interest. Lastly, the SNPs are related to the studied trait within a group of individuals (Womack *et al*., 2012; Patnala *et al*., 2013).

Hence, these markers may be used to predict the unique individuals in a population early in their life and to identify the biological pathways of the desired trait (Bush and Moore, 2012). The fatty acid binding protein 4 (*FABP4)* is a protein coding gene that binds long-chain fatty acids. They are recognized to be involved in the intracellular transportation. The gene was found to be highly up-regulated expressed during lactation in cattle (Bionaz and Loor, 2008). It has been mapped to the ovine chromosome 9 with 4 exons and spanning 4517 base pairs (www.ensembl.org). Selection for high fat contents in ewes' milk is of interest as it is important to fulfill the nutrition needs of the newborn lambs (Amel *et al*., 2019). The *FABP4* gene is well-known as a leading candidate gene for economically important traits, such as milk production in cattle (Zhou *et al*., 2015), carcass and growth

* **Corresponding author.**
E-mail address: ahmedsallam2@gmail.com
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traits in sheep (Yan *et al*., 2018). Nevertheless, only one report has correlated polymorphisms in this gene with milk traits in Sfakia sheep (Ibrahim *et al*., 2019) in general and it has not been investigated yet in Egyptian sheep. Thus, this is the first report to address the correlation between *FABP4* polymorphisms and milk traits in Egyptian sheep. This information will be used as a useful adjacent to improve milk production in Barki sheep.

MATERIALS AND METHODS

The studied population

This study included about 233 milk-producing ewes during the season of 2017 through 2018. The milk records were collected according to the test-day protocol. Briefly, ewes were milked monthly until full udder empty from the time of parturition till the 3rd or 4th month. After lambs separation from their dams for about 12h, the right half of the udder was milked in the morning, and the other half was left for feeding lambs. This approach was repeated in the evening starting with the other half of the udder. Afterwards, milk yield per day was measured by summation the two-milking multiplied by 2. In addition to the daily milk yield (*DMY*), the composition of the milk was identified by MilkoScan. This included total fat percent (*FP*), total protein percent (*PP*), lactose percent (*LP*), and total solid percent (*TSP*).

Genetic analysis

DNA extraction and *PCR* conditions: For each sample, genomic *DNA* was extracted from blood using Intron-bio (commercial kits, Germany) following the manufactures protocol. Exon3 of the *FABP4* in *Ovis aries* (GeneBank: JX409933.1) gene was amplified using thermal cycler polymerase chain reaction (PCR) using these specific primers; forward: 5`-TTAGATGAAGGTGCTCTGGTACA-3` and reverse: 5`-CTCAGGACTAAACAACTCATGGTT-3` (https:// www.ncbi.nlm.nih.gov/tools/primer-blast/). The amplification (522 bp) was conducted with the following PCR conditions; first step of one cycle of 5 min. at 95°C, then 35 cycles of 1 min at 95°C, 1 min at 59°C, 1 min 30 s at 72°C with final extension of 10 min at 72°C.

FABP4 genotyping and sequencing: *S*ingle strand conformation polymorphism (*SSCP*) technique was used to genotype the amplified sequences in the studied population. About 15-µL aliquot of each PCR product was heated for denaturation for 5 min at 95°C. Immediately, samples were placed in ice and then loaded onto 12% acrylamide, which consists of bis-acrylamide gels. Electrophoresis for 18 h in 0.5× Tris/borate/ethylene diamine tetra acetic acid (*TBE*) buffer at 300 V was conducted in Bio-Rad with water circulation at 10°C. The gels were silver-stained using the method of Zhou *et al*. (2015). PCR products representing different *SSCP* banding patterns were sequenced using the BigDye terminator protocol (Macrogen, Seoul, Korea) to identify the polymorphic *SNP*s,

Association analysis

To test the associations between the ovine *FABP4* genotypes and the studied traits, the following general liner model implemented in SAS (2004) was implemented:

$$Y_{ijklm} = \mu + G_i + P_{sj} + A_k + e_{ijklm},$$

Where, Y_{ijklm}**=the studied trait;** μ**=the overall mean;** G_i**=the effect of genotype;** P_{sj}**=the effect of parity (4 levels);** A_k**=the effect of age of the ewe (4 level: 1st level= animals at 2 and 3 years old, 2nd level= animals at 4 and 5 years old, 3rd level= animals at 6 and 7 years old, 4th level= animals above 8 years old; and** e_{ijklm}**=random errors.**

RESULTS AND DISCUSSION

Effect of age of ewe and parity on the studied traits

The effects of age of the ewe and parity were significant on *DMY, FP* and *TSP* (Table 1). Ewes at 3 and 4 years old tended to produce the highest DMY (286.87±31.37g/day) followed by ewes at 5 and 6 (253.47±41.76g/day), while the *DMY* was the lowest from ewes above 8 years old (174.01±82.68 g/day). Similarly, the *TSP* tended to decrease in milk produced from older ewes, as it was 17.81±1.46%, 17.43±1.95% and 10.66±3.53%, for the corresponding levels of age, respectively. Conversely, the *FP* in milk was the lowest at ages 3 and 4 years old (4.53±0.55%) followed by 5 and 6 years old ewes (4.59±0.41%) while it was the highest at the above 8 years old ewes (6.92±0.99%).

Likewise, the effect of parity of the ewe on milk traits was highly significant ($p<0.01$). Ewes at third parity tended to produce the highest *DMY* (340.62±32.46g/day) followed by ewes at the second parities (256.33±34.31g/day), while the ewes at the fifth parity produced the lowest *DMY* (127.36±94.21g/day). Similarly, ewes in the first parities produced the highest *TSP* in milk (21.78±2.11%) compared to those who are in the fifth parity, which produced the lowest *TSP* (3.60±4.05%). Conversely, ewes in the second parity produced the highest *FP* in milk (6.63±0.45%) and ewes at the third parity were the lowest *FP* producers (5.04±0.43%). Nevertheless, the *PP* and *LP* were not significantly influenced ($p<0.05$) by any of these factors.

Table 1. Effect of age of ewe and parity on milk traits in Barki ewes

Factor	N	Traits				
		DMY	FP	PP	LP	TSP
Age of ewe						
1st	17	253.47±41.76	4.53±0.55	6.35±0.54	6.97±0.65	17.43±1.95
2nd	48	286.87±31.37	4.59±0.41	6.29±0.41	7.08±0.48	17.81±1.46
3rd	13	137.12±40.9	6.18±0.54	5.97±0.54	7.37±0.64	17.40±1.93
4th	14	218.77±46.22	6.61±0.59	7.34±0.59	7.75±0.70	11.47±2.11
5th	4	174.01±82.68	6.92±0.99	7.63±0.99	6.96±1.17	10.66±3.53
Significance		0.031*	0.001**	0.15	0.87	0.039*
Ewe parity						
1st	14	206.38±43.03	5.87±0.59	6.11±0.59	6.73±0.70	21.78±2.11
2nd	32	256.33±34.31	6.63±0.45	6.54±0.45	6.99±0.53	20.58±1.61
3rd	30	340.62±32.46	5.04±0.43	5.83±0.42	7.0±0.50	18.93±1.52
4th	17	139.53±41.15	5.40±0.50	6.89±0.50	5.99±0.60	9.89±1.80
5th	3	127.36±94.21	5.90±1.14	8.20±1.14	9.37±1.35	3.60±4.05
Significance		0.001**	0.035*	0.28	0.17	0.0001**

[1] N=96 ewes. [2] MY=daily milk yield (g/day), FP=fat percentage, PP=protein percentage, LP=lactose content, TSP=total solid percentage. [1]Predicted least square means ± standard errors from GLMs. * Significance estimated at P < 0.05 and P < 0.01 for the highly significant.

Phenotypic correlations between the studied traits

Results of the phenotypic correlation between the studied milk production traits in Barki ewes are presented in Table 2. Negative correlations were reported between *DMY* and *FP* and *PP*, however, this correlation was not significant. In contrary, positive significant (P<0.01) correlations were reported between all of the studied traits. Overall, significant correlations between milk yield and composition were reported in different livestock species, which is consistent with those estimated in the current study in Barki sheep. Negative correlations were estimated between *DMY* and *FP* (r=-0.16) and *PP* (r=-0.12). However, slightly higher estimates, were reported in Najdi (Ayadi *et al*., 2014) and D'man sheep breeds (Amel *et al*., 2019).

Table 2. Phenotypic correlation among the production and composition of milk in Barki sheep[1]

Trait[2]	DMY	FP	PP	LP	TSP
DMY	1				
FP	-0.16 (0.07)	1			
PP	-0.12 (0.22)	0.61 (>0.001)	1		
LP	0.042 (0.6)	0.29 (0.001)	0.28 (0.001)	1	
TSP	0.33 (0.001)	0.16 (0.07)	0.24 (0.006)	0.29 (0.001)	1

[1] P-values for the correlation coefficients presented within brackets. [2] DMY=daily milk yield (g/day), FP=fat percentage, PP=protein percentage, LP=lactose percentage, TSP=total solid percentage. * Significance estimated at P <0.05.

Genetic polymorphisms of the *FABP4* in Barki sheep

The *PCR-SSCP* patterns identified three conformation banding patterns (Figure 1; A, B and C) in the FABP4 in the investigated ewes. The allele frequencies were 34.2%, 31.3%, and 34.5% for alleles A, B and C, respectively. Likewise, the genotypic frequencies were 21%, 11%, 14%, 23%, 16.6%, and 14.4% for the genotypes AA, AB, BB, BC, AC and CC, respectively. DNA sequencing results identified two variants in the intronic region of the gene (SNPs; *rs606077535* (ENSOART00000010169.1 :c.349- 108C > T; 57536683bp and *rs422473198* (ENSOART00000010169 .1:c.348+85G>A; 57536954 bp) in the amplified sequence. Our results showed quite lower polymorphism in the investigated region of FABP4 in Barki sheep. Only three DNA variants (A, B and C) were identified here compared with four variants identified in different sheep breeds (Yan *et al*., 2012) and Sfakia sheep (Ibrahim *et al*., 2019). However, the same polymorphism was identified in the same region in dairy cows in New Zealand (Zhou *et al*., 2015) with quite similar *SSCP* patterns. Furthermore, Yan *et al*. (2018) identified five variants in Romney sheep. This lower polymorphism may be due to higher selection pressure for the gene in Barki (Zhou *et al*., 2017; Sallam, 2021), which decreases the genetic variations in the studied individuals in the corresponding genomic region. It could be attributable also to the high similarity of the allelic frequency of *FABP4* reported in this study in Barki sheep, due to inbreeding (Abousoliman *et al*., 2020).

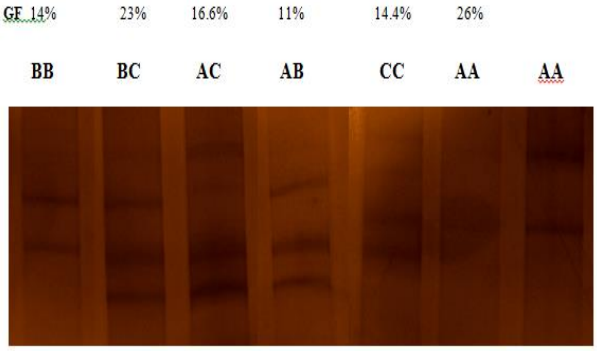

Figure 1. SSCP patterns of FABP4 gene in Barki sheep. It represents A, B and C alleles with different genotypes and the genotypic frequencies (GF) for the corresponding genotypes.

Effect of presence/absence of the *FABP4* gene variants on the studied traits

Effect of presence/absence model of the *FABP4* gene variants on milk traits is illustrated in Table 3. Absence of allele A significantly decreased *FP* (presence: 2.87±0.25, absence: 4.15±0.3; *P*=0.04), *PP* (presence: 3.49±0.29, absence: 4.85±0.35; *P*=0.01), *LP* (presence: 3.98±0.33, absence: 5.65±0.4; *P*=0.04) and *TSP* (presence: 9.96±1.01, absence: 16.62±1.22; *P*=0.01), while allele C tended to increase *TSP* (presence: 15.70±1.07, absence: 10.88±1.17; *P*=0.06). Nonetheless, this model did not show any significance with B allele. None of these variants influenced significantly *DMY* in Barki sheep.

Table 3. Association of presence or absence of the FABP 4 gene variants with milk traits in Barki ewes

Trait	Variant	Present	Absent	Effect[1]	P-value[2]
		Mean±SE	Mean±SE		
DMY	A	310.62±17.91	345.30±21.69	-35.1	0.42
	B	334.77±21.4	321.15±18.22	13.62	0.82
	C	336.22±18.95	319.63±20.79	16.59	0.78
FP	A	2.87±0.25	4.15±0.3	-1.28	0.04*
	B	3.65±0.3	3.37±0.25	0.28	0.77
	C	3.96±0.26	3.06±0.29	0.9	0.21
PP	A	3.49±0.29	4.85±0.35	-1.36	0.01**
	B	4.17±0.34	4.17±0.29	0	0.46
	C	4.59±0.35	3.74±0.33	1.19	0.64
LP	A	3.98±0.33	5.65±0.4	-1.67	0.04*
	B	5.03±0.39	4.6±0.33	0.43	0.77
	C	5.34±0.35	4.29±0.38	1.05	0.31
TSP	A	9.96±1.01	16.62±1.22	-6.66	0.01**
	B	14.2±1.2	12.38±1.03	1.82	0.45
	C	15.70±1.07	10.88±1.17	4.82	0.06

SE, standard errors; DMY, daily milk yield (g/d); FP, fat percentage; PP, protein percentage; LP, lactose percentage; TSP= total solid percentage, [1] Increase (+) or decrease (-) of the corresponding trait, [2] Predicted least square means±SE from general linear models. Significance estimated at * p< 0.05, ** p< 0.01.

Effect of variations in *FABP4* gene on milk traits

Significant association was found between the *FABP4* genotypes and *FP* (*P*=0.027), *LP* (*P*=0.015) and *TSP* (*P*=0.001) in milk. However, the effect of *FABP4* genotypes was not significant (*P*>0.05) on *DMY* or the protein percent in milk (Table 4). Results also suggested that ewes with the BC genotype tended to produce more milk (*DMY* = 363.48±27.74g/day; *P*=0.26) with higher

TSP (22.99±1.27%) and *LP* (7.88±0.33%) compared with ewes with other genotypes. Likewise, ewes that carry AC genotypes tended to produce milk with higher protein percent (6.70±0.40%; *P*=0.27) and fat percent (5.69±0.40%; *P*=0.025).

Table 4. Effect of the FABP4 genotypes on the production and composition of milk in Barki ewes[1].

Trait[2]	Genotypes						P-value*
	AA (48)	AB (25)	BB (33)	BC (55)	AC (40)	CC (32)	
MY (g/day)	267.63±30.81	267.57±63.79	349.73±43.58	363.48±27.74	318.57±36.83	312.62±42.19	0.26
FP	4.12±0.33	3.78±0.69	4.71±0.47	5.46±0.3	5.69±0.4	4.14±0.46	0.027*
PP	5.11±0.33	5.15±0.69	5.44±0.44	6.24±0.3	6.7±0.4	5.61±0.46	0.27
LP	5.62±0.37	6.86±0.77	6.32±0.52	7.88±0.33	7.36±0.44	5.77±0.51	0.015*
TSP	12.96±1.96	16.54±2.94	17.45±2.00	22.99±1.27	21.7±1.69	18.01±1.94	<0.001**

[1]N=233 Number of ewes. [2]MY=daily milk yield (g/day), FC=fat percentage, PC=protein percentage, LC=lactose percentage, TSC=total solid percentage. * Significance estimated at P <0.05. ** Significance estimated at P <0.001.

The association analyses showed that polymorphisms in the investigated region of *FABP4* significantly influenced *FP*, *LP* and *TSP* but no significant effect on DMY, in Barki ewes. Ewes that carry BC genotypes tended to produce more milk that contains higher *TSP* (22.99±1.27%) and *LP* (7.88±0.33%) compared to ewes with other genotypes carriers. Likewise, ewes that carry AA genotypes tended to produce milk with the lowest *PP* (5.11±0.33%), *LP* (5.62±0.37%) and *TSP* (12.96±1.96%) and the lowest *FP* (3.78±0.69%) was produced from the AB carriers. These results were quite consistence with the presence-absence model. The absence of A allele influenced negatively *FP* (-1.28%; *P*=0.04), *PP* (-1.36; *P*=0.01), lactose percent (-1.67%; *P*=0.04) and total solid percent (-6.66%; *P*=0.01), while allele C tended to increase *TSP* (+4.82%; *P*=0.06).

In agreement, Ibrahim *et al.* (2019) reported the same effects of *FABP4* in Sfakia sheep. Confirmation of the same effects of the *FABP4* variants in two different population strongly emphasizes the gene as a putative genetic marker to select the elite individuals in milk traits. Importantly, this is consistent with the physiological function of the gene, which is known as it binds long-chain fatty acids. Moreover, they are involved in their intracellular transport. It was suggested as one of the most interesting candidate gene affecting fat composition in milk (Marchitelli *et al*., 2013). Similarly but not directly equivalent, the *FABP4* haplotypes were correlated with fatty-acid profiles with no significant effect on milk yield in bovine milk (Nafikov *et al*., 2013). Despite the negative effect of *FABP4* on *DMY* in Barki sheep, the significant associations with other milk composition make it particularly appropriate for manufacturing of dairy products (Balthazar *et al*., 2017).

In conclusion, our results showed a significant influence (P<0.05) of the *FABP4* polymorphisms on milk composition (*FP*, *LP* and *TSP*). However, the influence of these variants on *DMY* may be detected by further investigations with a larger population number. Accordingly, the *FABP4* may be putative candidate gene to improve milk production in sheep.

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الأنماط الجينيه لجين *FABP4* وعلاقتها بصفات اللبن في نعاج البرقي

أحمد محمد عبدالعظيم سلام و عادل حسيني محمد إبراهيم

قسم تربيه الحيوان والدواجن، مركز بحوث الصحراء – المطريه - مصر

تم التعرف علي جينات *FABP4* كجينات مرشحه محتمله لصفات متنوعه في الحيوانات وخاصه صفات اللبن. ومع ذلك فان المعلومات المتاحه عن مساهمه هذه الجينات في صفات اللبن في الأغنام تعتبر محدوده علي الرغم من تزايد الاهتمام بمنتجات الالبان من الأغنام علي مستوي العالم. استخدمت طريقه Single Strand Conformation Polymorphism لتحديد التعدد الاليلي في سلاله الاغنام البرقي. تم التعرف علي ثلاثه اليلات هي A وB وC وكان تكرارها كالتالي ٣٤,٥٥٪ و٣١,٣٣٪ و٣٤,١٢٪، علي الترتيب. كان للتركيب الوراثي تأثيرا معنويا على كل من نسبه الدهن واللاكتوز والجوامد الصلبة الكلية في اللبن، بينما لم يكن تأثيره معنويا على كل من كميه اللبن اليومية المنتجة ونسبه البروتين في اللبن. أظهرت النتائج أيضا الي ان غياب الاليل A ادي الي خفض نسب كل من الدهن (-١,٢٨٪) والبروتين (-١,٢٦٥٪) واللاكتوز (-١,٦٧٪) والجوامد الصلبة الكلية (-٦,٦٦٪) في اللبن، بينما تسبب وجود الاليل C في زيادة نسبه الجوامد الصلبة الكلية (+٤,٨٢٪) في اللبن. الجدير بالذكر ان الحيوانات التي تحمل التركيب الوراثي BC تميل الي انتاج كميه أكبر من اللبن (٣٦٣,٤٨ ± ٢٧,٧ جرام / يوم) وبمحتوي اعلي من الجوامد الصلبة الكلية (٢٢,٩٩ ± ١,٢٧٪) واللاكتوز (٧,٨٨ ± ٠,٣٣٪) مقارنه بالحيوانات التي تحمل تراكيب وراثيه اخري. بالمثل فان الحيوانات التي تحمل التركيب الوراثي AC تميل الي انتاج لبن به نسب اعلي من الدهن (٥,٦٩ ± ٠,٤٪) والبروتين (٦,٧ ± ٠,٤٪). تشير النتائج أيضا الي ان كل من عمر الام وعدد مرات الانجاب له تأثيرا معنويا على كل من كميه اللبن اليومية ونسبه الدهن والجوامد الصلبة الكلية في اللبن. تقدم هذه الدراسة معلومات مهمه متعلقة بإمكانيه اعتبار جين *FABP4* كجين مرشح محتمل للانتخاب لصفات انتاج اللبن في اغنام البرقي.