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DNA Methylation Level of Gene *SIRT1* in Ram Spermatozoa and Relationship with Fertilizing Ability According to Breed and Age

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ABSTRACT

***Background*:** Effect of the epigenetic factors on the male fertility is well proofed. Sperm acts as a carrier of genetic material, and its DNA methylome can affect maternal pregnancy rate and offspring phenotype. However, the research on the DNA methylation in the spermatozoids of livestock males, in particular rams, is still limited. To best of our knowledge the data about as a global as well as gene specific DNA methylation in ram spermatozoa from different breeds and ages are missed in the scientific literature. The present study was designed to analyze the relationship between methylation levels of the important for spermatogenesis gene *SIRT1* in spermatozoa and fertilizing ability of sperm in rams from different breeds and ages.

***Materials, Methods & Results*:** The ejaculates of 16 rams from Lacaune, East Friesian and Assaf breeds at age between 18 to 96 months were evaluated. The kinematic parameters of 2 semen samples from each animal were estimated by CASA. The separated spermatozoa were used for DNA extraction followed by bisulfite conversion. The DNA methylation of *SIRT1* was detected through quantitative methylation-specific PCR using 2 sets of primers designed specifically for bisulfite-converted DNA sequences to attach methylated and unmethylated sites. The breed and age effect on the gene *SIRT1* methylation by ANOVA was estimated. Experimental females included 393 clinically healthy milk ewes (Lacaune, n = 131; East Friesian sheep, n = 100 and Assaf, n = 162) in breeding season. Reproductive performances (conception rate at lambing, lambing percentage and fecundity) of ewes, inseminated by sperm of the investigated rams, were statistically processed. ANOVA showed that the animal breed influences significantly on the level of DNA methylation of gene *SIRT1* in ram spermatozoa ($P = 0.002$) An average value of DNA methylation of *SIRT1* in ram sperm from Lacaune breed was significantly higher than in Assaf and East Friesian (81.21 ± 15.1% vs 36.7 ± 14.2% and 38.3 ± 18.6 respectively, $P < 0.01$). The highest percent of *SIRT1* methylation was observed in old animals compared to the young and middle-age. Moderate and strong correlations (r from 0.44 to 0.71, $P < 0.05$) between the methylation level of the *SIRT1* gene in rams' sperm and reproductive parameters of inseminated ewes in all breeds were established.

***Discussion*:** Our data are the first message about the effect of breed on the specificity of DNA methylation of gene *SIRT1* in ram spermatozoa. These results demonstrated an existence of the sheep breeds with high and low level of DNA methylation of gene *SIRT1* in ram sperm. Although the effect of age on the methylation level in sperm is still discussable, our results showed a moderate correlation between age and methylation level of *SIRT1* in spermatozoa of rams. Taking into account that DNA methylation in sperm is stabilized with puberty onset and is a heritable epigenetic modification, it can be a promising marker of sperm quality in animal breeding. In all investigated breeds the rams with relatively high level of DNA methylation of gene *SIRT1* in spermatozoa (50-68%) demonstrated a high conception rate at lambing (> 70%). In conclusion, the DNA methylation level of the *SIRT1* gene in ram spermatozoa is determined by both the breed and the age of the animals and correlates with fertilizing ability of sperm.

Keywords: *SIRT1* methylation, ram spermatozoa, Lacaune, East Friesian, Assaf breeds.

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INTRODUCTION

Effect of the epigenetic factors on the male fertility is well proofed [3,14,19,26,32,33]. DNA methylation is one epigenetic event that controls gene expression, without altering the DNA sequence [10]. Sperm acts as a carrier of genetic material, and its DNA methylome can affect maternal pregnancy rate and offspring phenotype [18]. However, the studies on the DNA methylation in the spermatozoids of livestock males, in particular rams, are still limited [23,24,27]. There are few investigations in boars and bulls, but not in rams, confirmed correlation between site-specific sperm DNA methylation and reproductive efficiency [8,15,33].

The gene *SIRT1* codes a nicotinamide adenine dinucleotide (NAD)-dependent deacetylase that belongs to the Sirtuin family and regulates different transcription factors, and signal transduction molecules in a variety of cellular processes [25]. *SIRT1* also plays a very important role in the synthesis and secretion of sex hormones, participates in several key stages of spermatogenesis and sperm maturation [13] and its repression disturbs sperm morphology and acrosome biogenesis, and leads to male sterility [2,9,17]. The data on *SIRT1* methylation status in sperm is limited.

The DNA methylation in sperm, as the most stable epigenetic modification, is important for livestock genomic selection [5,6,20]. However, the data about as a global as well as gene specific DNA methylation in ram spermatozoa from different breeds and ages are missed. The present study was designed to analyze the relationship between methylation level of the important for spermatogenesis gene *SIRT1* in spermatozoa and its fertilizing ability in ram from different breeds and ages.

MATERIALS AND METHODS

Experimental animals

The experiment was carried out in an agreement with all requirements for welfare and animals protection showed in Bulgarian legislation The study was performed during breeding season (September - October).

Males

Semen were collected from 16 clinically healthy milk breed rams (Lacaune, n = 6; East Friesian sheep, n = 4 and Assaf, n = 6), housed in intensive sheep farms, located in south Bulgaria. Animals were aged from 14 to 96 months, body weight according to the breed (75-90 kg, 80-85 kg and 70-80 kg for Lacaune, East Friesian and Assaf breed, respectively), and housing technology free-stalls in boxes. The feeding was in accordance with the recommendations for specific breed and age of the males with water intake *ad libitum*.

Females

Experimental females included 393 clinically healthy milk ewes (Lacaune, n = 131; East Friesian sheep, n = 100 and Assaf, n = 162) in breeding season, belonging to the same sheep farm, aged 24-36 months, weighing from 65-70 kg, housed in groups in free-stalls technology. The feeding was in accordance with the recommendation for specific breed and age of animals with water intake *ad libitum*. All ewes were at the end of lactation with milk production < 200 mL per day.

Semen collection, primary assessment and CASA analysis

Two ejaculates per ram through 30 min interval were collected by the artificial vagina method in presence of sheep in estrus. Immediately after collection each ejaculate was submitted to primary assessment by experienced operator and only these responding to requirements for fresh ram semen (volume > 0.5 mL normal color and transparency, consistency dense and smell specific for animal species) were used. After that the semen samples were placed in a water bath at 37°C, equal quantities of each ejaculate were analyzed by CASA (SCA® 2002)[1] and the rest semen was used for DNA analysis. The concentration of spermatozoa (x109/mL) and the most important motility parameters were evaluated.

Analysis of DNA methylation of SIRT1 in ram spermatozoa

Two semen samples from each ram were used for analyzing the methylation status of gene *SIRT1*. Each sample was divided in 2 parts for the analyzing of intra-sample variations. In total 64 probes were investigated. The spermatozoa were separated by centrifugation, mixed with somatic cell lysis buffer to avoid DNA contamination, and washed twice with PBS.

The detailed method is described by Abadjieva *et al*. [1]. Briefly, the genomic DNA was extracted from the spermatozoa pellets using Qiazol Lysis Reagent[2], following the manufacturer's instructions. Spectrophotometric control of the quantity and purity of genomic DNA was done by NanoDrop 1000[3]. The extracted

DNA samples were treated with sodium bisulfite using the EpiTectBisulfite Kit[2] in accordance with producer's recommendations. DNA methylation of the *SIRT1* gene was analyzed using SYBR green-based quantitative methylation-specific PCR [31]. Two sets of primers, designed specifically for bisulfite-converted DNA sequences to attach the methylated and unmethylated sites, were used as follow: *SIRT1* Ram MSP Methylated, forward GTATGTTGTAGTTTATGGGGTCG and reverse TACTCCTTTTAATCTTAAATTCGCT-3, and SIRT1 Ram MSP Unmethylated forward GGTATGTTGTAGTTTATGGGGTTGT and reverse ACTCCTTTTAATCTTAAATTCACT [21].

For the PCR, the bisulfite-treated DNA template was mixed with the SYBR qPCR Kit[4] and a pair of primers. The PCR conditions included initial denaturation at 95°C for 10 min and 40 cycles of 2 step PCR at 95°C for 15 s and 60°C for 60 s. The PCR reactions were performed using a cycler real-time PCR instrument (Agilent Stratagene Mx3005P)[5]. After PCR amplification, a dissociation curve was generated to confirm the PCR methylated and unmethylated products. The percentage of DNA methylation in a sample was estimated using the following formula: Methylated DNA (%) = 1/(1+2-ΔCt)*100, where ΔCt = CtU–CtM, CtU being the threshold cycle for the unmethylated primers and CtM being the threshold cycle for the methylated primers [12]. Each measurement was performed in triplicate. Data are presented as a mean ± standard error of mean (SEM).

Estrus synchronization, artificial insemination and reproductive parameters recording and calculation

All animals were submitted to estrus synchronization by intravaginal sponges with 30 mg flurogeston acetate (Syncro-part® 30 mg)[6] for 12 days and injection of 500 UI PMSG (Folligon®)[7] on day of sponge removal. The artificial insemination with fresh-diluted semen was made from 56 to 58 h after the sponge withdraw. A single inseminating dose of 0.2 mL contained 200 x 10^6 motile spermatozoa, deposed into the cervix.

After lambing of ewes the following parameters were recorded: number of ewes with a single fetus, twins or triplets and a total number of lambs born. Conception rate at lambing (number of fertilized ewes/number of inseminated ewes), lambing percentage(total number of born lambs/number of inseminated ewes) and fecundity (total number of born lambs/number of lambed ewes) were calculated according to Olivier [22]. Also a percent of ewes with multiply pregnancy was estimated. The conception rate at lambing was used as an indicator of ram fertility.

Statistical analysis

The data obtained from analyses of semen parameters and *SIRT1* methylation level were processed by the statistical program Statistica (Data Analysis Software System) version 10.08. The parameters are presented as mean ± standard error of mean. Initially, the data were tested for normal dispersion distribution by the Kolmogorov-Smirnov test. Analysis of variance (ANOVA) was used to assess the statistical significance of the influence of breed and age on the methylation level of the *SIRT1* gene. The strength of the relationship between these traits was assessed by Pearson's linear correlation test. The significance of the differences between the mean values for different breeds was compared using the posthoc Tukey test. Statistical significance is considered at $P < 0.05$.

RESULTS

Significant difference between means of the semen parameters such a volume, sperm concentration and motility were not found (Table 1). A lower percentage of progressively motile sperm was observed in Lacaune animals compared to the East Friesian and Assaf breeds.

The distribution of the DNA methylation level of gene SIRT1 in ram's spermatozoa from all investigated breeds is presented on (Figure 1). The variation of this parameter waves widely from 6 to 100% within whole ram's group (Coefficient of variation =56.03%) while the most part of samples range from 60 to 100%.

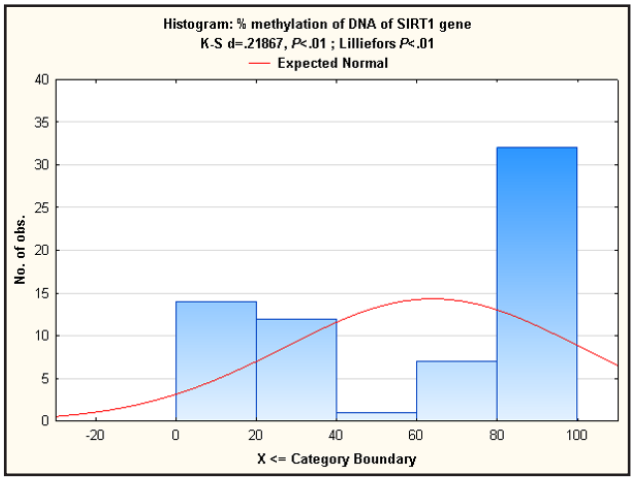

Figure 1. Distribution of variances of DNA methylation of gene *SIRT1* in spermatozoa of investigated rams.

ANOVA showed that the animal breed influences significantly on the level of DNA methylation of gene *SIRT1* in ram spermatozoa (Figure 2). Lacaune males have higher levels of DNA methylation of *SIRT1* gene in sperm compared to Assaf and East Friesian breeds (Table 2). Although the analysis of variance did not show the reliability of the effect of age on the methylation of *SIRT1* (Figure 2, $P = 0.63$), the significant difference between these parameters in adult and middle-aged animals was proved by a post-hoc Tukey test (Table 3).

Additionally, the correlation analysis showed a moderate positive relationship ($r = 0.3$, $P < 0.05$) between the age and the level of methylation of *SIRT1* in ram sperm (Figure 3).

No close correlation between the kinematic parameters of the spermatozoa and the DNA methylation level of *SIRT1* gene was established. However, the lambing results of ewes, inseminated with semen from the investigated rams, showed the presence of certain relationships between the DNA methylation level of *SIRT1* and reproductive parameters. Taking into account the breed's effect on the DNA methylation level of *SIRT1*, the data were analyzed for each breed separately. The individual variation of this parameter in rams allowed the distribution of animals into groups with high and low methylation of *SIRT1*, according to the absolute values of the parameter in each breed (Tables 4, 5 and 6).

The results show that most of the reproductive parameters differ significantly in ewes inseminated with sperm of high and low DNA methylation levels of gene *SIRT1*. Both very high (> 80%) and very low levels (< 30%) lead to decrease in the conception rate at lambing (Tables 4, 5 and 6).

The higher level of DNA methylation of gene *SIRT1* corresponds in all breeds with higher percent of lambing, fecundity and percent of ewes with multiply pregnancies. The correlation analysis performed for the Lacaune breed showed that there is a moderate ($r = 0.44$, $P < 0.05$) and strong ($r = 0.52$, $P < 0.05$) relationship between the level of DNA methylation of *SIRT1* gene in sperm and the percentage of lambing and fecundity. For the Assaf breed and East-Friesian breed a strong relationship between the methylation of the *SIRT1* gene in sperm and conception rate at lambing was observed ($r = 0.53$, $P < 0.05$ and $r = 0.71$, $P < 0.05$ respectively).

The obtained data demonstrated that variations of the DNA methylation level of the *SIRT1* gene in ram spermatozoa are determined by both the breed and the age of the animals, with the effect of breed affiliation being higher (power of effect for breed = 0.85; for age = 0.25, $\alpha = 0.05$). Also, for the 3 breeds, correlations were found between the level of DNA methylation of the *SIRT1* gene in rams' sperm and the reproductive parameters of inseminated ewes.

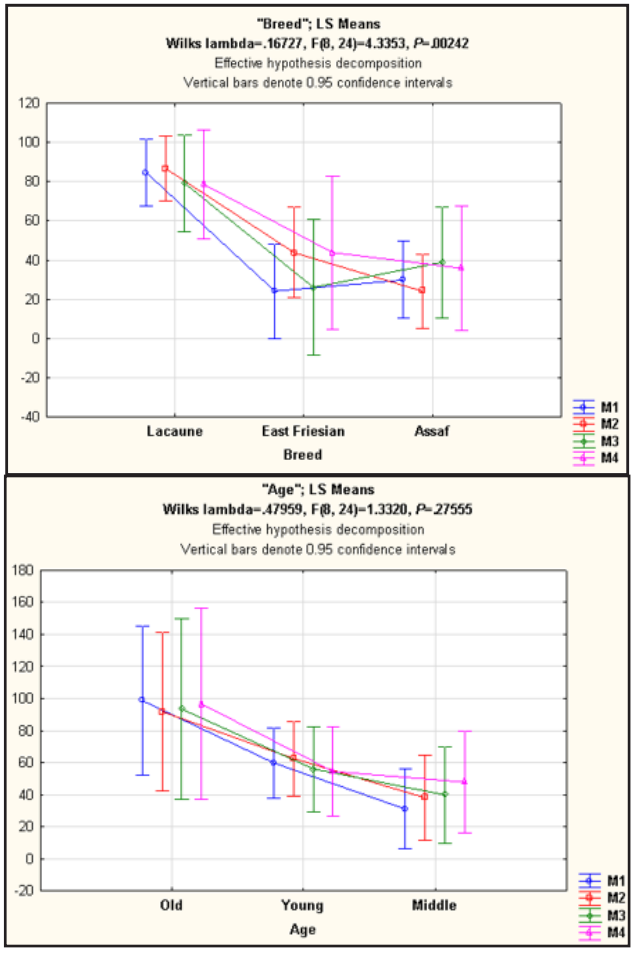

Figure 2. Effect of breed and age on the DNA methylation level of gene *SIRT1* in ram spermatozoa. M1-M4 - number of measurements per ram.

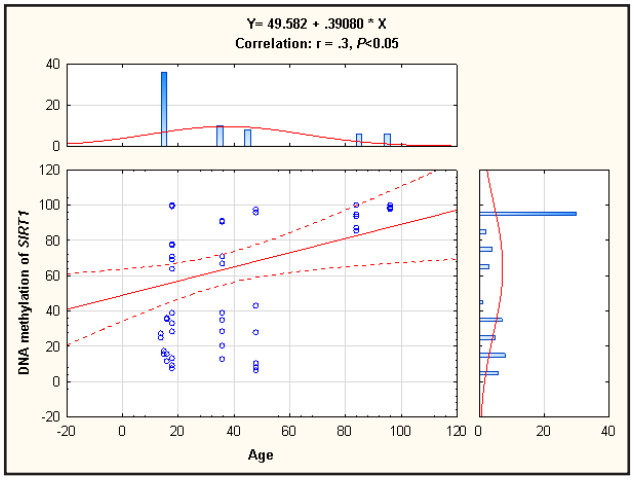

Figure 3. Relationship between DNA methylation level of gene *SIRT1* in spermatozoa and age in whole group of rams.

Table 1. Semen parameters of rams from Lacaune, East-Friesian and Assaf breeds.

Breed	Ejaculate parameters		Kinematic parameters of spermatozoa			
	Volume (mL)	Concentration (10^9/mL)	Motility (%)	Progressive motility (%)	Non-progressive motility (%)	Static (%)
Lacaune (n = 6; N = 12)	1.22 ± 0.33	2.21.6 ± 0.21	76.5 ± 16.8	22.06 ± 3.5*	54.4 ± 13.4	23.4 ± 16.5
East-Friesian (n = 4; N = 8)	1.22 ± 0.36	2.68 ± 1.14	93.8 ± 5.57	70.27 ± 19.70	23.5 ± 14.27	6.22 ± 5.47
Assaf (n = 6; N = 12)	1.24 ± 0.55	2.1 ± 0.84	86.8 ± 5.6	64.9 ± 3.74	21.8 ± 6.14	13.2 ± 5.61

n: number of animals; N: number of samples; *$P < 0.05$

Table 2. DNA methylation of gene *SIRT1* in spermatozoa of rams from different breeds.

Breed	Average age (months)	DNA methylation of gene SIRT1 (%)						*P* value (Posthoc Tukey test)
		mean	SEM	CV	min	max	median	
Lacaune (n = 6; N = 24)	39.6	81.21[a]	15.1	37.35	7.5	99.9	98.85	
East Friesian (n = 4; N = 16)	40.8	38.3[ab]	18.6	99.8	5.9	90.7	16.2	0.0016
Assaf (n = 6; N = 24)	30.4	36.7[ac]	14.3	80.69	11.1	97.4	28.05	0.0013

n: number of animals; N: number of samples; CV: coefficient of variation; [a,b,c]Within the same column, groups labelled with 2 different superscript are significantly different.

Table 3. DNA methylation level of gene *SIRT1* in spermatozoa of rams at different age.

Age, months	DNA methylation of gene SIRT1 (%)					*P* value (Posthoc Tukey test)
	mean	SEM	CV	min	max	
Young, 14 -18 (n = 8; N = 32)	64.35[a]	19.5	60.48	7.5	99.9	
Middle-aged, 36 - 48 (n = 6; N = 24)	43.24[b]	16.7	77.53	7.76	97.4	
Old, 84 - 96 (n = 2; N = 8)	95.99[cb]	2.5	5.24	85.3	99.9	0.0093

n: number of animals; N: number of samples; CV: coefficient of variation; [a,b,c]Within the same column, groups labelled with 2 different superscript are significantly different.

Table 4. Reproductive parameters of ewes from Lacaune breed inseminated with sperm of high and low methylated DNA of gene *SIRT*.

Rams with different level of DNA methylation of gene *SIRT1*	Average DNA methylation of gene *SIRT1* in group	Conception rate at lambing (%)	Percent of lambing (%)	Fecundity	Ewes with multiply pregnancy (%)
High (> 80%)	95.38 ± 8.84	67.3 ± 11.81	110.11 ± 24.45	1.63 ± 0.17	50.53 ± 5.54
Low (< 80%)	59.96 ± 8.34	78.28 ± 12.73	95.2 ± 23.73	1.21 ± 0.02	20.99 ± 2.9
P value	< 0.001	0.02	0.06	< 0.001	< 0.001

All results represent the mean ± standard error of mean (total number of inseminated ewes = 131).

Table 5. Reproductive parameters of ewes from Assaf breed inseminated with sperm of high and low methylated DNA of gene *SIRT1*.

Rams with different level of DNA methylation of gene *SIRT1*	Average DNA methylation of gene *SIRT1* in group	Conception rate at lambing (%)	Percent of lambing (%)	Fecundity	Ewes with multiply pregnancy (%)
High (> 30%)	50.54 ± 28.78	72.47 ± 12.69	124.75 ± 3.53	1.71 ± 0.04	63.52 ± 8.54
Low (< 30%)	22.89 ± 9.45	49.8 ± 6.17	86.03 ± 14.94	1.72 ± 0.09	61.5 ± 2.78
P value	0.02	0.0004	0.0015	> 0.05	> 0.05

All results represent the mean ± standard error of mean (total number of inseminated ewes = 162).

Table 6. Reproductive parameters of ewes from East-Friesian breed inseminated with sperm of high and low methylated DNA of gene *SIRT1*.

Rams with different level of DNA methylation of gene *SIRT1*	DNA methylation of gene *SIRT1* in group	Conception rate at lambing (%)	Percent of lambing (%)	Fecundity	Ewes with multiply pregnancy (%)
High (> 35%)	68.6 ± 12.7	70.8 ± 1.97	70.8 ± 2.05	1.85	71.8 ± 3.67
Low (< 35%)	10.71 ± 5.05	60.7 ± 2.82	63.8 ± 4.16	1.95	80.45 ± 9.01
P value	< 0.05	< 0.05	> 0.05	> 0.05	> 0.05

All results represent the mean ± standard error of mean (total number of inseminated ewes = 100).

DISCUSSION

Our investigations have shown that the methylation of the *SIRT1* gene in rams' sperm varies widely across the 3 breeds. The obtained mean level of DNA methylation of gene *SIRT1* in the present study (65.2 ± 36.53%) and the range of a trait variation correspond to our previous results in rams [1] and to the claim that the total DNA methylation in ram sperm is higher compared to other livestock males [23]. The results on rams from Lacaune demonstrated a level of *SIRT1* comparable with those in Synthetic Population Bulgarian Milk breed (78.5 ± 23.9%) [1]. Although the wide individual variation of this trait, analysis of variance showed a statistically significant effect of the breed on the level of DNA methylation of the *SIRT1* gene in ram sperm. There are only few data about breed specific DNA methylation in livestock. Zheng *et al*. [34] comparing the DNA methylation characteristics of testicles and *longissimus dorsi* of Bamaxiang and Large White pigs, found that the DNA methylomes differed among different breeds but were stable within a single breed. A study of total methylation in boar sperm from 3 breeds reported about presence of 1,040-1,666 breed-specific hypomethylated regions (HMRs) that are associated with embryonic developmental and economically complex traits for each breed [7]. Our data are the first message about the effect of breed on the specificity of DNA methylation of gene *SIRT1* in ram sperm. These results demonstrated an existence of the sheep breeds with high and low level of DNA methylation of gene *SIRT1* in ram spermatozoa.

The effect of age on the methylation level in sperm is still discussable. The variance analysis of our results did not show significant influence of age on the level of DNA methylation of gene *SIRT1* in sperm of rams from different breeds. Although this finding, the methylation in old rams (84-96 months) was higher than in young and middle age animals. The intra-individual variations affecting DNA methylation in semen collected at different ages have recently been reported [16,29]. The difference methylation at the 37 224 CpGs was analysed between peripubertal (10-10.5 months) and mature (15-20 months) semen samples from bulls [29]. In addition, they established that one CpG located in the mitochondrial glutamate carrier 1 gene displayed a marked increase in DNA methylation with age. The results of Lambert *et al*. [16] demonstrate important plasticity of the sperm DNA methylome during the peripubertal period. The semen samples collected from the same bulls at 10, 12, and 16 mo of age did not display any significant differences in DNA methylation between 12 and 16 mo, whereas many differential methylated regions (DMRs) can distinguish the 10 and 16 mo stages [16]. Our results also demonstrated

any significant difference in the DNA methylation of *SIRT1* in sperm between young (18 mo) and middle age rams (36 mo), because both groups were reached a sexual maturity. Taking into account that DNA methylation in sperm is stabilized with puberty onset and is a heritable epigenetic modification, it can be a promising marker in animal breeding.

In contrast with Capra *et al*. [4], pointed at differential methylation profile in high and low motile sperm, we did not find the correlation between level of DNA methylation of gene *SIRT1* and such sperm characteristics as a concentration of spermatozoa and main kinematic parameters. However, the close relationship between DNA methylation level of *SIRT1* in sperm and conception rate at lambing was established in all investigated breeds. The rams from each breed were divided in 2 groups - with relatively high and low level of DNA methylation of gene *SIRT1* in spermatozoa according to the absolute average meaning of the parameter for breed. Interesting was that an optimal level of *SIRT1* methylation, leading to high conception rate at lambing (70-79%), was between 50-68% for all breeds. The lower and higher levels resulted in a decrease of the conception rate at lambing. The results for 3 investigated breeds in this study confirmed our findings observed for Synthetic Population Bulgarian Milk breed where the rams with higher level of DNA methylation of gene *SIRT1* in spermatozoa also had better fertilization ability of sperm [1]. Fang *et al*. [11] mentioned that bovine sire's sperm DNA hypermethylation is associated with low fertility traits. Another authors reported about 10 fertility-related DMRs in sperm of Japanese Black bulls, which methylation levels were significantly different between sires with high fertility (> 50%) and low fertility (< 40%) [30]. Moreover, a regression line indicated that methylation levels at the DMRs increased as sire conception rate (SCR) increased [30]. In accordance with [15], assessment of the epigenetic signature of spermatozoa between high and low fertility bulls revealed 76 differentially methylated regions while 60 of them had enriched methylation levels in sperm of high fertility sires. Mentioned above corresponds with our results that showed a higher conception rate in ewes inseminated by rams with high level of DNA methylation of gene *SIRT1* in sperm. The fertility-related differences in spermatozoa methylation levels could be new epigenetic biomarkers for malefertility despite the discussable and limited information about this parameter. Analyzing of methylation status of individual genes in buffaloes spermatozoa also identified a significant difference in methylation of 96 genes between the high and sub-fertile bulls [34]. However, in contrast of our results, they established a hyper methylated status of *SIRT1* in sperm of sub-fertile buffalo bulls. It was studying the *SIRT1* role in the spermatogenesis on mouse mode [2], confirmed a lack of the *SIRT1* mRNA expression in the late round and early elongated spermatids. It is possibly due to fact that genome-wide DNA methylation occurs prior to meiosis in male germ cells [28]. In our opinion, the high methylation level of *SIRT1* in the ejaculated spermatozoa may be a result of the successful maturation and properly epigenetically reprogramming of germ cells during the spermatogenesis. Our experimental results confirmed this hypothesis, because in all investigated breeds the rams with relatively high level of DNA methylation of gene *SIRT1* in spermatozoa (50-68%) demonstrated a high conception rate at lambing.

CONCLUSION

The established effect of breed on the methylation level of gene *SIRT1* in ram sperm and correlation between this indicator and the reproductive parameters of inseminated ewes give reason to assume this trait as a breed specific marker of ram semen fertilizing ability. Identifying the specific methylation of individual genes in sperm is important for improving the reproductive and semen quality traits in livestock. Because of the sperm-related traits are usually low-heritability, DNA methylation of individual genes, as a stable and heritable epigenetic marker, may give effective information for early in- or excluding the rams in the breeding programs.

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***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

REFERENCES

1 **Abadjieva D., Stancheva N., Marchev Y., Yotov S. & Kistanova E. 2020.** *SIRT1* gene methylation in sperm differs in rams with high and low fertility. *Acta Phytotechnica et Zootechnika*. 23: 156-161.

2 **Bell E.L., Nagamori I., Williams E.O., Del Rosario A.M., Bryson B.D., Watson N., White F.M., Sassone-Corsi P. & Guarente L. 2014.** *SirT1* is required in the male germ cell for differentiation and fecundity in mice. *Development*. 141(18): 3495-504. DOI: 10.1242/dev.110627.

3 **Boissonnas C.C., Jouannet P. & Jammes H. 2013.** Epigenetic disorders and male subfertility. *Fertil Sterility*. 99: 624-631.

4 **Capra E., Lazzari B., Turri F., Cremonesi P., Portela A.M.R., Ajmone-Marsan P., Stella A. & Pizzi F. 2019.** Epigenetic analysis of high and low motile sperm populations reveals methylation variation in satellite regions within the pericentromeric position and in genes functionally related to sperm DNA organization and maintenance in *Bos taurus*. *BMC Genomics*. 20: 940.

5 **Champroux A., Cocquet J., Henry-Berger J., Drevet J.R. & Kocer A. 2018.** A decade of exploring the mammalian sperm epigenome: paternal epigenetic and transgenerational inheritance. *Frontiers in Cell and Developmental Biology*. 6: 50.

6 **Chavatte-Palmer P., Tarrade A., Kiefer H., Duranthon V. & Jammes H. 2016.** Breeding animals for quality products: not only genetics. *Reproduction, Fertility and Development*. 28: 94-111.

7 **Chen S., Liu S., Mi S., Li W., Zhang S., Ding X. & Yu Y. 2021.** Comparative Analyses of Sperm DNA Methylomes Among Three Commercial Pig Breeds Reveal Vital Hypomethylated Regions Associated With Spermatogenesis and Embryonic Development. *Frontiers in genetics*. 12: 740036.

8 **Congras A., Yerle-Bouissou M., Pinton A., Vignoles F., Liaubet L., Ferchaud S. & Acloque H. 2014.** Sperm DNA methylation analysis in swine reveals conserved and species-specific methylation patterns and highlights an altered methylation at the GNAS locus in infertile boars. *Biology of Reproduction*. 91(6): 137. DOI: 10.1095/biolreprod.114.119610.

9 **Coussens M., Maresh J.G., Yanagimachi R., Maeda G. & Allsopp R. 2008.** Sirt1 deficiency attenuates spermatogenesis and germ cell function. *PLoS One*. 13(2): e1571. DOI: 10.1371/journal.pone.0001571.

10 **Donkin I. & Barres R. 2018.** Sperm epigenetics and influence of environmental factors. *Molecular Metabolism*. 14: 1-11.

11 **Fang, L., Zhou Y., Liu S., Jiang J., Bickhart D.M., Null D.J., Li B., Schroeder S.G., Rosen B.D., Cole J.B., Van Tassell C.P., Ma L. & George E.L. 2019.** Integrating signals from sperm methylome analysis and genome-wide association study for a better understanding of male fertility in cattle. *Epigenomes*. 3: 10. DOI: 10.3390/epigenomes3020010.

12 **Islam S., Uehara O., Matsuoka H., Kuramitsu Y., Adhikari B.R., Hiraki D., Seiko T., Asiri J., Saito I., Muthumala M., Nagayasu H., Yoshihiro A.Y. & Chiba I. 2020.** DNA Hypermethylation of Sirtuin 1 (*Sirt1*) Caused by Betel Quid Chewing-A Possible Predictive Biomarker for Malignant Transformation. *Clinical Epigenetics*. 12(1): 12. DOI: 10.1186/s13148-019-0806-y.

13 **Khawar M.B., Sohail A.M. & Li W. 2022.** *SIRT1*: A Key Player in Male Reproduction. *Life*. 12(2): 318. DOI: 10.3390/life12020318.

14 **Kiefer H. & Perrier J.P. 2020.** DNA methylation in bull spermatozoa: evolutionary impacts, interindividual variability, and contribution to the embryo. *Canadian Journal of Animal Science*. 100: 1-16.

15 **Kropp J., Carrillo J.A., Namous H., Daniels A., Salih S.M., Song J. & Khatib H. 2017.** Male fertility status is associated with DNA methylation signatures in sperm and transcriptomic profiles of bovine preimplantation embryos. *BMC Genomics*. 18: 280.

16 **Lambert, S., Blondin P., Vigneault C., Labrecque R., Dufort I. & Sirard M.A. 2018.** Spermatozoa DNA methylation patterns differ due to peripubertal age in bulls. *Theriogenology*. 106: 21-29. DOI: 10.1016/j.theriogenology.2017.10.006.

17 **Liu C., Song Z., Wang L., Yu H., Liu W., Shang Y., Xu Z., Zhao H., Gao F., Wen J., Zhao L., Gui Y., Jiao J., Gao F. & Li W. 2017.** *Sirt1* regulates acrosome biogenesis by modulating autophagic flux during spermiogenesis in mice. *Development*. 144(3): 441-451. DOI: 10.1242/dev.147074.

18 Liu S., Fang L., Zhou Y., Santos D.J.A., Xiang R., Daetwyler H.D., Chamberlain A.J., Cole J.B., Li C.J., Yu Y., Ma L., Zhang S. & Liu G.E. 2019. Analyses of inter-individual variations of sperm DNA methylation and their potential implications in cattle. *BMC Genomics*. 20: 888. DOI: 10.1186/s12864-019-6228-6

19 McSwiggin H.M. & O'Doherty A.M. 2018. Epigenetic reprogramming during spermatogenesis and male factor infertility. *Reproduction (Cambridge, England)*. 156(2): 9-21.

20 Mi S., Chen S., Li W., Fang L. & Yu Y. 2021. Effects of sperm DNA methylation on domesticated animal performance and perspectives on cross-species epigenetics in animal breeding. *Animal frontiers : the review magazine of animal agriculture*. 11(6): 39-47.

21 Nayak K., Walsh J., Wilson J., Mirmina A., Cole K., Wei W. & Sartini B.L. 2016. Epigenetic regulation of gene expression during spermatogenesis. [Source: <https://digitalcommons.uri.edu/srhonorsprog/491/ >].

22 Olivier W.J. 2014. Calculation of reproduction parameters. Info pack ref: AP 2014/032, Grootfontein Agricultural Development Institute. [Source: <http://gadi.agric.za/InfoPacks/2014032%20Calculation%20of%20reproduction%20parameters.pdf>].

23 Perrier J.P., Sellem E., Prézelin A., Gasselin M., Jouneau L., Piumi F., Al Adhami H., Weber M., Fritz S., Boichard D., Le Danvic C., Schibler L., Jammes H. & Kiefer H. 2018. A multi-scale analysis of bull sperm methylome revealed both species peculiarities and conserved tissue-specific features. *BMC Genomics*. 19(1): 404. DOI: 10.1186/s12864-018-4764-0.

24 Pool K.R., Rickard J.P. & Graaf S.P. 2020. Global Methylation and Protamine Deficiency in Ram Spermatozoa Correlate with Sperm Production and Quality but Are Not Influenced by Melatonin or Season. *Animals*. 10: 2302. DOI:10.3390/ani10122302.

25 Rahman S. & Islam R. 2011. Mammalian *Sirt1*: insights on its biological functions. *Cell Communication and Signaling*. 9: 11.

26 Schagdarsurengin U. & Steger K. 2016. Epigenetics in male reproduction: effect of paternal diet on sperm quality and offspring health. *Nature Reviews Urology*. 13: 584-595.

27 Sharafi M., Rahimi M., Shahverdi A. & Esmaeili V. 2017. Epigenetic modulation of ram sperm during cryopreservation. *Reproduction in Domestic Animals*. 52(S3): 133.

28 Stewart K., Veselovska L. & Kelsey G. 2016. Establishment and functions of DNA methylation in the germline. *Epigenomics*. 8(10): 1399-1413.

29 Takeda K., Kobayashi E., Nishino K., Imai A., Adachi H., Hoshino Y., Iwao K., Akagi S., Kaneda M. & Watanabe S. 2019. Age-related changes in DNA methylation levels at CpG sites in bull spermatozoa and *in vitro* fertilization-derived blastocyst-stage embryos revealedby combined bisulfite restriction analysis. *Journal of Reproduction and Development*. 65: 305-312.

30 Takeda, K., Kobayashi E., Ogata K., Imai A., Sato S., Adachi H., Hoshino Y., Nishino K., Inoue M., Kaneda M. & Watanabe S. 2021. Differentially methylated CpG sites related to fertility in Japanese Black bull spermatozoa: epigenetic biomarker candidates to predict sire conception rate. *The Journal of Reproduction and Development*. 67: 99-107. DOI: 10.1262/jrd.2020-137.

31 Tolić A., Grdović N., Dinić S., Rajić J., Đorđević M., Sinadinović M., Arambašić Jovanović J., Mihailović M., Poznanović G., Uskoković A. & Vidaković M. 2019. Absence of PARP-1 affects Cxcl12 expression by increasing DNA demethylation. *Journal of Cellular and Molecular Medicine*. 23(4): 2610-2618.

32 Urdinguio R.G., Bayón G.F., Dmitrijeva M., Toraño E.G., Bravo C., Fraga M.F., Bassas L., Larriba S. & Fernández A.F. 2015. Aberrant DNA methylation patterns of spermatozoa in men with unexplained infertility. *Human Reproduction*. 30(5): 1014-1028. DOI: 10.1093/humrep/dev053.

33 Verma A., Rajput S., De S., Kumar R., Chakravarty A.K. & Datta T.K. 2014. Genome-wide profiling of sperm DNA methylation in relation to buffalo (*Bubalus bubalis*) bull fertility. *Theriogenology*. 82: 750-759.

34 Zheng M., Xiao S., Guo T., Rao L., Li L., Zhang Z. & Huang L. 2020. DNA methylomic homogeneity and heterogeneity in muscles and testes throughout pig adulthood. *Aging (Albany, NY)*. 12: 25412-25431. DOI: 10.18632/aging.104143.