Leptin gene polymorphism in Romanian cattle breeds and associations with milk production traits

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Abstract. The leptin *(LEP)* gene, mapped on chromosome 4q32 in cattle, is considered a potential QTL, influencing different traits, including those related to milk. Various single nucleotide polymorphisms (SNPs) were reported, several of them being associated with milk, fat, and protein yields or fat and protein percentages. One of them, A1620G, was chosen as the aim of our investigation, two alleles (A and G) and three genotypes (AA, AG, GG) being identified in a population of 82 Romanian Spotted and 55 Romanian Brown cattle. The G allele was predominant in both breeds (62.20% in Romanian Spotted and 73.64% in Romanian Brown), the AG genotype in Romanian Spotted (68.29%), whereas both AG and GG in Romanian Brown cattle (each of them, 49.09%). For 23 Romanian Spotted and 29 Romanian Brown cows, associations of *LEP A1620G* genotypes with milk, fat, and protein yields, fat and protein percentages were investigated. No significant differences were found among genotypes, but differences were found among breeds. This might be due to the influence of a small number of the individuals included in this second part of research and is the reason why we consider an enlargement of the base study for further studies, including here individuals` number and *LEP* loci number.
Key Words: chromosome 4q32, nucleotide polymorphisms, milk fat, milk protein, Romanian Spotted cattle, Romanian Brown cattle.

Introduction. Leptin is a 16-kDa hormone-like non-glycosylated polypeptide consisting of 146 amino acids. It belongs to the cytokine family, adopting a helical, tertiary structure similar to that of various interleukins (IL-2, IL-6, IL-11, IL-12, and IL-15) (Dridi et al 2000; Kulig et al 2010; Santos-Alvarez et al 1999). It exhibits high homology in mammalian species but is somehow different in chicken by the lack of one amino acid (Dridi et al 2000).

Leptin, also known as Obese protein, is encoded by the Leptin gene *(LEP)* or Obese gene (*Ob*), highly conserved in mammalian species, with three exons separated by two introns, coding regions being found in exon 2 and exon 3 (Komisarek et al 2005; Moravčíková et al 2012), while exon 1 is non-coding (Buchanan et al 2002). The coding region of these two exons is by 501 nucleotide length (Trakovická et al 2013) and translates into a precursor form of 167 amino acids, with 21 amino acid signal sequence (Giblin et al 2010; Komisarek et al 2005). The active form of the hormone consisting of 146 amino acids is the result of subsequent cleavage of amino-terminal secretory signal that affects the translocation of polypeptide into microsomes (Komisarek et al 2005).

In cattle, *LEP* gene maps to chromosome 4 (4q32), consisting of 16735 kb, and is considered a potential QTL (Quantitative Trait Locus) influencing different traits, such as meat production, milk performance and reproduction (De Matteis et al 2012; Giblin et al 2010; Madeja et al 2004). Together with serum amylase-1 gene, it is a part of a region on chromosome 4 (*BTA 4 – Bos taurus* autosome 4), which is considered a QTL for milk production traits; there were also reports of other QTLs affecting milk protein and fat

percentage both together (*BTA 3* and *BTA 6*), or independently, *BTA 14* for milk fat percentage and *BTA 20* for milk protein percentage (Madeja et al 2004).

Although various polymorphisms were reported in the bovine Leptin gene, four of them are well-known to be related to amino acid sequence changing in the protein: (i) *Kpn*2I RFLP recognition site corresponds to *R4C, R25C* or *C73T* single nucleotide polymorphisms (SNPs), is a result of C→T substitution located 73 bp from the start of exon 2, involving a change from arginine to cysteine at position 4 of the secreted peptide (*Arg4Cys*) or at position 25 of the encoded protein (*Arg25Cys*); (ii) *Hph*I RFLP recognition site, also named *A59V* or *A80V* SNPs, is a result of C→T substitution located 95 bp from the start of exon 3, involving a change from alanine to valine at position 59 of the secreted protein (*Ala59Val*), and at position 80 of the encoded protein (*Ala80Val*); (iii) *Cla*I RFLP recognition site, also named *T7F* SNP, is a result of A→T substitution located in exon 2, involving a change from tyrosine to phenylalanine at amino acid position 7 within peptide signal sequence; (iv) *Nru*I RFLP recognition site is a result of C→T substitution located in exon 3, involving a change from valine to alanine (Banos et al 2008; Buchanan et al 2003; Clempson et al 2011; Giblin et al 2010; Komisarek et al 2005; Kulig 2005a; Madeja et al 2004).

Other investigated *LEP* gene SNPs often refer to *C207T* or *UASMS-1*; *C528T*, *UASMS-2* or *LEP-2470*; *A1457G*; *C963T*; all of them located in *LEP* promoter region (Banos et al 2008), *A252T* or *E2JW* (*Tyr* to *Phe* changing), *C305T* or *E2FB* (*Arg* to *Cys* changing), both located in *LEP* exon 2 (Banos et al 2008; Clempson et al 2011); *Sau*3AI polymorphism in intron 2 due to C→T substitution, resulting in amino acid changing (*Arg* to *Cys*) at position 2059 of the secreted protein (Madeja et al 2004; Moravčíková et al 2012; Trakovická et al 2013).

Up to date, associations of bovine *LEP* gene locus and milk and meat yields and quality, metabolic and reproductive traits, or immune functions were investigated, revealing possible interaction models of dominance, epistasis or pleiotropic effects (Giblin et al 2010; Szyda & Komisarek 2007). The product of *LEP* gene is mainly but not exclusively secreted in white adipose tissue (white adipocytes) and also: (i) in the mammary gland tissue during lactation and on colostrum and/or milk of cattle, goats, ewes, sows, mares, and even in human milk; (ii) in placenta and fetal tissues; (iii) in muscle and brown adipose tissue; (iv) in stomach; (v) in rumen, abomasum, duodenum, and pituitary gland in ruminants (Feuermann et al 2004; Kulig 2005a; Kulig et al 2010; Moravčíková et al 2012; Pinotti & Rosi 2006). However, both the *LEP* gene expression and subsequent tissue leptin concentration are closely related to the amount of adipose tissue (Kulig 2005a). Basically, leptin produced in adipose tissue inhibits feed intake and down-regulates adipose tissue deposition (Liefers et al 2003) in a feedback loop involving key metabolic regulators including insulin, glucocorticoids, and the sympathetic nervous system (Buchanan et al 2002). Its mechanism of action is related to the protein binding to a Neuropeptid-Y (*NPY*) receptor mainly located in hypothalamic neurons, resulting in a reduction of feed intake (central appetite suppression) and increased thermogenesis (increased energy expenditure) (Anton et al 2012; Liefers et al 2002). The higher the body fat stores, the lower concentrations of *NPY* as a result of their capture; depleted body fat stores are related to elevated concentrations of *NPY* (Clempson et al 2011). Plasma leptin levels linearly increase with body fat mass and energy balance (Buchanan et al 2002, 2003).

Leptin is involved in reproductive performance since neuropeptide *Y* is also involved in the control of reproductive function (Liefers et al 2002). Although the infertility associated with leptin deficiency could be attributed to the excess of adipose tissue, it seems to be rather caused by the insufficiency of hormones at the hypothalamic-pituitary level since leptin stimulated the release of *GnRH, FSH*, and *LH* (Liefers et al 2002; Moravčíková et al 2012).

The effect of leptin on local tissues, such as the mammary gland, ovary, pancreas or muscle tissue for example, is not only a result of its action on the central nervous system through *NPY*, but also centrally and locally, through specific receptors (Silva et al 2002; Trakovická et al 2013). In cattle, for example, the leptin receptor gene (*LEPR*) is located on autosome 3 (3q33), with a different position than the *NPY* gene located on

autosome 4 (Clempson et al 2011). The bovine *LEPR* gene contains 20 exons divided over 1.75 Mb (Liefers et al 2004), its product of synthesis being a 1165 amino acids glycoprotein, a member of the class I cytokine receptor family (De Matteis et al 2012). Due to alternative RNA splicing, the resulting protein is expressed in five isoforms (a-e), included in three classes: long, short, and secretory. For two of them, the location and effect were reported. The long and fully active isoform b (*LEPR-b*) is closely related to IL-6 receptor, it has a long intracellular domain of 302 amino acids, is mainly expressed in the hypothalamus and is essential for the weight-reducing effect of leptin. Other *LEPR* isoforms are characterized by the lack of some "-b" variant domains. In cattle, the *LEPR-a* short variant was found to be expressed in the pituitary gland, liver and spleen, without any contribution in mammary gland, since it is not expressed there (De Matteis et al 2012; Silva et al 2002). A well-known missense mutation inside the *LEPR* gene was reported by Liefers et al (2004). This polymorphism in exon 20 (*T945M*), involving a C→T missense mutation at position 115 that causes a *Threonine→Methionine* amino acid substitution in the intracellular domain of the *LEPR-b* isoform (residue *945*), was reported to be associated with leptin concentrations only during late pregnancy, but not during lactation.

Various reports showed different associations of *LEP* and/or *LEPR* SNPs` genotypes with milk yield and quality. Therefore, these traits, hormonally controlled, may be influenced in their expression by the selection of individuals with preferred genotypes, the resulted genetic progress having an impact on economic effectiveness increasing both in cattle breeding and dairy industry. These facts were actually assumed to justify the importance of our study, whose aim was to investigate the polymorphism at the *LEP* gene locus in a population of cattle bred in Romania and to seek associations between genotypes which were found for the investigated SNP and milk production, milk fat and protein yields and percentages.

Material and Method

Animals and DNA extraction. Blood samples were collected from 137 cattle belonging to the Bovine Research and Development Station, located in Arad, Romania, an institution which is a part of Romanian Academy of Agricultural Sciences. 82 samples were collected from Romanian Spotted cattle breed or Romanian Simmental (*RS*), and 55 from Romanian Brown or Romanian Brown of Maramureș (*RB*); both breeds are considered to be genetically improved by matings of absorption between females of native breeds, such as Romanian Grey Steppe cattle, and bulls of western European origins.

Genomic DNA was extracted from blood collected from tail vein in vacutainers containing K3EDTA as anticoagulant (Vacutest Kima®, Italy). Individual DNA was extracted using the manual kit Wizard Genomic DNA Purification (Promega®, USA). After extraction, all DNA samples were spectrophotometrically evaluated with NanoDrop-2000 (Thermo Fisher Scientific®, MA, USA) and diluted to 50-100 ng. Genomic DNA was also evaluated visually by standard agarose gel electrophoresis (1% agarose (w/v) in TBE). All DNA samples were stored at -20°C prior genotyping.

Ethics statement. The research activities were performed in accordance with the European Union's Directive for animal experimentation (Directive 2010/63/EU).

Milk production data collection. The animals involved in the present research were included in the Official Performance and Recording Scheme. All cattle were milked twice per day in a "herringbone" milking parlour (2 sides x 14 units). The milking parlour was equipped with AfiMilk 3.076 A-DU® software. Furthermore, all cattle were fitted with AfiTag® pedometers. Cattle were housed in groups of 40 to 50 animals, according to lactation stage and productivity, regardless of breed.

Genotyping. The genotypes for *LEP* gene (*A1620G*) (GenBank Acc. No. GQ411537) were identified based on the PCR-RFLP method, using specific primers to amplify fragments of

522 bp as previously reported by Lien et al (1997). The PCR amplification was carried out in 25 µL reaction containing 1 µL of genomic DNA, 25 pmoL concentration of forward and reverse primers and 2× Green PCR Master Mix (Rovalab GmbH, Germany). The PCR conditions consisted of first denaturation for 5 min at 95°C, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 62°C for 30s, and extension at 72°C for 30s. The final extension step was done at 72°C for 7 min. Amplification was performed in 48-well PCR plates using a C1000 Thermal Cycler PCR System (Biorad, California, USA). Genotype identification was carried out using the RFLP technique. The LEP amplicons were digested for two hours with the *Bsa*AI endonuclease (also named *Ppu*21I). Restriction fragments from the above PCR reactions were separated on 3.5% agarose gel and stained with Midori Green Advance dye (Nippon Genetics®, Japan).

The primers used for the amplification of *LEP*, annealing temperatures of the primers, expected size of the amplification products, restriction enzyme used, size of the PCR-RFLP products and corresponding genotypes are presented in Table 1.

Table 1

Primers used for the PCR amplification of *LEP*, annealing temperatures of the primers, PCR product size, restriction enzyme and genotypes according to the obtained digestion fragments.

Gene	Primers (5'-3')	Annealing temp. (°C)	Amplicon PCR (bp)	Restriction endonuclease	Digestion product size (bp)
LEP	F: 5`-GTC TGG AGG CAA AGG GCA GAG T-3; R: 5`-CCA CCA CCT CTG TGG AGT AG-3`	62	522	*Bsa*AI	(AA) 522 (AG) 522,441,81 (GG) 441,81

Data analysis. Milk production data were available for the second lactation of a total of 52 individuals (23 Romanian Spotted and 29 Romanian Brown), normalized for standard lactation length (305 days), mature equivalent. The following phenotypic traits were investigated: the amount of milk (kg); the amount of fat (kg); the fat percentage; the amount of protein (kg) and the percentage of protein. Further, the production and milk quality data were analyzed statistically and expressed as mean ± SD. Descriptive statistics were computed using the R package psych v. 1.9.12.31 (Revelle 2020) for the whole dataset and for each of the two breeds, respectively. Genotype and allelic frequencies were computed for each gene using a custom R script. Scatterplots and correlations were plotted for the whole dataset and for the two breeds, using the pairs.panels function from the R package psych and the ggpairs function from the R package GGally v.2.0.0 (Schloerke et al 2020), respectively (data not shown). Comparisons between the three genotypes for production level and quality of milk were carried out using ANOVA and Tukey's test using the corresponding base R methods. Associations with $p<0.05$ were deemed significant.

Results and Discussion

Alleles and genotypes frequency. The genotyping of *LEP* locus revealed for the 522 bp fragment the same digestion patterns in both investigated cattle, represented by a single 522 bp fragment which corresponds to AA genotype, two different fragments of 441 bp and 81 bp, corresponding to GG genotype, and three different fragments of 522 bp, 441 bp, and 81 bp, corresponding to heterozygous individuals, AG. Allele and genotype frequencies individually expressed for each investigated breed and at the level of the entire population are presented in Table 2.

The predominance of G allele was observed in both investigated breeds, with a slightly lower predominance in Romanian Spotted compared to Romanian Brown cattle (0.6220 vs 0.7364). In Romanian Spotted investigated individuals, 56 cows were found to be heterozygote, while 23 were homozygote for GG genotype and 3, for AA genotype. In Romanian Brown individuals, the frequency of GG and AG genotypes was the same, including 27 cows in each of them; a single individual of Romanian Brown was

homozygote AA at this locus. At the level of the entire population, 83 individuals were heterozygous, 50 were homozygous for the GG genotype, and 4 for the AA genotype.

Table 2

Distribution of the allele and genotype frequencies for *LEP* locus in the in Romanian Spotted (*RS*) and Romanian Brown (*RB*) cattle breeds and total investigated population (*RS+RB*)

Breed	*n*	*Allele frequency*		*Genotypes frequency (n)*		
		A	*G*	*AA*	*AG*	*GG*
RS	82	0.3780	0.6220	0.0366 (3)	0.6829 (56)	0.2805 (23)
RB	55	0.2636	0.7364	0.0182 (1)	0.4909 (27)	0.4909 (27)
Total	137	0.3321	0.6679	0.0292 (4)	0.6058 (83)	0.3650 (50)

Researches on the 522 bp fragment amplifying of the *LEP* gene comprising the partial intron 2 and exon 3 and using the same primer pair as Lien et al (1997) reported, were not as common as those of other SNPs` polymorphisms. Interestingly, similar polymorphisms as that detected in our research, with two alleles and three genotypes at its locus, were reported not only in other *Bos taurus* breeds (Choudhary et al 2005) but also in various *Bos indicus* breeds (Choudhary et al 2005; Rambachan et al 2017; 2019), *Bos taurus* and *Bos indicus* crossbreed (Choudhary et al 2005), and even in *Bos frontalis* (Mukherjee et al 2013), and different types of buffalo (*Bubalus bubalis*) (Marrero et al 2016; Yadav et al 2015). This suggests that, at least for *Bos taurus* and *Bos indicus*, *LEP* gene mutation occurred far back in evolution, before the divergence of cattle into taurine and indicine (Choudhary et al 2005). Contrary to our findings, Choudhary et al (2005) reported the highest frequencies for the GG homozygous genotype in the Holstein Friesian and Jersey cow breeds, of 0.67 and 0.57, respectively, these being followed by the AG genotype, whose reported frequencies in the investigated breeds were of 0.3 and 0.38, respectively. A similar situation was reported for *Bos frontalis*, with G allele (Mukherjee et al 2013). Slightly higher frequencies of the GG genotype compared to AG were reported in the *Bos indicus* Gir and Nimari breeds, as well as in the *Bos taurus x Bos indicus* crossbreds, while the reverse situation was found in the Hariana and Sahiwal *Bos indicus* breeds (Choudhary et al 2005). However, excepting the reported *LEP* (*A1620G*) gene frequencies in different types of buffalo, where A was prevalent over G (Marrero et al 2016; Yadav et al 2015), for different breeds of *Bos indicus*, those of *Bos taurus* (Holstein Friesian and Jersey), and crossbreds *Bos indicus* x *Bos taurus*, the frequency of the G allele being more or less prevalent over that of A allele. As a personal observation, the difference between the frequencies of the G and A alleles was more pronounced in the case of the Holstein Friesian and Jersey *Bos taurus* breeds, which were improved for milk production. In our study, this difference was in favor of the G allele in Romanian Brown individuals compared to those of Romanian Spotted, the frequencies recorded in Romanian Spotted individuals being comparable to those in some *Bos indicus* breeds. The higher frequency of the G allele and GG genotype is related to the selection made over time for the G allele and against the A allele at this locus.

The LEP genotypes influences on milk production and chemical composition. In the current study, the influence of the *LEP* genotypes on milk production and chemical composition was investigated. The effects of the *LEP* locus on milk production and chemical composition in Romanian Spotted and Romanian Brown breed are presented in Table 3.

The highest milk production was recorded for the AG genotype in both breeds (5902.08±1540.20 and 5426.86±952.64 kg). However, no significant differences (P>0.05) were recorded between the genotypes for this trait. An average fat percentage in milk of 4.33±0.08% was associated with AA genotype in RS breed, while values of 3.90±0.41 and 4.14±0.46% (P=0.45) were found for the AG genotype and 3.88±0.51 and 4.26±0.38% (P=0.85) for the GG genotype in RS and RB breeds. This suggests that the genotypes have no significant influence on the fat content of milk. No significant

differences were recorded between the AA, AG and GG genotypes (3.32±0.11, 3.37±0.29 and 3.27±0.17%) on protein percentage in milk in Romanian Spotted breed or in Romanian Brown breed (3.46±0.32 and 3.50±0.37%). Moreover, in our study we did not find any *LEP* genotype influence on investigated traits. This may be attributed to the relatively low number of production data available for the second lactation (RS=23 and RB=29).

Table 3

Mean ± SD for milk production and chemical composition according to the *LEP* locus in Romanian Spotted (RS) and Romanian Brown (RB) cattle breeds.

Breed	*Genotype (n)*	*Milk (kg)*	*Fat (kg)*	*Fat (%)*	*Protein (kg)*	*Protein (%)*
RS	AA (2)	5119.00±141.42^a	221.50±2.12^a	4.33±0.08^a	170.00±9.90^a	3.32±0.11^a
	AG (12)	5902.08±1540.20^a	230.67±66.48^a	3.90±0.41^a	198.92±57.62^a	3.37±0.29^a
	GG (9)	5254.11±1105.91^a	203.11±45.91^a	3.88±0.51^a	171.67±35.3^a	3.27±0.17^a
RB	AA (0)	NA	NA	NA	NA	NA
	AG (14)	5426.86±952.64^a	222.14±30.86^a	4.14±0.46^a	186.64±29.96^a	3.46±0.32^a
	GG (15)	5015.47±1130.85^a	211.73±43.54^a	4.26±0.38^a	173.00±32.00^a	3.50±0.37^a
Total RS		5580.43±1323.29^a	219.09±56.17^a	3.93±0.44^a	185.74±48.12^a	3.33±0.24^a
Total RB		5214.07±1050.97^a	216.76±37.66^a	4.20±0.42^b	179.59±31.25^a	3.48±0.34^a
Total population		5376.12±1181.33	217.79±46.27	4.08±0.45	182.31±39.30	3.41±0.31

Columns means with different superscript differ significantly at $P \leq 0.05$, within the same variation source.
NA - no available data.

When comparing the *LEP* locus influence on the two investigated breeds, the Romanian Brown breed favored a significantly higher fat percentage in milk (4.20±0.42%) compared to the Romanian Spotted breed (3.93±0.44%, P=0.0308), with a difference of 0.268%. In our study, there were no significant differences for the milk yield between the breeds (5580.43±1323.29 and 5214.07±1050.97kg; P=0.27). Also, our results highlight a small influence of breed on protein percent of RS and RB cattle (3.33±0.24 and 3.48±0.34%; P=0.082).

Over time, various associations with milk traits and not only of the *LEP* gene polymorphisms have been studied, both for our investigated SNP and others, in *Bos taurus* and *Bos indicus* individuals. Some of the reported results are the presented below.

In 2019, Rambachan et al, studying the influence of the same locus polymorphism of *LEP* gene as we did, but in Hariana cattle from India (*Bos indicus*), found longer lactation period and higher total milk yield and milk yield in 300 days for AA genotype compared to AB or BB genotypes in first lactation (B allele is the same with the G allele found in our study). The authors reviewed different reported results such as a significant association of AB genotype with higher milk yield, less fat and protein percentage, and of AA genotype with higher fat and protein percentage, whereas in their own study the A allele was considered favorable for higher milk yield. In another study of theirs, published in 2017, the authors reported that the AA genotype was associated with a significantly lower dry period compared to BB and AB genotypes in second and third lactation of Hariana cattle.

In 2004, Madeja et al (2004) studied the influence of *Kpn*2I, *Hph*I and *Sau*3AI polymorphisms on milk production in Polish Black and White cattle. Among them, only *Hph*I polymorphism was associated with milk production traits. Three genotypes were found at this locus (CC, CT, and TT), the TT genotype being associated with about 2-fold higher Estimated Breeding Values (EBVs) for milk and protein yields; fat yield also tended to be higher for this genotype. These results were somehow unexpected since the *Hph*I polymorphism includes a change from alanine to valine at the conserved region of the leptin protein β-helix, the involved amino acids having similar nonpolar aliphatic R-groups. Although the authors did not find associations between *Kpn*2I and *Sau*3AI polymorphisms and milk productions traits, other of their cited reports proved otherwise, for example a strong influence of the TT genotype of *Kpn*2I locus on milk and protein yield, and also of the rare C allele of *Sau*3AI locus to fat and protein content in a different Polish Black and White cattle population.

In 2005, Komisarek et al studied the associations of *Kpn*2I and *Hph*I restriction sites with various milk traits in Black and White cattle (96.4% Holstein Friesian genes). These polymorphisms are related to a C/T substitution in exon 2 of the gene that causes an amino acid change from *Arg* to *Cys* at position 4 of *LEP* protein (*Arg4Cys*) and a C/T replacement in exon 3 with a change of *Ala* to *Val* at position 59 of *LEP* protein (*Ala59Val*), respectively. They found the T allele and its TT genotype of *Arg4Cys* locus with a highly significant increasing effect on milk yield compared to the C allele and CC genotype, and a significant increasing effect on protein yield, with a highly significant effect on milk fat content decreasing. However, although the involved substitution of *Ala59Val* locus was considered unlikely to alter the hormone functioning since both *Ala* and *Val* amino acids belong to the same group of non-polar aliphatic amino acids, the reported polymorphism at this locus was responsible for the control of the produced butterfat amount.

In 2010, Kulig et al studied the influence of three SNPs of *LEP* gene (namely *R4C* in exon 2, *Sau*3AI in intron 2, *A59V* in exon 3) on somatic cell count (SCC) in milk of Jersey cows, reporting no influence of *A59V* polymorphism but significant ones for *R4C* (C desirable allele and CC genotype) and *Sau*3AI (T desirable allele and TT genotype) polymorphisms; a selection in this way was considered to contribute to a reduction of SCC in Jersey cattle. In a previous study in 2005, Kulig (b) reported a significant influence of *LEP*/*Hph*I AA and *LEP*/*Sau*3AI BB genotypes on milk, protein, and fat yields (B allele of the last locus is the same with previous reported T allele). The *LEP*/*Sau*3AI BB positive influence was also reported in Kulig (2005a). In 2009, Kulig`s team found a significant influence of A59V polymorphism of LEP gene on milk, protein, and fat yield in Jersey cows (with CC and CT favorable genotypes), although this time no associations were reported for *Sau*3AI polymorphism and these investigated traits.

Studying the influence of 6 SNPs of *LEP* gene (*C207T=UASMS-1; C528T=UASMS-2; A1457G; C963T; A252T=E2JW; C305T=E2FB=Exon-2-FB*) on milk production, feed, and body energy traits in Holstein cows, Banos et al (2008) found a significant alteration of daily milk yield due to substitutions of A with T at the *A252T* SNP and of A with G at the *A1457G* locus. In fact, there was an increased daily milk yield by 2.3 kg in favor of thymine and of 0.7 kg in favor of guanine, respectively; due to the complete dominance of A over G at *A1457G* locus, the milk yield of AG genotype was reported to be more similar to that of AA than GG animals. The A allele of *LEP/A252T* SNP was suggested to be an indicative of compromised body condition, while *C528T* and *A1457G* were associated with significant dominant effects on body condition.

The influence of C to T transition in *LEP* gene that results in an *Arg25Cys* change in the *LEP* protein was found by Buchanan et al (2003) to be associated with increased fat deposition in beef cattle. Genotyping Holstein cows and comparing lactation performance, the authors observed that TT animals produced more milk than the CC ones (with 1.5 kg day^{-1}, with a prominent increasing in the first 100 day of lactation) and had higher somatic cell count linear scores, but without fat or protein percent being significantly affected over the entire lactation.

In 2011, Clempson et al, investigating various associations of *LEP* gene SNPs with fertility, growth, and milk production in Holstein cows, reported influences of the *A59V* SNP on milk production.

In 2010, Giblin et al investigated the associations of various SNPs of *LEP* gene with performance traits in Holstein Friesian cattle. They reported associations of *LEP 2470* SNP with milk protein concentration, and a tendency of association with milk yield. Reduced milk and protein concentrations and a tendency for somatic cells in the milk were associations established for *LEP 963* and *R25C* SNPs, respectively. The *LEP/Y7F* T allele was reported to be associated with reduced milk protein yield, and with a tendency of association with reduced milk yield.

Trakovická et al (2013), verifying associations of *LEP* gene polymorphism with production traits in Slovak Spotted and Pinzgau cows, found that *LEP*/*Sau*3AI AA genotype was characterized by the highest milk protein and fat yields, the opposite relationship being reported for the BB genotype at this locus.

The results of our research are important in terms of the cattle included in the study, which are part of the genetic heritage of our country (Romania). The associations found between the genotypes of the investigated *LEP* SNP and the milk traits showed significant differences only between the breeds and not between the genotypes. Of course, this is mainly attributed to the small number of animals included in the present investigation, but a future reorientation towards other *LEP* SNPs, which have been shown to be associated with milk indicators, will be considered for further studies.

Conclusions. The results of genotyping of 82 blood samples of Romanian Spotted cattle and of 55 Romanian Brown at the *LEP locus (A1620G)* showed the predominance of the G allele in both breeds, of AG genotype in Romanian Spotted and of both AG and GG genotypes in Romanian Brown cows. Associations with milk traits were investigated on 23 Romanian Spotted and 29 Romanian Brown individuals, with no significant associations among genotypes but only between breeds. Further investigations in this aim should consider a higher number of individuals and other *LEP* SNPs reported as associated with milk traits in cattle.

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