The influence of CSN3 and LGB polymorphisms on milk production and chemical composition in Romanian Simmental cattle

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The aim of this study was to assess the influence of genotypes from the CSN3 and LGB loci on milk production and chemical composition in Romanian Simmental cattle (n=114). For the CSN3 locus, a significantly higher frequency (P<0.001) was observed for the allele A (0.785) compared to allele B (0.215) and for AA (0.631) compared to AB (0.307) and BB (0.062) genotypes, respectively. The AA genotype was associated with a higher milk production (5887.76±115.7 kg) compared to the yields associated with the BB genotype (5619±86.34 kg, P=0.003), as well as a higher fat percentage (4.19±0.05% vs. 4.01±0.02%, P≤0.001). A higher milk protein percentage was associated with BB (3.4±0.02%) compared to AA (3.27±0.03) and AB (3.29±0.03) genotypes, respectively (P<0.05). For the LGB locus, the frequency of allele A was significantly higher (0.606, P<0.008) than allele B (0.394). The AB genotype was the most prevalent in the herd (0.579) compared to AA (0.316) and BB genotypes (0.105). The AB genotype was associated with a higher milk production (5906.54±166.76 kg) compared to the other two genotypes (P<0.05). The BB genotype was associated with a higher fat percentage in milk (4.23±0.06%) compared with AA (4.17±0.06) and AB (4.2±0.08) genotypes, respectively (P<0.01). For the LGB locus, no significant differences (P>0.05) were observed for milk protein percentage. In order to increase the quantity and quality of milk, the outcomes obtained in this study encourage improving genetic structure in cattle based on marker assisted selection for genes with economic values.

Key words: κ-casein; β-lactoglobulin; Romanian Simmental cattle breed; milk production traits

INTRODUCTION

The Romanian cattle population is quite high. There are 2092000 cattle in Romania according to the National Institute of Statistics (I.N.S., 2015). Thereby, Romania is among the top ten countries in the EU regarding the cattle population. From the total breed structure, a third of the population is represented by the Romanian Simmental (Bâlțată românească – BR) breed (30.96%), according to the National Agency for Animal Improving and Reproduction from Romania. The Romanian Simmental breed is preferred by farmers due to high organic resistance, high longevity (8–10 lactations) and an excellent adaptive capacity to the environment and rearing systems. Being a dual purpose breed, the genetic improvement schemes are focused on milk production (60%), meat (35%) and fitness (5%). An Official Performance Recording Scheme reports an average milk production level of 5000 to 5500 kg/lactation. Chemical composition of milk is characterized by an average percentage of 3.5 to 4.3% fat and 3.3 to 3.5% protein (Acatincai & Cziszter, 2010). The Romanian Simmental breed is suitable for extensive, semi-intensive and even intensive rearing systems having good aptitudes for mechanical milking (1.9–2.2 kg/min). The growing interest of the dairy industry for high biological value products motivates this study, requesting a good knowledge related to genetic polymorphism, milk’s chemical composition and processing properties. The dominance of the BR breed and milk obtained from that cattle, among other breeds reared in Romania, requires a good knowledge of the genetic structure and polymorphism of genes with an economical importance. Furthermore, improving the breed’s genetic structure is necessary to improve the overall quality of milk and related products.

Increasing a given farm’s efficiency through rapid genetic gain is a current goal imposed by the competitive milk market on one hand, and an increased consumer demand for products with high biological value on the other hand. The major selection traits for improving cattle during the last 70 years were milk production and lactation length. However, during the last decades, genetic polymorphism studies of milk protein genes attracted the interest of the dairy industry and the marker assisted selection (MAS) started to be more and more frequently included in the selection programs. Researchers have identified different genetic markers in cattle that contribute to both, achieving a higher milk production and a better milk quality. In dairy cattle, studies have been focused on the most important economic traits: milk yield, fat and protein content, somatic cells count, fertility, longevity, health traits, temperament and conformation. The recent MAS studies regarding the quantity and quality of milk were focused on the genotypes of the CSN3 and LGB loci, encoding κ-casein and β-lactoglobulin proteins which play an important role in determining the milk’s biological value (Singh et al., 2015).

Knowledge of the genetic structure of cattle allows implementation of modern and fast methods in the genetic improvement programs that will lead to an increase
in the dairy farms’ efficiency. Furthermore, by using parental favourable genotypes for future breeding, farmers might create the premises to obtain milk with high biological value, according to the current needs of the market. Most of the studies have focused on favourable genotypes and alleles for milk yields in the dairy breeds. Worldwide, outcomes obtained regarding the correlations between the CSN3 and LGB genotypes and milk performances are contradictory. Currently, there is a lack of knowledge for dual purpose breeds, particularly in Romanian Simmental, about the CSN3 and LGB genetic variants (Ilie et al., 2009; Balteanu et al., 2010; Gradinaru et al., 2013, 2015), and there are very few outcomes regarding correlations between genetic polymorphism, milk yield, chemical composition and processing properties (Ilie et al., 2009; Gradinaru et al., 2013). Therefore, the importance of indigenous breeds as gene reservoirs is recognized worldwide and requests more precise outcomes (Kusza, et al., 2015). The results found on individual genotypes bring important insights that can be used in the future breeding and marker assisted selection programs for the Simmental cattle breed group.

The aim of the research presented here was to determine the genotypes and allele frequency of the CSN3 and LGB loci, and to correlate these results with the milk yield and milk chemical composition in the Romanian Simmental dual purpose cattle breed.

MATERIALS AND METHODS

General description. This study was carried out at the Research and Development Station for Bovine Arad-Romania (46°10′36″N 21°18′4″E) for 114 Romanian Simmental (national name: Bălțată românească; other English names: Romanian Spotted or Romanian Fleckvieh) dual purpose cows reared year around under the loose system, with no access to grazing. Cows included in the study were randomly selected, being between their 1st and 6th lactation (1st, n=22; 2nd, n=28; 3rd, n=14, 4th, n=19; 5th, n=16; 6th, n=15). All cows were included in the Official Performance Recording Scheme through which the milk production and chemical composition data were registered. Cows were milked twice per day in a ‘herringbone’ milking parlour (2 sides×14 units). The milking parlour was equipped with Afimilk 3,076 A-DU® software.

The research activities were performed in accordance with the European Union’s Directive for animal experimentation (Directive 2010/63/EU). All procedures performed on cows in this study were approved by the Scientific Committee of the Research and Development Station for Bovine Arad.

Genetic analysis. Blood samples used for DNA genotyping were collected from the tail vein (vena cava) into 2 mL vacuum tubes containing the anticoagulant K<sub>3</sub>EDTA. Genomic DNA was extracted from blood samples using two types of kits: a manual kit- Wizard Genomic DNA Purification Kit (Promega, Madison, USA), and an automated kit – InnuPREP Blood DNA Mini Kit – IPC 16 (Analytikjena, Berlin, Germany) using an automatic extractor InnuPure C16, according to the manufacturers’ instructions. After extraction, the DNA concentration was spectrophotometrically evaluated with NanoDrop-2000 (Thermo Fisher Scientific, MA, USA). For the genotyping of animals, the Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR-RFLP) technique was used (Medrano & Cordova, 1990 a,b). The genotypes for CSN3 were identified based on the amplification of a 350 bp fragment, using the forward primer 5′-ATC ATT TAT GGC CAT TCC ACC AAA G-3′ and the reverse primer 3′-GCC CAT TTC GCC TCC TCT GTA ACA GA-5′. For the identification of the A and B alleles of LGB, a 247 bp fragment was amplified, using the forward primer 5′-TGT GCT GGA CAC CGA CTA CAA AAA G-3′ and the reverse primer 5′-GCT CCC GGT ATA TGA CCA CCC TCT-3′.

The PCR amplification was performed in a 25 μl reaction containing: 1 μl (50–100ng) of genomic DNA as template, 20 pmol concentration of forward and reverse primer, and 2× Go TagGreen Master Mix (Promega, Madison, WI, USA) for the CSN3 amplification, and 2× DreamTaq Green Master Mix (Fermentas, Thermo Fisher Scientific, MA, USA) for the LGB amplification. Amplification was carried out with the C1000 Thermal Cycler PCR System (Biorad, California, USA) in 48-well PCR plates. PCR conditions for CSN3 were as follows: 5 min at 95°C, 35 cycles of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C and a final extension cycle of 7 min at 72°C. For LGB, the reaction was subjected to 5 min at 95°C, 35 cycles of 30 sec at 95°C, 30 sec at 63°C and 30 sec at 72°C and a final extension cycle of 7 min at 72°C. Genotype detection was performed using the RFLP technique. The obtained PCR amplicons were digested for three hours at 37°C with the HindIII restriction enzyme for CSN3, and with HaeIII endonuclease for LGB. The PCR-RFLP fragments for CSN3 and LGB were separated on 3.5% agarose gels.

Data recording and statistical analysis. Production data collection. Milk yield and chemical composition (total fat yield and percentage, total protein yield and percentage) were recorded by the Official Performance Recording Scheme technicians. The alternative milking AM/PM at a 28 day interval protocol was used for this purpose. Milk yield was standardized for mature equivalent (cow’s parity) using correction coefficients (Acatincai & Cziszter, 2010).

Statistical analysis. Production and milk quality data were analyzed statistically, being expressed as LSM ± S.E.M. Comparisons between the three genotypes for production level and quality of milk were carried out using a one-way ANOVA protocol with the genotype of cows considered as the categorical factor. Differences between the three genotypes were statistically tested using the Shapiro-Wilks test (Shapiro & Wilk, 1965). Decisions about the acceptance or rejection of the statistical hypothesis have been made at the 0.05 level of significance. All the statistical inferences were carried out using Statistica software v.13.0 (Hill & Lewicki, 2007).

RESULTS AND DISCUSSION

In the study presented here, the CSN3 and LGB loci have been genotyped using the PCR-RFLP method. For both loci, three genotypes (AA, AB, BB) and two alleles (A, B) have been identified. The alleles and the genotype frequency for the CSN3 and LGB loci of the Romanian Simmental breed are presented in Table 1.

Alleles and the genotype frequency for the CSN3 locus

Within the studied herd, a significantly higher frequency of allele A (0.785) compared with the allele B (0.215) for the CSN3 locus was recorded. The AA genotype prevailed in the studied herd (0.631) compared to AB (0.307) and BB genotypes (0.062), respectively. Similar results were obtained in the few studies conducted for the local
cattle breeds in Romania (Romanian Simmental) where an increased frequencies of allele $A$ (from 0.579 up to 0.761) compared to $B$ (from 0.239 up to 0.313) was recorded (Balteanu et al., 2010; Gradinaru et al., 2013, 2015). The frequencies of genotypes reached 0.461, 0.427, and 0.097 for $AA$, $AB$ and $BB$, respectively (Balteanu et al., 2010). For the Romanian local Brown breed, in successive studies conducted by Ilie et al. (2008, 2015), an increased frequency of the $B$ allele (0.625 to 0.63) compared with the $A$ allele (0.37) in the CSN3 locus highlighted, although the frequency of the $BB$ genotype was to have a lower value (0.3 to 0.33) when compared to the $AB$ genotype (0.63 to 0.58). The Romanian milk production specialized breed (Romanian Black and White, Holstein-Friesian type) had an increased frequencies of the $A$ allele (0.761), as well as $AA$ (0.699) and $AB$ (0.304) genotypes when compared to the values obtained for the $B$ allele (0.239) and $BB$ homoygous genotype (0.087) in the CSN3 locus (Ilie et al., 2008, 2015). A recent study conducted by Bartonova and coworkers (2012) for the Czech Fleckvieh breed, revealed a lower frequency of the $AA$ genotype (0.296) and an increased share of the $BB$ (0.158) genotypes, respectively. Bonfatti and coworkers (2010) found higher frequencies of the $AA$ (0.438) and $AB$ (0.432) compared to the $BB$ (0.13) genotypes, in a study conducted on Simmental cows. An unbalanced distribution was recorded for the allele's frequencies, 0.652 for $AA$ and 0.348 for allele $B$.

Higher frequency of allele $B$ was found within genetically unimproved breeds (0.3) when compared to a reduced frequency of this allele (0.01–0.02) found in their hybrids with milk production specialized breeds (Holstein-Friesian) (Azevedo et al., 2008).Variable frequencies of allele $B$ (0.16–0.5) and homozygous $BB$ genotype (0.02–0.25) were found in local breeds from the Russian Federation (Sulimova et al., 2007). Several studies reported a low frequency of allele $B$ in specialized breeds for milk production. Specific variations were also calculated by Strzalkowska and coworkers (2002) for dairy breeds (Polish Black and White). Low frequency was recorded for homozygous $BB$ genotype (0.04) when compared to the $AA$ (0.90) and $AB$ genotypes (0.39), respectively. The frequency of allele $A$ was significantly higher (0.77) compared with the frequency of allele $B$ (0.23) for the CSN3 locus (Strzalkowska et al., 2002). Significant variations in the frequency of alleles $A$ and $B$ and default for the three genotypes related to the CSN3 locus were calculated by Tsaras and coworkers (2005). Studies conducted for the Holstein Friesian cows had revealed a frequency of 0.81 for the homozygous $AA$ genotype, 0.14 for the heterozygous $AB$ genotype and 0.05 for the homozygous $BB$ genotype. The results obtained by Sitkowska (2008) for the Holstein Friesian cows confirm the earlier outcomes. For the herd included in the evaluation, frequencies of 0.71 for $AA$ genotype, 0.23 to 0.06 for the $AB$ and $BB$ genotypes, respectively, were calculated in the CSN3 locus. The frequency of allele $A$ was higher (0.83) when compared to that of allele $B$ (0.17) (Sitkowska et al., 2008). Similar outcomes were found by Lara et al. (2002). Conversely, Benesik and coworkers (2007) recorded an increased frequency of the homozygous $BB$ genotype (0.33), as well as 0.5 for $AB$, and 0.17 for $AA$ genotypes, in the CSN3 locus for a Holstein Friesian herd. The frequency of allele $A$ was 0.43 in comparison to 0.57 for allele $B$ (Benesik et al., 2007).

### Table 1. Polymorphism of the CSN3 and LGB loci for the studied Romanian Simmental cow herd

<table>
<thead>
<tr>
<th>Locus</th>
<th>Genotype</th>
<th>Frequency</th>
<th>Allele (frequency)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSN3</td>
<td>AA</td>
<td>0.631+</td>
<td>$p_1$-0.785+</td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>0.307+</td>
<td>$q_1$-0.215+</td>
</tr>
<tr>
<td></td>
<td>BB</td>
<td>0.062+</td>
<td></td>
</tr>
<tr>
<td>LGB</td>
<td>AA</td>
<td>0.316+</td>
<td>$p_1$-0.606+</td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>0.579+</td>
<td>$q_1$-0.394+</td>
</tr>
<tr>
<td></td>
<td>BB</td>
<td>0.105+</td>
<td></td>
</tr>
</tbody>
</table>

Different superscript per column (a, b, c) differ significantly at $P<0.05$, within the same locus

### Table 2. Least square means (± S.E.M.) for milk production and chemical composition according to the CSN3 and LGB loci in the Romanian Simmental dual purpose breed

<table>
<thead>
<tr>
<th>Variation source</th>
<th>Milk (kg)</th>
<th>Fat (%)</th>
<th>Fat (kg)</th>
<th>Protein (%)</th>
<th>Protein (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSN3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>5887.76±115.7*</td>
<td>4.19±0.05*</td>
<td>246.68±6.83*</td>
<td>3.27±0.03*</td>
<td>192.52±4.12*</td>
</tr>
<tr>
<td>AB</td>
<td>5839.37±117.8</td>
<td>4.08±0.06*</td>
<td>238.24±9.1*</td>
<td>3.29±0.03*</td>
<td>192.11±6.6*</td>
</tr>
<tr>
<td>BB</td>
<td>5619±86.34b</td>
<td>4.01±0.02b</td>
<td>225.32±4.69b</td>
<td>3.4±0.02b</td>
<td>191.04±3.61b</td>
</tr>
<tr>
<td>LGB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>5809±117.58*</td>
<td>4.17±0.06*</td>
<td>242.23±9.96*</td>
<td>3.31±0.04*</td>
<td>192.27±5.43*</td>
</tr>
<tr>
<td>AB</td>
<td>5906.54±166.76b</td>
<td>4.2±0.08b</td>
<td>248.07±5.93b</td>
<td>3.34±0.05b</td>
<td>197.27±6.19b</td>
</tr>
<tr>
<td>BB</td>
<td>5812±115.11a</td>
<td>4.23±0.06a</td>
<td>245.84±7.71a</td>
<td>3.33±0.01a</td>
<td>193.53±3.94a</td>
</tr>
</tbody>
</table>

Columns means with different superscript (a, b, c) differ significantly at $P<0.05$, within the same variation source.
For a Romanian local Brown breed, studies conducted by Ilie and coworkers (2009), reported a fairly close frequency for the homozygous genotypes, 0.13 for AA and 0.17 for BB genotype, respectively. In a study conducted by Bonfatti and coworkers (2010) for the Simmental cows, a similar distribution of the genotype frequency was reported, providing a higher share of the AB genotype (0.466) compared to the AA (0.306) and BB (0.213) genotypes. In other works on dairy breeds, it was found that the frequency of allele A was 0.57 and for allele B was 0.43. The genotype frequency was 0.43 for AA, 0.29 for BB and 0.28 for AB (Yahya et al., 2013).

The effects of the CSN3 and LGB loci on milk production and chemical composition are presented in Table 2.

The CSN3 genotype’s influence on milk production and chemical composition

The highest milk production was recorded for the AA genotype in the CSN3 locus (5887.76±115.7 kg). This production was significantly higher (>0.003) than that observed for the BB genotype (5619.28±164.3 kg). No significant differences (P>0.37) were recorded between the AA and AB (5839.37±117.8 kg) genotypes. Milk production associated with the AB genotype proved to be significantly higher (P<0.007) when compared to that associated with the BB genotype (5619±86.34 kg). These results confirm previous studies conducted by Lara and coworkers (2013) with a higher production of milk being associated with the BB genotype. No significant differences were observed between AA and BB genotypes in other studies conducted by Strzalkowska and coworkers (2002) for Holstein Friesians and Felenczak and coworkers (2008) for the Simmentals. Research conducted by Gradinaru and coworkers (2013) for the Romanian Simmental cattle, recorded a higher milk production associated with the BB, while the lowest production level was associated with the AA genotype. Bugac and coworkers (2013) found an increase in milk production associated with the AB genotype in a study conducted for the Montbeliarde breed.

The AA genotype favoured a higher fat percentage in milk (4.19±0.05%) compared to the AB genotype (4.08±0.06%, P<0.004) and BB genotype (4.01±0.02%, P<0.008). These outcomes were consistent with previous results found by Tsiaras and coworkers (2005), Benesik and coworkers (2007) and Sitkowska and coworkers (2008). In a study conducted for the Simmental cows, Felenczak and coworkers (2008), found opposite outcomes, with a higher production of milk being associated with the BB genotype. Scientific literature provides some conflicting with results obtained by Tsiaras and coworkers (2005) and Sitkowska and coworkers (2008) for dairy breeds. Felenczak and coworkers (2008) found that a higher value for milk fat content is associated with the BB genotype in a study conducted with Simmental cattle, 3.53±0.18% for BB when compared to 3.46±0.19% for the AA genotype. Scientific literature provides some outcomes that highlight no significant influence of genotypes on milk protein content for the Romanian Simmental breed (Gradinaru et al., 2013). In our study, the total protein yield was not influenced by the CSN3 genotype. Average values for milk protein yield were 192.52±4.12 kg for AA, 192.11±6.6 kg for AB, 191.04±3.61 kg for the BB genotype, respectively. These outcomes were consistent with results previously found by Gradinaru and coworkers (2013) and Felenczak and coworkers (2008).

The LGB genotype’s influence on milk production and chemical composition

The productive traits were assessed according to the three genotypes in the LGB locus. The highest milk production was recorded for the AB genotype (5906.54±166.76 kg), being significantly different from that produced by the AA (5890.1±117.58 kg, P<0.033) and BB genotypes (5812±115.11 kg, P<0.038). This result was consistent with previous findings of Ilie and coworkers (2009) for the Romanian Brown breed, and Strzalkowska and coworkers (2002) and Tsiaras and coworkers (2005) for dairy breeds. Conflicting outcomes were found by Ojala and coworkers (1997) whose studies recorded no significant association between genotypes and milk production. Some studies found a positive association between the BB genotype and milk yield (Yahya et al., 2013). For the Romanian Simmental breed, the results provided by Gradinaru and coworkers (2013) did not record a significant influence of LGB genotypes on milk production level, 6289.9±134.1, 6540.1±97.1, 6537.8±149.9 kg/lactation for AA, AB and BB, respectively.

A significantly higher fat percentage was recorded for the BB genotype (4.23±0.06%) when compared to the AA (4.17±0.06%, P<0.003) and AB genotypes (4.2±0.08%, P<0.011), as previously reported by Ahmadi and coworkers (2008) for the Holstein cows. Gradinaru and coworkers (2013) in a study conducted for the Romanian Spotted breed had found a similar fat content for the AA, AB and BB genotypes. In our study, there were no significant differences in the fat yield between the three genotypes: AA (248.07±5.93 kg), AB (242.23±9.96 kg, P>0.42) and BB (245.84±7.71 kg, P>0.31). Similar results were reported by Gradinaru and coworkers (2013).

An average protein percentage in milk of 3.34±0.05% was associated with the AB genotype, while a value of 3.31±0.04% (P>0.57) was associated with the AA and 3.33±0.01% (P>0.081) with the BB genotype. This highlights that the LGB genotype has no significant influence on the protein content of milk. These results are similar to previous findings by Gradinaru and coworkers (2013) obtained for the Romanian Simmental cows and are conflicting with results obtained by Ahmadi and coworkers (2008). In our study, the total milk protein yield was significantly lower in AA (192.27±5.43 kg, P<0.042) and BB (193.53±3.94 kg, P<0.026) homozygous genotypes when compared to the AB heterozygous genotype (197.27±6.19 kg). No significant differences (P>0.34) were recorded between the AA (192.27±5.43 kg) and BB (193.53±3.94 kg) homozygous genotypes, for the total milk protein yield.

In some previous studies regarding frequencies of alleles and genotypes in CSN3 and LGB different results were re-
corded probably due to genetic improvement schemes applied into herds. Moreover, along the same breed, the results obtained by different authors are inconsistent due to many potentially influential factors, such as the rearing areas and rearing conditions, weather conditions, nutritional support, age of cows, animal’s welfare state, accuracy of data recording, data analysis methods etc. Due to these inconsistent results, more studies regarding these issues are necessary, particularly for the local dual-purpose breeds.

CONCLUSIONS

The current study offers preliminary results regarding the influence of CSN3 and LGB genotypes on the milk yield and milk chemical composition.

For the CSN3 locus, higher milk productions were associated with the AA and AB genotypes. The homozygous BB genotype was negatively associated with the milk yield. The fat percentage was positively influenced by the AA genotype, which was significantly higher when compared to the values associated with the AB and BB genotypes. The AA and AB genotypes were negatively associated with the protein percentage when compared to the BB genotype.

For the LGB locus, the AB genotype was positively associated with the milk production. Significantly higher fat percentage for the BB homozygous genotype was recorded. Milk protein percentage was negatively associated with the LGB genotype. No significant differences were recorded between the three genotypes.

The importance of study is that it proves the influence of individual genotypes on milk quality. The studies focusing on the frequency of alleles and genotypes in the CSN3 and LGB loci have offered various results according to the cows’ breed. Nevertheless, more studies are necessary due to contradictory outcomes found within the literature. The future plan is to characterize a larger number of animals from different areas of the country, for milk proteins’ genetic variants in order to contribute to a better knowledge of the breed genetic structure. Furthermore, individual genotypes could be used in the future for the marker assisted selection programs, and could be included in the future selection scheme of the breed.

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For the LGB locus, the AB genotype was positively associated with the milk production. Significantly higher fat percentage for the BB homozygous genotype was recorded. Milk protein percentage was negatively associated with the LGB genotype. No significant differences were recorded between the three genotypes.

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