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β-LACTOGLOBULIN GENE POLYMORPHISM IN HOLSTEIN-FRIESIAN CATTLE REARED IN BULGARIA: A POPULATION GENETIC PERSPECTIVE

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ABSTRACT

β-lactoglobulin (LGB) is a major whey protein in ruminant milk. Its genetic polymorphism is of great interest due to its influence on the qualitative and technological properties of milk. The experiment was conducted within a small Holstein-Friesian population reared at the Academic Technological Complex of Trakia University – Stara Zagora and aims to determine the gene and genotype frequency distribution of the two major alleles of the β-lactoglobulin locus – A and B. The molecular analyses of the LGB gene were completed using the Axiom Bovine Genotyping Array v3. The observed frequencies were compared to the expected values according to the Hardy-Weinberg principle. The results obtained demonstrate that the frequency of allele A is 0.73, while allele B has a frequency of 0.27. The performed χ^2 test revealed the studied population is in a state of genetic equilibrium ($\chi^2 = 0.0189332$, df=1, P>0.05).

Key words: β-lactoglobulin, LGB gene, Holstein-Friesian cattle, milk.

Introduction

Milk proteins are divided into two major groups – caseins and whey proteins (*Thompson et al.* 2009). The casein fraction in cattle milk is over 80% of the total protein, which makes it one of the most commonly investigated polypeptides in dairy cattle. Due to its impact on the technological properties of milk, β-lactoglobulin is the second protein under the radar of modern research (Hernández-Herrera *et al.* 2025). Since they serve as an excellent tool for marker-assisted selection, polymorphisms of milk proteins are of great importance to modern farmers. The whey proteins group is mainly represented by α-lactalbumin and β-lactoglobulin families. Although other proteins contribute to the overall protein profile of milk, they do not have a significant impact on its technological properties.

In *Bos taurus*, the β-lactoglobulin locus is found on chromosome 11 and is about 4kb long (Boushaba *et al.* 2024). The gene consists of 7 exons separated by 6 introns (Windarti *et al.* 2022). β-lactoglobulin is the main whey protein and is found in milk in many species, but is not found in the human milk (Nguyen *et al.* 2015). Its molecular mass is about 18kDa and contains 162 amino acid residues. This protein represents about 12% of the total protein in milk and about 50-60% of the whey protein. β-lactoglobulin serves as a major indicator of milk quality, where its average concentration in cow's milk is about 2-4g/L (Ruprichova *et al.* 2014).

β-lactoglobulin belongs to the group of so-called lipocalins, which are known for their ability to transport small hydrophobic molecules. A number of studies have shown that β-lactoglobulin has the ability to bind to various types of molecules such as fatty acids, cholesterol, retinol, vitamin D, etc. (Le Maux *et al.* 2014). According to some studies, it exhibits ACE inhibitory properties, making it a potential tool for antihypertensive therapy purposes (Daliri *et al.* 2018).

Furthermore, according to Chaneton *et al.* (2011), it also exhibits antimicrobial capabilities against the bacteria *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus uberis*, which are known to be the main causes of mastitis in dairy cows. In addition, β-lactoglobulin demonstrates antimicrobial properties against bacteria that cause infections in humans (Pan *et al.* 2007).

There are several variants of this protein in cow's milk, but the most abundant are variant A and variant B. According to the literature, the total number of β-lactoglobulin variants is 11 (A, B, C, D, E, F, G, H, I, J and W), represented in different species of the genus *Bos* (Gai *et al.* 2021). The differences between variants are result of amino acid substitutions in the polypeptide chain (Farrell *et al.* 2004).

In 1955, Aschaffenburg and Drewry discovered the first two forms of β-lactoglobulin, currently known as variant A and variant B. The two variants differ in only two amino acids (Aschaffenburg and Drewry, 1955). Numerous studies have demonstrated that variants A and B of β-lactoglobulin have a significant impact on the composition of cow's milk, as well as on its processing capacity. Variant A is associated with higher total whey protein and milk yield, while variant B correlates with higher fat and casein content, making it better for cheese production. Furthermore, variant B is more heat stable, while variant A is more prone to deposit formations during heat treatment (Boye *et al.* 2004).

The aim of the present study was to determine the allele and genotype distribution of the β-lactoglobulin locus in a small population of Holstein-Friesian cattle.

Materials and Methods

Experimental animals and sample collection

The experiment included a total of 30 Holstein-Friesian cattle, reared at the Academic Technological Complex of Trakia University – Stara Zagora, Bulgaria. Animals were in their first, second or third lactation and were randomly selected to avoid related individuals. Tissue samples were obtained from the ear cartilage using a TSU sampling device and stored in special TSU tubes until DNA extraction. All sampling procedures were in accordance to the Veterinary Medical Activity Act (VMA) and Regulation No. 20/12 on the minimum requirements for the protection and humane treatment of experimental animals.

Genotyping

Molecular analyses of the LGB gene were performed using the Axiom Bovine Genotyping Array v3. The chip contains 630,000 informative single nucleotide polymorphisms (SNPs), which are evenly distributed across the bovine genome. The v3 version provides a significantly higher density of markers, which allows for increased accuracy and broader detection of alleles, including the LGB gene. The animal genotyping procedure consists of three main stages: DNA extraction and purification; genotyping of the animals with the Affymetrix Axiom Bovine Array; and data processing with subsequent sending to the CDCB – the Council on Dairy Cattle breeding. The first stage includes preparation and lysis of the samples with subsequent DNA purification. At the end of the stage, a control test is performed to quantify the concentration of obtained DNA, as well as checking for the presence of PCR inhibitors. In the second stage, the animals are genotyped using the Axiom Bovine Genotyping Array v3 chip. The third stage consists of processing the received data and sending them to CDCB. All procedures were performed at Genetic Visions-ST, LLC (8137 Forsythia St, Suite 100, Middleton, WI 53562, USA). Results generated by Affymetrix after July 30, 2019 are accredited according to ISO17025:2017 (Cert 5436.01), which guarantees their compatibility with international standards for genetic analysis.

Allele and genotype frequency calculations

The observed allele and genotype frequencies of the LGB gene were processed according to the Hardy-Weinberg principle. This allows assessment of the correspondence between the observed and expected genotype distributions under random distribution of alleles. The data were processed according to the following equation:

$$p = 2(AA) + (AB) / 2N; q = 1 - p$$

where "p" is the frequency of allele A, "q" is the frequency of allele B, N is the total number of animals tested, "AA" is the number of animals that are homozygous for allele A, "AB" is the number of heterozygous individuals, and "BB are the animals that are homozygous for allele B.

Results

The obtained results for the allele and genotype distributions among the analyzed Holstein-Friesian population can serve as a prerequisite for the development of MAS program, targeting specific subtype of the LGB gene.

In the studied group of 30 animals, we observed the following genotypes: 16 cows (53.33%) were homozygous for allele A (genotype AA), 12 cows (40%) were heterozygous carriers (genotype AB) and 2 cows (6.67%) were homozygous for allele B (genotype BB). Based on these data, the allele frequencies were calculated: Allele A: $p = (2 \times 16 + 12) / (2 \times 30) = 44 / 60 = 0.73$; Allele B: $q = (2 \times 2 + 12) / (2 \times 30) = 16 / 60 = 0.27$. The expected genotype frequencies according to the Hardy-Weinberg principle ($p^2 + 2pq + q^2 = 1$) were as follows: for genotype AA: $p^2 = 0.73^2$ x N = $0.53 \times 30 \approx 15.99$ animals, for genotype AB: 2pq x N = $(2 \times 0.73 \times 0.27)$ x 30 = $0.39 \times 30 \approx 11.82$ animals and for genotype BB: q^2 x 30 = 0.27^2 x 30 = $0.07 \times 30 \approx 2.19$ animals.

A χ^2 test was performed to statistically assess the deviations between the observed and expected genotype frequencies (Table 1). The χ^2 value was calculated using the equation $\Sigma (O - E)^2/E$, where: O is the observed number of animals and E is the expected number according to the Hardy-Weinberg principle. The calculations for each genotype are as follows: for genotype AA: $(16 - 15.99)^2/15.99 = (0.01)^2 / 15.99 \approx 0.0000063$; for genotype AB: $(12 - 11.83)^2 / 11.83 = (0.17)^2/11.83 \approx 0.0024429$ and for genotype BB: $(2 - 2.19)^2 / 2.19 = (-0.19)^2 / 2.19 \approx 0.0164840$. The overall value of χ^2 is $= 0.0000063 + 0.0024429 + 0.0164840 = 0.0189332$. With a degree of freedom of 1 (df = number of genotypes − number of alleles = 3 − 2 = 1) and a critical value of χ^2 at $\alpha = 0.05$ equal to 3.84, the calculated value of χ^2 is significantly below this threshold. The result confirms that the distribution of genotypes in the studied population does not differ significantly from the expected one and indicates that the population is in genetic equilibrium according to the Hardy-Weinberg rule with respect to the LGB locus. The insignificant deviation in the frequency of heterozygotes may be due to the limited sample size or to the breeding practices on the farm. The results demonstrate a predominance of the A allele, which allows its use as a selective marker for the production of milk with the preferred type A β-lactoglobulin.

Table 1: Comparison between observed and expected genotype distributions for the LGB locus and results of the χ² test for Hardy–Weinberg equilibrium in Holstein-Frisian cattle reared at the Academic Technological Complex of Trakia University.

Genotype	Observed (O)	Expected (E)	$(O-E)^2/E$
AA	16	15.99	0.0000063
AB	12	11.83	0.0024429
BB	2	2.19	0.0164840
Σ			**0.0189332**

df = 1; χ^2 critical (0.05) = 3.84

Discussion

Allele A and allele B encode a protein composed of 162 amino acid residues, with the two variants differing by two amino acids. At position 64 variant A contains the amino acid Asparagine (Asp), while variant B encodes Glycine (Gly) the same position, g. 3983 A>G (p. Asp64Gly) (GenBank X14710.1). At position 118 variant A includes the amino acid Valine (Val), while variant B holds the amino acid Alanine (Ala), g. 5263 T>C (p.Val118Ala) (GenBank X14710.1) (Figure 1).

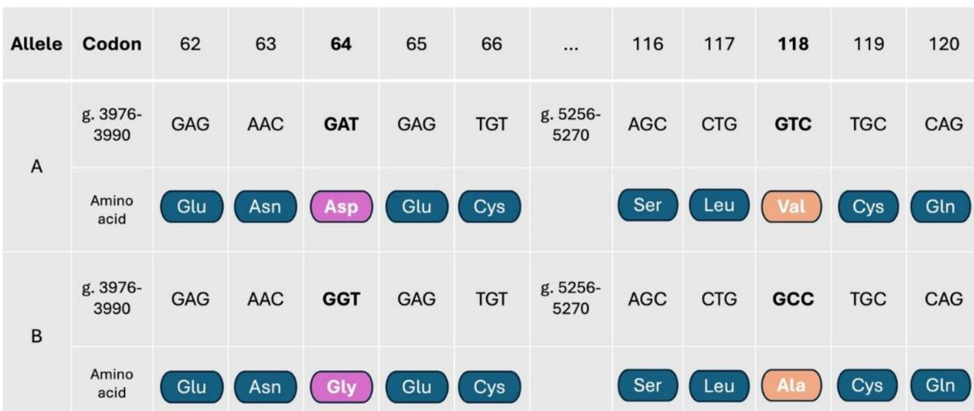

Figure 1: Genetic polymorphism of the β-lactoglobulin (LGB) gene in *Bos taurus*.

Polymorphisms in β-lactoglobulin are associated with a number of milk parameters, where milk processability is clearly related to the different responses to heat treatment of different variants. Studies by Ng-Kwai-Hang *et al.* (1990), have shown that the B variant of β-lactoglobulin (β-LG) is associated with higher milk fat content, while the A variant is associated with higher protein content. Specifically, cows with the β-LG BB genotype are characterized by a higher percentage of fat in milk compared to the carriers of the AA or AB genotypes.

The existence of such correlation between the alleles of the LGB gene and productive parameters in different cattle breeds can serve as a prerequisite for further studies regarding the relationship between gene polymorphisms and milk productivity in Holstein-Friesian cattle.

An interesting correlation was found in a study by Ost`erhoff *et al.* (1973) between the polymorphism of the LGB gene and the manifestation of mastitis. Authors documented very low incidence of the condition among the carriers of the heterozygous AB genotype. The results of Rodriguez-Serrano *et al.* (2025) demonstrate that the presence of allele A is associated with the highest concentration of milk protein (ideal for cheese production), while genotype BB is associated with a higher fat content in the milk.

The frequencies of the most common LGB alleles are of great interest and the results of different studies show a wide variation in these values. Such fluctuations can be explained with the different breeding practices as well as the variation of the selection pressure of the local markets.

Angelova *et al.* (2021) reported a frequency of allele A of 0.42 and allele B of 0.58 in Brown cattle. Authors report genotype frequencies of 15.9 for the AA genotype, 52.9 for the heterozygous carriers (genotype AB) and 31.8 for the BB genotype, respectively. Kučerová *et al.* (2006) reported a frequency of allele A and allele B in Flekvich cattle bred in the Czech Republic of 0.511 and 0.489, respectively.

A study by Yordanova *et al.* (2021) documented frequencies of 0.575 for allele B and 0.425 for allele A in Bulgarian Black and White cattle showed. The most common genotype in their experiment was the heterozygous variant AB (49.25%), followed by genotype BB (32.84%). Rincon-Florez *et al.* (2024) reported a frequency of allele B of 0.657, and of genotype BB of 0.389.

Using the same SNP chip, Lihodeevskaya *et al.* (2025) reported allele A frequency of 0.375, and the frequency of allele B was 0.625. Shah *et al.* (2024) obtained a genotype frequency of AA of 0.68 in Jersey and 0.56 in Holstein-Friesian populations, respectively. The frequency of the A allele in Jersey was 0.66 and in Holstein-Friesian cattle 0.72, respectively. The later results is very close to the data from the current experiment of ours.

In a large-scale study by Khudyakova *et al.* (2023) on the frequency of alleles and genotypes of the LGB gene in Black and White cattle and Simmental in Italy, India and the Czech Republic, revealed that the frequency of the B allele in the Black and White breed varies in the range from 0.21 to 0.64, while in Simmental the frequency of this allele varies from 0.42 to 0.65. Such findings indicate that in certain areas artificial selection benefits the frequency of the B allele.

Kamiński *et al.* (2023) noted that for the period from 1991 to 2014 the frequency of the B allele in Holstein-Friesian cattle decreased from 63% to 40%, which may be the result of increased selection towards the A allele of the LGB gene. The results from our experiment confirm the same trend. The observed changes in allele frequencies could be a result of breeder decisions and/or a positive correlation between certain milk protein variants and milk production traits.

Conclusion

The present study provided a detailed analysis of allele and genotype frequencies, as well as genetic polymorphism of the LGB gene in the studied group of animals. The results obtained confirm the presence of significant genetic diversity, which is directly related to the selection processes and technological qualities of milk.

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