GENE AND GENOTYPIC FREQUENCIES OF β-CASEIN VARIANTS A1 AND A2 IN DAIRY CATTLE

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ABSTRACT

Taking into account the possible benefits that milk containing only β-casein A2 can bring to the intestinal health of individuals and milk constituents, the objective of this work was to identify the allelic and genotypic frequency of variants A1 and A2 of the β-casein gene and their influence on the physicochemical composition of milk from crossbred cows. To identify the genetic profile, tail hairs were collected from 51 crossbred cows for DNA extraction and genotyping, and the gene and genotypic frequencies of the A1 and A2 variants of beta-casein were calculated. The genotypes were associated with the following milk

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characteristics: fat, total protein, lactose, casein, total solids and defatted dry extract, density and pH. The data from the physicochemical analysis were submitted to the t-test with Bonferroni correction at 5% significance ($P<0.05$). The content of total solids (12.42 and 15.31%) and fat (2.14 and 4.05%) were different between genotypes A1A2 and A2A2, respectively ($P<0.05$). The herd is in Hardy-Weinberg equilibrium.

Keywords: Beta-casomorphin-7. CSN2. Milk A2. Polymorphism.

INTRODUCTION

Dairy cattle farming is considered an activity that has a high possibility of growth and plays a significant economic role in developing countries. Brazil is in 3rd place in the ranking of the world's largest milk-producing countries (ROCHA et al., 2020).

Given the growing association of milk consumption with adverse effects in susceptible individuals and the increased interest in the consumption of milk alternatives, it has stimulated researchers, farmers and consumers to a new trend in the dairy market: the production of milk from cows with only the A2 allele of β-casein, called Milk A2A2. Consumption of A2 milk has been linked to beneficial effects on human health and is easier to digest in sensitive individuals.

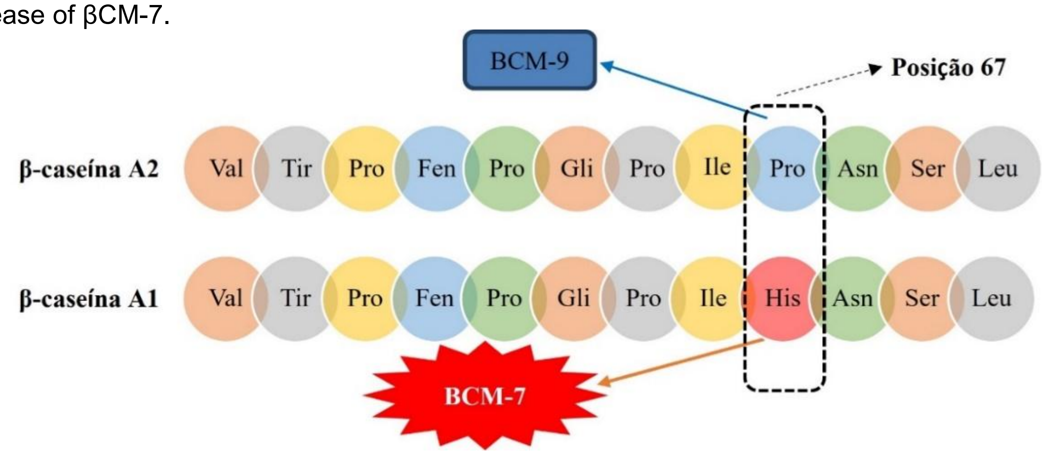
Dairy proteins are classified into two major groups: caseins and whey proteins (alpha-lactalbumin and beta-lactoglobulin). Caseins make up about 80% of bovine milk proteins and come in four forms: α S1 (ranging from 12 to 15 g/L or 38%), α S2 (3 to 4 g/L or 10%), β-casein (9 to 11 g/L or 34%), and k-casein (2–3 g/L or 15%) (RASIKA, 2021). These proteins differ in amino acid sequence and are encoded by four genes (CSN1-S1, CSN1-S2, CSN2, and CSN3), respectively (VISKER et al., 2011).

β-casein contains 209 amino acids and fifteen variants (A1, A2, A3, A4, B, C, D, E, F, G, H1, H2, I, J, K) (GAZI et al., 2022), however, only seven of these (A1, A2, A3, B, C, I and E) have been detected in European cattle breeds, with variants A1 and A2 being more common (MONTENEGRO et al., 2022).

Studies indicate that β-casein A2 is the oldest variant, present in the cattle herd since its domestication, being considered the original form of the protein, from which the others originated by mutation and spread with the directed reproduction of animals to increase milk production. It is considered a mutation via natural selection (MONTENEGRO et al., 2022).

Casein A1 and A2 are differentiated by the polymorphism of a nucleotide in the CSN2 gene located on chromosome 6, which results in the substitution of only one amino acid at the 67th position of the 209 amino acids that make up this protein. The A1 variant has a histidine, while the A2 variant has a proline, as shown in Figure 1. (JIAQUIN et al., 2016). This difference affects how these proteins will be hydrolyzed in the gastrointestinal tract. (VITTE et al., 2022).

Figure 1 – Fragment of the genetic variants A1 and A2 of β-casein, highlighting the differentiation at position 67 and release of βCM-7.



Source: adapted from Daniloski et al., (2021).

The consumption of milk from cows carrying the A1 variant of β-casein allows the cleavage of the amino acid peptide chain at the 67th position, releasing Beta-Casomorphin-7 (βCM-7). βCM-7 is composed of seven amino acids (Tyr-Pro-Phe-Pro-Gly-Pro-Ile), which act as a source of opioid peptides in the body during digestion, known as exorphins (AYAZ et al., 2022). The presence of proline in the 67th position of the A2 variant of β-casein prevents the breakdown of the polypeptide sequence at this point, forming another peptide called Beta-Casomorphin-9 (BCM-9) (MARKO et al., 2020).

Gastrointestinal digestion of β A1 casein through proteolytic degradation with the help of different enzymes such as pepsin, trypsin, alkalase, chymotrypsin, pancreatin and thermolysin releases βCM-7 into the intestinal epithelium, which due to its low molecular weight, crosses the blood-brain barrier and is distributed in various organs and tissues of the body including ear, brain, pancreas and kidneys (VISKER et al., 2011). βCM-7 is an opioid agonist that has a wide variety of potential health effects, including immunosuppressive activity, however, more research is needed in this area (SWINBURN, 2004).

The hypothesis that a high intake of milk containing CNS2-A1 promotes cardiovascular disease (ischemic heart disease, atherosclerosis), type 1 diabetes mellitus, and neurological problems (schizophrenia and autism) is intriguing and premature. There is some very suggestive evidence from ecological studies for type 1 diabetes mellitus and ischemic heart disease, and there is certainly a possibility that milk variants A1/A2 are involved in the etiology of these diseases. However, this hypothesis does not yet present sufficient scientific evidence. The evidence for schizophrenia is minimal (SWINBURN, 2004).

Recent studies have shown that the consumption of milk containing the A1 variant of β-casein increases the activity of MPO (Myeloperoxidase), MCP-1 (monocyte chemotactic protein), IgE (immunoglobulin E), IgG (immunoglobulin G) and IL-4 (interleukin 4) in the gastrointestinal tract along with the infiltration of leukocytes into the intestinal villi. The increase in these molecules, which act as markers of inflammatory processes and play an important role in allergic reactions, is attributed to the release of βCM-7 (HAQ et al., 2014).

According to Jianquin et al. (2016), based on the effects of βCM-7 on the gastrointestinal tract, it is possible that part of the discomforts attributed to dairy product intolerance may be due to the effects of βCM-7 on the gastrointestinal tract, rather than lactose itself.

Due to its inflammatory characteristic, βCM-7 can affect the production and decrease in the activity of the enzyme lactase, consequently, reduction in motility and inflammation, altering lactose metabolism and promoting similar symptoms of lactose intolerance in individuals sensitive to βCM-7 (BARBOSA et al., 2019).

Recent research has also detected the influence of the A2 variant of β-casein on the increase of milk constituents, especially in relation to protein concentration, fat and volume (VISKER et al., 2011). Therefore, the preference for the A2 allele in dairy cattle may have several positive implications not only for humans, but also for milk production and composition. However, to clarify the definitive effects of β-casein variants A1 and A2 on human health and milk production characteristics, further investigations are needed.

Considering that crossbred breeds are responsible for a large part of milk production in the country, and that with the expansion of crossbreeding, the levels of mixing between breeds are unpredictable and the lack of pedigree registration can lead to a lack of genetic information about the herds, the importance and usefulness of the study of these herds for their characterization is highlighted (KHAN et al., 2023).

In view of the above, the objective of this study was to verify the genetic and genotypic frequencies of the A1 and A2 variants of β-casein and their effect on the milk composition of crossbred cows.

METHODOLOGY

OPINION OF THE ETHICS COMMITTEE

This study was submitted for evaluation and appreciation by the Ethics Committee on the Use of Animals of the Federal University of Piauí (CEUA/UFPI) and approved on March 18, 2022, through protocol No. 709/2022.

STUDY SITE AND HERD

The present experiment was carried out from April 2021 to December 2022. The animals used in this experiment come from the herd of a dairy farm, located in the municipality of Paragominas - Pará. The studied herd was composed of 51 crossbred Holstein x Gir cows, with five genetic compositions: 1/2 HO + GL; 3/4 HO + GL; 5/8 HO+GL; 9/16 HO + GL and 13/16 HO + GL in lactation. The property uses the Compost Barn installation system with a diet based on corn silage, corn and soybean meal and supplementation with Nutron Core. Milking is carried out twice a day with an interval of 12 hours (4 am and 4 pm).

GENOTYPIC ANALYSIS OF COWS

The animals were genotyped for the A1 and A2 variants of β-casein from DNA extracted from hair follicles. The hairs were removed from the broom of the animal's tail, lacing between 15 and 25 hairs on the fingers of the hand, pulling them firmly and then checking if the bulbs were intact. Then, they were placed in envelopes, identified and stored at room temperature until they were sent to the Institute of Animal Science in Nova Odessa-SP, responsible for performing DNA extraction and genotyping.

DNA extraction from hair follicles was performed using the Easy-DNA™ kit (Cat. No. K1800-01—Protocol No. 1—Small samples of blood and hair follicles; Invitrogen, Carlsbad, USA), as recommended by the manufacturer. The DNA was diluted in 20 μL of Tris-EDTA. The quantification and purity of the extracted DNA were estimated by spectrophotometric readings in the proportions of 260 nm and 260/280 nm, respectively. The DNA concentrations of all samples tested were adjusted to 5 ng μL^{-1}. A novel real-time PCR using a combination of modified nucleic acid-blocked conjugated probes (LNA) was developed to genotype the A1 and A2 alleles of the β-casein (CSN2) gene, according to the methodology of Giglioti et al., (2021). Based on the control samples (genotypes A2A2, A1A1 and A1A2), the evaluated samples were compared. Subsequently, upon receipt of the results, the allele and genotypic frequencies for beta-casein were calculated.

CALCULATION OF GENE AND GENOTYPIC FREQUENCIES

The allele (Xi and Xj) and genotypic (Xii, Xij and Xjj) frequencies were determined by the following equations:

Allele frequencies $X_i = \frac{2N_{ii} + \sum N_{ij}}{2N} \times 100$ $Xj = \frac{\sum N_{ij}}{2N} \times 100$

Genotypic frequencies $X_{ii} = \frac{N_{ii}}{N} \times 100 \quad X_{ij} = \frac{N_{ij}}{N} \times 100 \quad X_{jj} = \frac{N_{jj}}{N} \times 100$

Where, Nii, Njj and Nij represent the number of homozygotes and heterozygotes observed in the i and j alleles; and N corresponds to the number of individuals observed.

To test the observed frequencies, a Hardy-Weinberg equilibrium test calculation was performed, which is given by the expansion of the described binomial: $(Xi + Xj)^2$ = Xi2 + 2XiXj + Xj2

Where, 2Xi is the expected frequency of homozygotes for the i allele; 2XiXj = expected frequency for ij heterozygotes; 2Xj = expected frequency of homozygotes for the j allele.

PHYSICOCHEMICAL ANALYSIS OF MILK

For the milk collections of the physicochemical analyses, 20 crossbred cows were separated and selected, among the 51 genotyped animals, being 10 cows with heterozygous genotype A1A2 and 10 cows with homozygous genotype A2A2 for beta-casein with three genetic compositions: 1/2 HO+GL, 3/4 HO+GL and 5/8 HO+GL. The samples were collected 72 hours postpartum, during the manual milking of the cows, packed in sterile collection bottles with a volume of 50ml, properly identified and frozen at -20ºC.

Subsequently, the samples were thawed at refrigerated temperature and packed in bottles with bronopol preservative, and sent to the Milk Quality Laboratory of the Food Research Center of the School of Veterinary and Animal Science of the Federal University of Goiás - LQL/CPA/EVZ/UFG, responsible for the determination of fat, total protein, lactose, casein, total solids and defatted dry extract in an infrared absorption instrument. The determination of density at 15ºC and pH were carried out at the Center for Studies, Research and Food Procedures of the Federal University of Piauí – NUEPPA/UFPI, by means of the portable ultrasonic milk analyzer equipment, the Ekomilk® (Cap-Lab Ind.e Com. Ltda.). Following the manufacturer's recommendations, the raw milk analysis mode was selected in the device.

STATISTICAL ANALYSIS

To adjust the generalized linear model (GLM), the GENMOD procedure was used to analyze the association between the variables (fat, protein, lactose, total solids, non-fat solids, pH, casein and density) and factors: genotypes (A1A2, A2A2) and blood grade (1/2 HO, GL; 3/4 HO, GL; 5/8 HO, GL), in which the age of the animals was considered as a covariate. The means were compared using the t-test with Bonferroni correction at 5%

probability, using the Statistical Analysis System® Academy (SAS, 2023) computer program. The chi-square test was performed to determine whether the observed genotypic frequencies deviated significantly from the Hardy-Weinberg Equilibrium.

RESULTS AND DISCUSSION

ALLELE AND GENOTYPIC FREQUENCIES

Zebu breeds have been successfully used in the generation of crossbred and synthetic breeds in Brazil, mainly in milk production. The animals studied showed promise for milk production containing only β-casein A2, as shown in Table 1.

Table 1. Genetic and genotypic frequency of beta-casein in crossbred cows.

	Observed Genotypes			Allele Frequencies	
	A1A1	A1A2	A2A2	A1	A2
Observed frequency	0,019	0,27	0,70	0,156	0,843
Expected frequency	0,024	0,26	0,71		
N	1	14	36		

Source: The authors (2024).

Of the 51 genotyped animals, only 1% were homozygous for the A1 allele, 27% were heterozygous (A1A2) and 70% were homozygous for the A2 allele. For the allelic frequency, 16% was observed for the A1 allele and 84% for the A2 allele. The allele frequencies observed showed that the A2 allele is more frequent than the A1 allele in crossbred animals, as well as the frequency of the A2A2 genotype prevailed over the A1A1 and A1A2 genotypes.

The results presented corroborate the results found in the national literature (Sousa et al., 2019; Paschoal et al., 2017; Schettini et al., 2020), which in general have a higher frequency of the A2 allele, and its A2A2 genotype, to the detriment of the A1 allele and the A1A1 genotype in the most varied breeds.

Sousa et al. (2019) and Paschoal et al. (2017) working with Gir cows observed a higher frequency for the A2A2 genotype, representing 91.2% for the first author and 59% for the second author. Such results are in agreement with studies in the literature and indicate an association of genetic polymorphism with the breed, with zebu breeds having a higher prevalence of the A2A2 genotype compared to taurine. This information values the Zebu breeds that, associated with animals of European origin, give rise to crossbred cattle, responsible for approximately 70% of milk production in Brazil.

For the Sindhi breed, Schettini et al. (2020) found a frequency of 94% for the A2 allele and 90% for the A2A2 genotype of the same breed. In addition, Silva et al. (2022)

evaluated 114 animals of the Curraleiro Pé Duro cattle breed and found a frequency of 40% for the A1 allele and 60% for the A2 allele and the frequencies of the A1A1, A1A2 and A2A2 genotypes were 20%, 39% and 41%, respectively.

Several studies have been carried out to quantify allele and genotypic frequencies in the most varied breeds. However, there is still little data on the frequencies of the CSN2 gene in crossbred breeds, mainly Holstein x Gir animals of varied genetic compositions.

Therefore, 84% of the animals in this research are carriers of the A2 allele, and can transmit this allele to their offspring, producing milk with lower levels of βCM-7, since this peptide has the potential to affect the opioid receptors of the nervous, immune, endocrine and gastrointestinal systems.

In addition, it was possible to conclude that the observed frequencies are in accordance with what was expected, there is constancy of gene and genotypic frequencies over the generations, that is, the population is in Hardy Weinberg equilibrium.

POLYMORPHIC ASSOCIATION OF THE CNS2 GENE WITH MILK COMPOSITION CHARACTERISTICS

The median, interquartile distance (1st and 3rd quartiles) and minimum and maximum values of the milk composition characteristics of crossbred cows associated with the genotypes (A1A2 and A2A2) of β-casein were presented in the form of a boxplot and are presented in Figure 2. The results related to the two genotypes (A1A2 and A2A2) and blood grade (1/2 HO, GL 3/4 HO, GL and 5/8 HO, GL) in relation to the physicochemical components of milk from crossbred cows are described in Table 2.

Figure 2. Estimates of means, medians, minimum, maximum and coefficient of variation (CV) related to the compositional parameters of milk from crossbred cows A1A2 and A2A2.

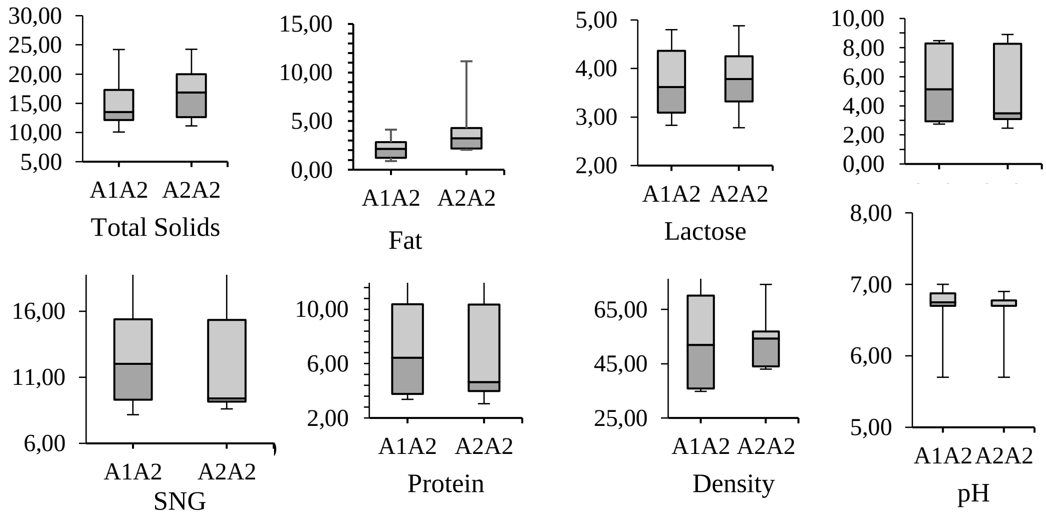


Table 2. Physicochemical composition of milk as a function of genotypes A1A2 and A2A2 for β-casein and blood grade of crossbred cows.

Variables	Genotypes		Degree of Blood			EPM	p-value1	p-value2
	A1A2	A2A2	1/2 HO, GL	3/4 HO, GL	5/8 HO, GL			
Fat (%)	2,14	4,05	2,84	3,86	2,33	0,77	0,019*	0,536
Protein (%)	4,95	5,69	7,69	5,90	3,32	1,33	0,549	0,203
Lactose (%)	4,12	3,88	3,63	4,08	4,31	0,21	0,460	0,404
ST (%)	12,42	15,31	16,14	15,58	10,42	1,35	0,046*	0,182
Casein (%)	3,91	4,46	6,16	4,63	2,56	1,10	0,574	0,189
SNG (%)	10,14	10,99	13,25	11,01	8,07	1,34	0,526	0,161
Density (g/mL)	47,38	49,7	56,97	43,79	45,80	5,37	0,728	0,289
pH	6,82	6,77	6,76	6,72	6,91	0,03	0,232	0,209

Source: The authors (2024). ST = Total Solids; SNG = Non-greasy solids; HO = Dutch; GL = Dairy Gyr; EPM = Standard error of the mean; p-value [1] = probability of t-test corrected for genotype; p-value [2] = probability of t-test corrected for blood grade.

Among the components, fat showed the greatest variability, with a coefficient of variation of 78.3%, followed by casein (61.1%) and protein (59.2%). The lactose content showed the lowest variation in composition (18.7%), as its amount is always constant because it is responsible for the osmotic balance of the milk, results that are in agreement with those published in the literature (GONZÁLEZ, 2001).

The interquartile range (difference between the third and first quartile) for the fat data was 1.59 and 2.18 for genotypes A1A2 and A2A2, respectively. For the position measures, the following values were found: 2.46 and 3.20 for median and mean. The maximum value was 4.12 and 11.16 and the minimum was 0.89 and 2.05 for genotypes A1A2 and A2A2, respectively (Figure 2.) This high range of variation is due to the variable nature of the component itself.

The contents of fat, protein, lactose, total solids, casein, non-fat solids, density and pH were associated in two ways: the first within the genotypes A1A2 and A2A2 of β-casein, the second taking into account the blood grade of the animals. The analysis of the parameters of the first revealed statistically significant differences (P<0.05) for the yield of fat and total solids, while in the second there were no significant differences.

The influence of the CSN2 gene on milk production parameters has been evaluated by different authors (Nilsen et al., 2009; Hanusová et al., 2010; Visker et al., 2011; Gustavsson et al., 2014; Oleński et al., 2010; Hallén et al., 2008).

For the total solids content, according to the results of the mean test, there was a significant difference (P<0.05), with an average of 12.42% for the A1A2 genotype and 15.31% for the A2A2 genotype. Fat, protein, and lactose are the main constituents of total solids, and this positive result is likely to correlate with higher fat concentration. The non-significant values of lactose and protein are possibly due to these parameters negatively correlating with fat. Lactose is the main osmotic agent in milk and the greater the amount of water removed by lactose, the lower or more diluted the fat content will be and vice versa.

The positive correlation of fat content is related to the amount of protein, indicating that the increase in fat yield will negatively influence milk protein levels.

In the herd studied, the fat concentration for genotype A1A2 was 2.14% and 4.05% for genotype A2A2. This means that, for each A2A2 animal in the herd, there is an increase of 1.91% in the milk fat content.

This result is in line with the results reported by Marko et al. (2020) who when evaluating the effect of β-casein genotypes on milk production, protein and fat traits in a population of Holstein-Friesian cows, found significantly higher fat concentrations in A2A2 animals compared to A1A1 and A1A2 genotypes.

Kumar et al. (2022) when studying the association of β-casein polymorphism and economically important production traits in a population of crossbred Vrindavani cows, revealed that genotype A1A1 had significantly lower fat percentage compared to genotypes A1A2 and A2A2, while genotype A2A2 had the highest fat percentage.

Milk fat has important technological consequences, especially associated with performance in cheese making, in addition to being a source of several bioactive fatty acids with beneficial properties to health.

However, our results differ from those obtained by Ikonen et al. (2001); Nilsen et al. (2009); Olenski et al. (2010); Ivánkovi´c et al. (2020); and Miluchová et al. (2023) who associated the influence of the A2 allele of β-casein with high protein concentration and low fat content. Miluchová et al. (2023) when analyzing the effect of beta-casein gene polymorphism and the association of genotypes with milk production traits in Holstein cattle herds, found a significant positive effect of the A2A2 genotype for protein content and a slightly negative trend for milk fat content.

The differences found are probably due to the fact that fat is the most variable component of milk, and can be affected by the particularities between breeds, the season of calving, or even the type of feeding.

The results were contradictory, possibly due to the small sample size and offers room for further investigation to provide clarity and robustness in the association of the A2 allele of β-casein and milk production and composition characteristics.

Although research is still ongoing to provide a concrete answer on the safety of the A1/A2 variants of β-casein, the demand and prices of products containing exclusively the A2 allele are increasing very rapidly at the national and international level due to the reported adverse impact of the A1 variant of β-casein on human health. The present investigation, however, sheds light on the comparative performance of β-casein A1/A2 variants in crossbred animals, warranting further genomic-association studies that may lead to the establishment of a reliable marker to improve herd profits, composition traits, milk quality, and human health.

CONCLUSION

The allelic frequency of the A2 allele and the genotypic frequency of the A2A2 genotypes for the β-casein gene in the crossbred animals evaluated indicate that these herds can produce less allergenic milk for individuals sensitive to the β-casein protein.

The investigation highlights the lack of superiority of the A1 variant of β-casein over the A2 variant, predominant in most of the breeds studied nationally and internationally, scoring positively on the characteristics of milk.

Due to the inconsistency in the literature related to the effects of the A1 and A2 alleles of β-casein, especially in relation to the protein and fat contents of milk, a more extensive investigation with a larger number of animals is necessary.

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