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The effects of κ-casein, β-lactoglobulin, prolactin and DGAT1 polymorphisms on milk yields in Turkish Holstein cows

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Abstract: The aim of this study was to determine the effects of κ-casein, β-lactoglobulin, prolactin, and DGAT1 (diacylglycerol acyltransferase1) gene polymorphisms on milk yields in Holstein cows raised in Antalya province, Turkey. A total of 517 cows were genotyped by the PCR-RFLP method to detect κ-casein/*Hinf*I, β-lactoglobulin/*Hae*III, prolactin/*Rsa*I, and DGAT1/*Cfr*1 polymorphisms. In the study, two types of alleles were identified as A and B for κ-casein, β-lactoglobulin, and prolactin loci, while K and A alleles were detected for DGAT1 locus. The frequencies of A and B allele were calculated as 0.8279 and 0.1721 for κ-casein, 0.4662 and 0.5338 for β-lactoglobulin, 0.8762 and 0.1238 for prolactin, respectively. The frequencies of the K and A allele were 0.6441 and 0.3559 for DGAT1. The studied population was found to be in Hardy-Weinberg equilibrium for all of the loci. Association analysis revealed a significant effect of prolactin-*Rsa*I polymorphism on milk yield ($p < 0.05$) in which animals with the B allele showed superior value than the A allele. On the other hand, no statistically significant relation effects of κ-casein-*Hinf*I, β-lactoglobulin-*Hae*III, and DGAT1-*Cfr*I polymorphisms on milk yield were observed ($p > 0.05$). It would be beneficial to increase the number of studies that determine the variation of existing genes and investigate the effects of these polymorphisms on milk yield characteristics in Holstein cattle. Thus, obtained results can be used as a selection criterion in marker-assisted selection programs.

Key words: Holstein, genetic polymorphism, milk protein genes, PCR-RFLP

1. Introduction

Animal products are an indispensable food source for humans due to their biological properties. In this context, animal production, which is the source of foods such as meat, milk and eggs, has an important place in the agricultural sector across the world. Turkey is suitable for almost all kinds of animal breeding in which cattle breeding is the most important part of livestock production in the country. Cattle breeds in Turkey meet approximately 88% of red meat and 90% of milk production[1]. Holstein breed, which has the ability to adapt to a wide range of climatic zones, is mainly preferred by farmers in Turkey as in the world, therefore the number of pure and hybrid has increased rapidly.

Milk proteins contain all of the nine essential amino acids necessary for humans. Cow's milk contains 3%-5% proteins, which of casein consist of approximately 70%-80% of this protein, whey protein makes up the remaining 20%-30%. Casein is composed of four different types known as α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein. Determination of milk protein variants in different cattle breeds is an important application aiming to avoid the increase of mutations that negatively affect specifically cheese making.

Located on bovine chromosome 6, the κ-casein gene[2] encodes milk protein important for the structure and stability of casein micelles. Additionally, κ-casein is a very important protein for cheese making. The rennin enzyme necessary to make milk coagulate and curdle destabilizes this protein. In the light of previous studies, the κ-casein gene is seen as a strong candidate gene for MAS in dairy and beef cattle [1-13].

Whey proteins make up the soluble fraction and they are composed of several different proteins, the most important of which are α-lactoalbumin and β-lactoglobulin. Locating on bovine chromosome 11[3], β-lactoglobulin is one of the

[1] TUİK, Turkish Statistical Institute (2019). Website http://www.tuik.gov.tr [accessed 05.05.2021].

[2] NCBI, National center for biotechnology information (2021). Website https://www.ncbi.nlm.nih.gov/nuccore/AJ849456.1#goto54114662_0. [accessed 07.12.2021].

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major whey proteins of ruminant species. The biological functions of this protein are still unclear; however, it could have a role in the metabolism of phosphate in the mammary gland, on the transport of retinol and fatty acids within the gut [14]. Because of its potential for use in genetic selection of the β-lactoglobulin gene polymorphism, several studies have been carried out to identify genetic variation and frequencies of their variants in different cattle breeds [3-6,9,10,15-18].

Prolactin, known as a lactogenic hormone, is essential for the initiation and maintenance of lactation. It is mainly responsible for promoting the synthesis and secretion of milk proteins, lactose, lipids, and all other major components of milk [19]. The bovine prolactin gene is located on chromosome 23[4]. Allelic variation in the regulatory sequences of these genes could be of interest due to their possible direct or indirect effects on milk production and growth performance [19]. The prolactin gene, which has an important regulatory function in milk production, is an important candidate gene for QTL. Therefore, in many studies, the relationships between prolactin polymorphism and milk yield characteristics were studied [9-26].

The gene of acylCoA-diacylglycerol acyltransferase1 (DGAT1)[5] is located in the centromeric region of bovine chromosome 14 and the locus of DGAT1 was identified as that underlying the quantitative trait locus for milk production parameters. The DGAT1 is a positional candidate gene for milk fat percentage with K232A substitution associated with the higher fat percentage in Bos taurus [27]. DGAT1 gene polymorphism and the effect of this polymorphism on milk yield characteristics were investigated in many studies [21,27-33].

It is normal to observe different variants in the genome due to genetic variations within and between various species. It is possible that the presence and frequencies of alleles encoding milk protein are different among individuals belonging to different cattle breeds. Their polymorphisms partly explain the genetic variance and improve the estimated breeding value. Therefore, milk protein genes could be useful as genetic markers for additional selection criteria in dairy cattle breeding.

The aim of this study was to determine polymorphisms in κ-casein, β-lactoglobulin, prolactin, and DGAT1 loci and their effects on milk yields in Holstein cows registered to the Cattle Breeders' Association of Turkey in Antalya province.

2. Materials and methods

A total of 517 blood samples were randomly collected from seven different herds of Holstein cows raised in Antalya province, Turkey. According to rules of the law approving ethical handling of animals, the blood samples were taken from jugular vena into tubes with 5% EDTA and stored at 4 °C until DNA extraction was performed. Genomic DNA was extracted from blood using the GenElute Blood Genomic DNA kit (Sigma Aldrich, St. Louis, MO, USA). Agarose gel electrophoresis and spectrophotometric methods were used to determine DNA quality and quantity. The purified DNA solution containing 50 ng/µL of DNA was used for all the further analyses. In addition, milk yield records of 432 cows were obtained from the Cattle Breeders' Association of Turkey. According to Akman and Kumlu [34], cows with consecutive lactations and calving intervals of 300-650 days were taken into account for the variance analysis. Additionally, in the records of the cows to be included in the analysis, attention has been paid that the lactation period should not be shorter than 220 days and the milk yield should not be less than 2000 kg [34].

Allele discriminations were based on size differentiation of κ-casein, β-lactoglobulin, prolactin, and DGAT1 genetic markers. Identification of these genotypes was performed by the PCR-RFLP method. Extracted DNA samples were amplified by PCR technique, using the primers are given in Table 1. PCR amplifications were prepared in a 50 µL volume containing 0.2 µM of each primer, 4 µL 10X buffer, 25 mM $MgCl_2$, 10 mM dNTPs, 1 U Taq DNA polymerase, and 100 ng template DNA. PCR reaction steps were optimized for each locus considering the annealing temperatures of the used primers. Amplified PCR products were digested with specific restriction enzymes (Table 1) and then these digested products were separated by electrophoresis on 3% agarose gel and were visualized under ultraviolet light after staining with ethidium bromide.

Descriptive and dispersion of allele frequencies were performed by direct counting from the genotype identified in the photo documentation of electrophoresis of the PCR-RFLP. Direct counting was used to estimate phenotype and allele frequencies of β-lactoglobulin, κ-casein, prolactin, and DGAT1 genetic variants [35]. The probability of Hardy-Weinberg equilibrium associated with the observed genotypic frequencies was calculated using the chi-square (χ^2) test for the population. The POPGENE [36] package program was used to determine the observed and expected heterozygosity values. After standardizing the data, the relationships between genotypes and milk yield were analyzed using the SAS statistical package program [37] according to the model below.

$$Y_{ijkl} = \mu + a_i + b_j + c_k + \beta age_{ijkl} + e_{ijkl},$$

where Yijkl represents the record of 305 days milk yield, µ is overall the mean, a_i is the effect of the i-th farm, b_j is

[4] NCBI, National center for biotechnology information (2021). Website https://www.ncbi.nlm.nih.gov/nuccore/ NM_173953.2#goto46810276_0 [accessed 07.12.2021].

[5] NCBI, National center for biotechnology information (2021). Website https://www.ncbi.nlm.nih.gov/nuccore/AY065621.1#goto18642597_0 [accessed 07.12.2021].

Table 1. The primer sequences and restriction enzyme of each locus.

Gene	Sequences 5'-3'	Enzyme	Reference
κ-casein	F: ATCATTTATGGCCATTCCACCAAAG R: GCCCATTTCGCCTTCTCTGTAACAGA	*Hinf*I	Patel et al. (4)
β-lactoglobulin	F: TGTGCTGGACACCGACTACAAAAA R: GCTCCCGGTATATGACCACCCTCT	*Hae*III	Patel et al. (4)
Prolactin	F: CGGAAGTCCTTATGAGCTTGATTCTT R: GCCTTCCAGAAGTCGTTTGTTTTC	*Rsa*I	Dybus et al. (20)
DGAT1	F: GCACCATCCTCTTCCTCAAG R: GGAAGCGCTTTCGGATG	*Cfr*I	Kaupe et al. (30)

the effect of the j-th lactation number, c_k is the effect of the k-th genotype, β is the regression coefficient, age_{ijkl} is the effect of age, e_{ijkl} is random residual effects.

3. Results

Target fragments of cattle κ-casein, β-lactoglobulin, prolactin, and DGAT1 genes were amplified from the genomic DNA of 517 Holstein cows registered to the Cattle Breeders' Association of Turkey in Antalya province. After the PCR products obtained for each locus were treated with the appropriate RE enzymes, target RFLP fragments were visualized under ultraviolet light (Figure 1).

In the present cows, the κ-casein gene was amplified as a 350 bp fragment comprising a part of exon V of the genomic DNA. Digestion of the PCR products with *Hinf*I enzyme revealed three genotypes as AA (134/132 and 84 bp), AB (266, 134/132, and 84 bp) and BB (266 and 84 bp) (Picture 1a). In the β-lactoglobulin gene located in exon IV, digestion of the 247 bp PCR products with *Hae*III enzyme revealed three genotypes as AA (148 and 99 bp), AB (148, 99 and 74 bp), and BB (99 and 74 bp) (Picture 1b). The prolactin gene located in exon V, digestion of the 156 bp PCR products with *Rsa*I enzyme revealed three genotypes as AA (156 bp), AB (156 and 82/74 bp), and BB (82/74 bp) (Picture 1c). Digestion of the 411 bp PCR products with *Cfr*I enzyme revealed three genotypes as AA (208/205 bp), AK (411 and 208/205 bp), and KK (411 bp) for the DGAT1 gene located in exon VIII (Picture 1d). It is noteworthy that an unexpected fragment (411 bp) was observed for AA genotype in the DGAT1/*Cfr*I polymorphism. This could be attributed to different reasons such as higher volume of PCR products and/or lower volume of restriction enzyme.

Gene and genotype frequencies were calculated using the gene counting method of Nei [35] by taking into consideration the genotypes obtained after the PCR-RFLP method (Table 2). The calculated chi-square (χ^2) values showed that the current population was in Hardy-Weinberg equilibrium for all loci.

Although a total of 517 cows were genotyped for κ-casein-*Hinf*I, β-lactoglobulin-*Hae*III, prolactin-*Rsa*I, and DGAT1-*Cfr*1 polymorphisms, milk yield records of only 432 cows were accessible. Therefore, both genotype and milk yield data of 432 cows were utilized for association analysis using SAS MIXED procedure (37). Table 3 presents the mean milk yields according to genotypes obtained from Holstein cows for 305 days of milking. While the highest milk yield was found in cows with homozygous BB genotype in the prolactin gene region, the lowest milk yield was detected in cows with homozygous BB genotype in the κ-casein gene region. (Table 3).

The results of association analysis showed that the genotype differences of κ-casein, β-lactoglobulin, and DGAT1 loci had no effect on milk yield, whereas *Rsa*I polymorphism of the prolactin gene has a significant effect on milk yield ($p < 0.05$) (Table 4). In addition to the results above, it was seen that the differences between farms and lactations and lactation x farm and age x lactation interactions in terms of milk yield in existing all loci were significant ($p < 0.05$) (Table 4).

According to analysis via the POPGENE software package program, it can be said that the differences between the observed and expected genotype frequency values are not significant in the studied population (Table 5). It is seen that the heterozygosity observed in all loci in the current population in Hardy-Weinberg equilibrium is higher than the expected heterozygosity. This situation can be explained by the use of bulls with different genotypes in artificial insemination for the current population.

4. Discussion

When κ-casein gene *Hinf*I polymorphism is examined within the scope of the study, it is seen that the A allele (0.8279) is quite common compared to the B allele (0.1721) in the current population. Similar results were reported in previous studies conducted on Holstein-Friesian and its crossbreds [1-10,13]. Similarly, in most studies performed

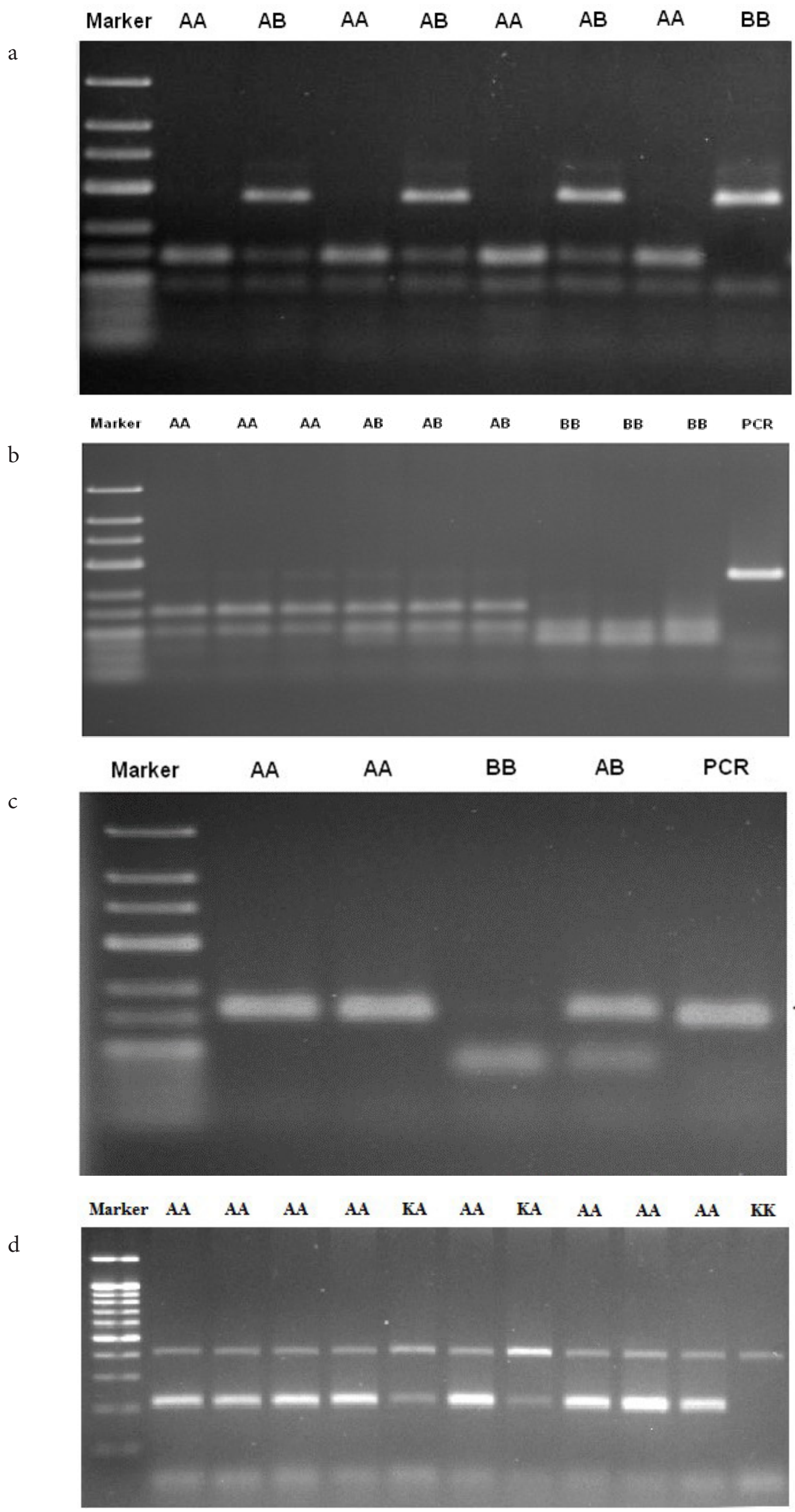

Figure 1. Agarose gel electrophoresis showing PCR-RFLP products (a) *Hinf*I polymorphism of κ-casein gene, AA genotype; 134/132:84 bp, BB genotype; 266:84 bp, AB genotype; 266:134/132:84 bp, (b) *Hae*III polymorphism of β-lactoglobulin gene, AA genotype; 148:99 bp, BB genotype; 99:74 bp, AB genotype; 148:99:74 bp, (c) *Rsa*I polymorphism of prolactin gene, AA genotype; 156 bp, BB genotype; 82:74 bp, AB genotype; 156:82:74 bp, (d) *Cfr*I polymorphism of DGAT1 gene, AA genotype; 208/205 bp, KK genotype; 411 bp. AK genotype; 411: 208/205 bp.

Table 2. Genotype and allele frequencies for studied loci.

Locus	Genotype frequencies					Allele frequencies			(χ^2)*
	AA	AB	BB	AK	KK	A	B	K	
κ-casein	0.6925	0.2708	0.0367	-	-	0.8279	0.1721	-	1.2875^{ns}
β-lactoglobulin	0.2302	0.4720	0.2978	-	-	0.4662	0.5338	-	1.3857^{ns}
Prolactin	0.7621	0.2282	0.0097	-	-	0.8762	0.1238	-	1.4022^{ns}
DGAT1	0.1374	-	-	0.4371	0.4255	0.3559	-	0.6441	1.1174^{ns}

* Chi-square values, ns: Not significant p > 0.005

Table 3. Milk yield obtained from Holstein cows for 305 days of milking according to loci and genotypes.

		Locus											
		κ-casein			β-lactoglobulin			Prolactin			DGAT1		
		MY	N	SD	MY	N	SD	MY	N	SD	MY	N	SD
Genotypes	AA	6296	303	52.56	6223	106	91.16	6261	333	50.35	-	-	-
	AB	6239	113	103.84	6323	202	66.21	6282	96	107.32	-	-	-
	BB	6111	16	180.82	6242	124	90.25	8311	3	1270.71	-	-	-
	KK	-	-	-	-	-	-	-	-	-	6318	188	73.54
	KA	-	-	-	-	-	-	-	-	-	6218	203	62.74
	AA	-	-	-	-	-	-	-	-	-	6376	41	169.90
	AA	-	-	-	-	-	-	-	-	-	6376	41	169.90

MY: Milk yield (kg), N: Number of animals (head), SD: Standard deviation

on native (Turkish gray, East Anatolian Red, South Anatolian Red, Anatolian Black) and other European (Brown Swiss) cattle breeds raised in Turkey, the A allele frequency of κ-casein gene has been reported to be high [6,8,10,12]. On the other hand, Akyüz et al. [8] reported that the A and B allele frequencies are close to each other for Anatolian Black and Brown Swiss cattle breeds.

The κ-casein B allele was found to be one of the most well-known alleles related to cheese quality [1]. Previous studies reported that cows with the AA genotype have higher milk yield [4,9], but they have a negative relationship with protein yield and protein percentage [2,4]. On the other hand, Hristov et al. [11] found that cows with the BB genotype have a 15% higher milk yield than cows with the AB genotype. It has been reported that the κ-casein gene B variant is associated with the increase in milk protein and fat content, which are important in cheese production [2,9]. However, Bartonova et al. [7] put forward that there are no effects of the *Hinf*I polymorphism of the κ-casein gene on the fat and protein yield.

In our study, the effect of the κ-casein gene polymorphism on milk yield is not statistically significant. Similar results were also found in some studies performed on breeds in Turkey [6]. The A allele of κ-casein gene was found to be associated with high milk yield and low protein content, while the B allele was associated with high protein content and milk quality, but low milk yield [1,2,4,9,12]. In the light of previous studies, it is thought that it will be useful to consider the positive effect of the κ-casein gene, especially the B allele, on cheese production for selection studies.

In the present population, the B allele frequency (0.5338) was relatively higher than the A allele frequency (0.4662), while the AA and BB genotype frequencies were almost the same distribution for the β-lactoglobulin/*Hae*III polymorphism. These results were similar to many studies conducted especially on Holstein Friesian and its crossbreds [4,6,9,10,16,17]. In some other studies on Holstein cattle, the β-lactoglobulin B allele was found quite higher compared to the A allele [3,5]. Different results

Table 4. Variance analysis results of loci.

		κ-casein	**β-lactoglobulin**	**Prolactin**	**DGAT1**
Fixed effects	DF	Pr > F	Pr > F	Pr > F	Pr > F
Age	1	0.1939	0.1837	0.2484	0.1999
Lactation	2	0.0047*	0.0058*	0.0012*	0.0084*
Farm	5	<.0001*	<.0001*	<.0001*	<.0001*
Genotype	2	0.6539	0.5341	0.0311*	0.9139
Lactation x farm	9	0.0006*	0.0011*	0.0020*	0.0005*
Age x lactation	2	0.0196*	0.0207*	0.0387*	0.0349*
Lactation x genotype	4	0.9862	0.5803	0.2496	0.5436

* Significant, $p < 0.05$, DF: Degree of freedom.

Table 5. Results of heterozygosity and homozygosity statistics of loci.

Loci	N	Observed heterozygosity (Ho)	Observed homozygosity	Expected homozygosity	Expected heterozygosity (He)	Mean He
κ-casein	517	0.5280	0.4720	0.5018	0.4982	0.4977
β-lactoglobulin	517	0.7292	0.2708	0.7147	0.2853	0.2850
Prolactin	517	0.7718	0.2282	0.7829	0.2171	0.2169
DGAT1	517	0.5416	0.4584	0.5657	0.4343	0.4407
Mean	517	0.6475	0.3525	0.6396	0.3604	0.3601
Standard Deviation		0.1209	0.1209	0.1312	0.1312	0.1311

N: Number of animals (head)

were found in studies investigated on other cattle breeds raised in Turkey. The A and B allele frequencies were found to be close to each other in Brown Swiss and Gray breeds [18]. While the B allele was found to be higher in South Anatolian Red [10,18], Brown Swiss [17], Anatolian Black [10], and East Anatolian Red [10] breeds, the A allele was detected at a higher frequency in Simmental cattle [6].

Similar to our study, it was found that the genotypes obtained by the *Hae*III polymorphism of the β-lactoglobulin gene had no effect on milk yield in the studies conducted by Ahmadi et al. [4] and Mohammadi et al [9]. In addition, it was reported that the effect of genotype differences of the β-lactoglobulin gene on fat content was not significant. It was reported that the protein content in the milk of animals with the BB genotype in terms of the β-lactoglobulin gene was higher than that of animals with other genotypes in Holstein cattle [4,9]. At the same time, Karimi et al. [15] reported that the difference in milk protein content among genotypes of the β-lactoglobulin gene was not statistically significant. Additionally, it was reported that the effect of the β-lactoglobulin AB and AA genotype had an effect on milk yield, but this effect was not statistically significant [15].

The present study showed that in terms of the prolactin/*Rsa*I polymorphism, the A allele (0.8762) is quite common in Holstein cows compared to the B allele (0.1238). This result was similar to previous studies on Holstein and its crossbreds [8,19-26]. Similarly, the A allele was detected at high frequencies in cattle breeds raised in Turkey (0.6617-0.8610), except for Anatolian Black (A and B frequencies are close to each other) [8,21,38].

After analyses made by the SAS program, it was determined that the effect of the *Rsa*I polymorphism of the prolactin gene on milk yield was significant ($p < 0.05$). In particular, it was detected that the milk yield of animals with the B allele was higher (Table 4). However, it should not be overlooked that animals with the BB genotype are very few. It is thought that it will be useful to evaluate this information obtained in selection studies in dairy cattle breeding. Similarly, in some studies, it has been determined that the BB genotype has a significant effect on milk yield or has a tendency to affect it positively [22,26]. On the other hand, Khatami et al. [20] and Kepenek [21] reported that the BB genotype formed by the *Rsa*I polymorphism of the prolactin gene has a negative relationship with milk yield, but this relationship is not statistically significant.

In some studies, it has been stated that the milk yield of Holstein cows with the AA genotype is higher than that of cows with other genotypes [21,23,26]. In other studies, it has been reported that the milk yield of Holstein cows with the AB genotype is higher than the milk yield of cows with other genotypes [20,25]. However, there are also studies reporting that the *Rsa*I polymorphism of the prolactin gene has no statistically significant effect on milk yield [19,20,24].

The significant effect of the prolactin/*Rsa*I polymorphism on milk fat content was reported in previous studies [19,25]. Dybus et al. [19] found that the effect of the prolactin gene on fat yield and fat percentage in milk was lower in animals with the AA genotype than animals with the AB and BB genotype, and this difference was statistically significant. Alipanah et al. [22] found that the milk of animals with the BB genotype had a higher fat yield compared to the others (BB > AA > AB), and the milk of animals with the AB genotype was higher in terms of fat content, but these results were not statistically significant. However, in some studies, it has been reported that the milk of animals with the AA genotype has higher fat content [20,25].

Dybus et al. [19] conclude that the effect of the prolactin gene *Rsa*I polymorphism on the protein content of milk is important, on the other hand, Alipanah et al. [22] argue that the effect of these genotypic differences is not statistically significant. Singh et al. [26] reported that the prolactin gene was associated with the number of somatic cells. They reported that the somatic cell count in the milk of cows with the AA and AB genotypes was lower than those with the BB genotype, depending on this result, cows with the BB genotype would have higher resistance to mastitis [26].

As a result of the *Cfr*I polymorphism of the DGAT1 gene, 3 genotypes (KK, KA, and AA) could be determined in this study where the K allele (0.6441), known as the lysine variant, was quite common in Holstein cattle breed compared to the A allele (0.3559) known as the alanine variant. This result was similar to some studies performed on Holstein Friesian breed [28,30]. On the other hand, there are many studies in which the A allele is quite high compared to the K allele for this breed [27,32,33,39]. In other studies, it has been reported that the K and A allele frequencies are close to each other in Holstein cattle breeds [21,23]. After analyses made by the SAS program, any effect of genotype differences of the DGAT1 gene on milk yield could not be determined ($p > 0.05$).

According to the study of Bal and Akyüz [39], A allele was detected at higher frequencies in Anatolian Black. On the other hand, the K allele was higher frequencies (0.7000-0.9250) in other native cattle [21,38,39].

In previous studies, it was concluded that the milk of animals with the K allele encoding lysine has a particularly high fat yield, but low protein and milk yield [21,28,30-32]. In addition, while the effects of the K allele on milk yield characteristics were found to be statistically significant, Nowacka-Woszuk et al. [30] and Bobbo et al. [33] reported that the effect of the K allele on fat yield and Spelman et al. [28] on protein yield was not statistically significant. It was reported that the A allele had a significant effect on milk and protein yield, while the fat content was low [27,29,30,32].

5. Conclusion

It is thought that polymorphisms in κ-casein, β-lactoglobulin, prolactin, and DGAT1 genes can be used as markers to improve milk yield characteristics in cattle breeds. Also, it was determined that the effect of the prolactin gene BB genotype on milk yield was significant. This result can be used as a selection criterion and it would also be beneficial to increase the number of studies investigating the effects of milk protein gene polymorphism on milk yield characteristics. In this context, it will be able to make significant contributions to milk processing technology by predetermining of the genetic structure of cows and candidate bulls to be used in breeding in terms of the gene regions in said and accordingly selecting them.

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