Veterinary Medicine and Science

WILEY

ORIGINAL ARTICLE OPEN ACCESS

Ruminants

Associations Between Polymorphisms of the *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3* Genes and Milk Composition Traits in Holstein Cattle

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Received: 10 July 2024 | **Revised:** 24 January 2025 | **Accepted:** 21 March 2025

Funding: This study was supported by Van Yüzüncü Yıl University Scientific Research Projects Coordination Unit via project TDK-2018-6796.

Keywords: *CSN1S1* | *CSN1S2* | *CSN2* | *CSN3* | Holstein cattle | PCR-RFLP

ABSTRACT

Background: Caseins are the major proteins found in cow's milk. There are four known casein fractions: α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein, encoded by the *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3* genes located on the sixth chromosome of cattle. These genes are considered candidate genes in marker-assisted selection. Therefore, it is essential to determine the relationship between these genes and quantitative characters.

Objectives: This study aimed to identify genotypes of *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3* genes and investigate their effect on milk components with the PCR-RFLP method in Holstein cattle.

Methods: The material of the study consisted of 519 Holstein cows that managed under intensive systems in Konya (*n*:189), Manisa (*n*:195) and Diyarbakır (*n*:135) provinces in Türkiye. Blood and milk samples from these cows were used in the study. The genetic structures of bovine *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3* genes were examined by PCR-RFLP in three Holstein cattle populations. A general linear model (GLM) was applied to analyse the effect of genotypic variants on phenotypic characters.

Results: Results indicated that milk solids-non-fat (SNF) ($p < 0.01$), protein ($p < 0.05$) and lactose ($p < 0.01$) percentages were significantly affected by the genetic variants of the *CSN2* gene of cow in general population. *CSN2* A2A2 genotype led to a significant increase in SNF, protein and lactose percentages by 0.14, 0.05 and 0.08 in comparison to other genotypes, respectively. Moreover, significant effect of the *CSN1S1* BC ($p < 0.05$) and *CSN3* AA ($p < 0.01$) genotypes on fat percentage were found in Konya province. Furthermore, a statistically significant genotype-by-environment interaction was identified in both the *CSN1S1* ($p < 0.05$) and *CSN3* ($p < 0.01$) genes in relation to milk fat content.

Conclusions: As a result, after increasing the number of studies that investigated the relationship between casein genes and milk traits and determined the genetic variation of *CSN1S1*, *CSN2* and *CSN3* genes of the Holstein cattle, these genes can be a strong genetic marker as marker-assisted selection programme in early selection.

1 | Introduction

Farm animals have been subjected to human-managed selection since they were first domesticated. Initially, selection was most likely confined to docility and manageability; however, over the last 60 years, breeding programmes have concentrated on the genetic enhancement of production traits, such as milk yield, growth rate and number of eggs (Oltenacu and Broom 2010). The selection effectiveness of complex quantitative characteristics in dairy cattle is dependent on the discovery of candidate

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genes responsible for these traits as well as the detection of causal DNA polymorphisms in those genes. Genetic diversity provides an opportunity to boost bovine milk output with genetic improvement programmes (Zaglool et al. 2016).

Caseins are the primary proteins of milk and are released during lactation as big calcium-containing clusters (micelles) (Lin et al. 1993). Caseins are phosphoproteins that account for roughly 80% of milk's protein composition. Casein consists of several components, including α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein (Phadungath 2005). α_{S1}-Casein, α_{S2}-casein, β-casein and κ-casein are encoded by the *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3* genes, respectively. These four genes are mapped on bovine chromosome 6 and are linked in a 250-kb cluster (Caroli et al. 2009). The casein gene cluster has a strong influence on casein synthesis in bovine milk, and polymorphisms in this region have significant effects on milk protein composition and cheese-making abilities (Zhou et al. 2019).

α_{S1}-Casein is a major fraction of casein (about 40%) and consists of 199 amino acid residues. There is a total of nine genetic variants (A, B, C, D, E, F, G, H and I), and the major variants are B and C variants. These variants differ in one amino acid substitution at 192 which is variant B has Glu (GAA), whereas variant C has Gly (GGA) (Caroli et al. 2009; Farrell et al. 2004).

α_{S2}-Casein consists of 207 amino acids and has four genetic variants (A, B, C and D) (Ibeagha-Awemu et al. 2007). The major variant is A. The D variant differs from αS_2-casein A by the deletion of 9 amino acid residues from positions 51–59 (Farrell et al. 2004).

β-Casein is the second most abundant casein behind αs1-casein (Tailford et al. 2003) and consists of 209 amino acids (Farrell et al. 2004). β-Casein has 13 genetic variants (A1, A2, A3, A4, B, C, D, E, F, H1, H2, I and G) but variant A4 is not yet recognized. The most common variants are A1 and A2 (Kamiñski et al. 2007).

κ-Casein has a polypeptide chain consisting of 169 amino acids (Swaisgood 1975). It encodes milk proteins that are critical to casein micelle stability and structure (Alexander et al. 1988). It constitutes about 13% of milk caseins. The κ-casein gene has a total length of approximately 13 kb and is in exon 4. There are 14 genetic variants of κ-casein: A, A1, B, B2, C, D, E, F1, F2, G1, G2, H, I and J. κ-Casein variants A and B are the two most common genetic variants (Caroli et al. 2009; Farrell et al. 2004).

Molecular genetic techniques are the emerging trend of the age. The latest advances in molecular knowledge and techniques have revealed a multitude of genetic polymorphisms at the DNA level, which are associated with animal performance. Single nucleotide polymorphisms (SNPs) have become a popular marker of phenotypic variation among individuals in a population (Younis et al. 2024).

Numerous researchers have investigated the frequency of variations in the genes that encode the milk protein in cows as well as the connections between these variations and the composition and yield of milk. Many studies were reported that *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3* are four important candidate genes associated with milk production and component as well as cheesemaking properties among dairy cattle breeds (Ibeagha-Awemu et al. 2007; Kamiñski et al. 2007; Caroli et al. 2009; Duifhuis-Rivera et al. 2014; Trakovická et al. 2017; Ardıçlı et al. 2018; Gürses et al. 2018; Čítek et al. 2019).

Despite the extensive analyses conducted in preceding studies, certain limitations pertaining to casein genes persist. In general, the studies have determined the genotypes of animals from a single farm, which may have resulted in diminished genetic variation due to the utilization of a limited number of bulls. Additionally, it is imperative to employ relatively large populations to analyse the relationship between genotype and phenotype. Therefore, this study is distinguished from other studies by virtue of its simultaneous evaluation of four casein fractions, the utilization of cows from three disparate rearing systems with regard to environmental conditions, and an examination of the interactions between casein genotypes and the environment. The current study aimed to identify genotypes of the *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3* genes using the PCR-RFLP method and investigate their effect on milk components in Holstein cattle. In addition, this study seeks to examine whether the interactions between casein genotypes and environment are important or not.

2 | Materials and Methods

2.1 | Animal Material

This research was carried out on Holstein cattle breeds raised in intensive dairy cattle breeding enterprises in Diyarbakır (Southeast Anatolia Region, *n*:135), Konya (Central Anatolia Region, *n*:189) and Manisa (Aegean Region, *n*: 195) provinces located in three different geographical locations of Türkiye (Figure 1). All lactating cows in the farms at the time of sample collection were included in the study. The cows were between 2 and 7 years of age and first to fifth lactation.

In all three farms, the cows were milked in a computer-controlled 2×15 herringbone milking parlour. The farms in Diyarbakır and Manisa were open barns with free movement on all four sides, whereas the farm in Konya was semi-open with free movement and each barn section had its own promenade area. The cows were permitted to access the promenade area at any time.

The feeding programme was implemented on each farm in accordance with the specific nutritional requirements of the animals in question. The animals were provided with a mixed ration twice a day, which was prepared and presented to them in a feed mixer that could grind and mix the ingredients, pulled by a tractor. The cows raised on the three farms were fed a diet comprising 8–13 kg concentrate feed, 15–25 kg maize silage and 3–5 kg dry alfalfa, vetch and hay, with an energy content ranging between 2500 and 2700 kcal and a protein content between 18% and 21%.

2.2 | Milk Analyses

Milk samples were collected uniformly from all milked milk from the beginning to the end of milking using the milk sample collection apparatus integrated into the milking system. The



FIGURE 1 | Location of farms sampled from three different provinces of Türkiye.

milk samples were transferred to 90 mL milk sample bottles for milk composition analysis and kept at room temperature for approximately 2 h. The milk analyses were conducted using a milk analyser device (Milkotester Master PRO, Bulgaria) that employs ultrasonic measurement technology. The solids-not-fat (SNF), fat, protein and lactose contents were determined from milk samples.

2.3 | Genomic DNA Isolation and PCR

Blood samples were collected from the coccygeal veins of cows using disposable needles in 10 mL K_3EDTA vacuum tubes. They were transferred to the laboratory via the cold chain and kept in a deep freezer at −20°C until DNA isolation. Total genomic DNA isolation from bovine blood samples was carried out according to the protocol determined by the manufacturer, using the GF-1 Blood DNA Extraction (Vivantis Technologies, Selangor, Malaysia) commercial kit.

Four casein gene regions were amplified in a Thermal Cycler (Applied Biosystems, SimpliAmp, Singapore) according to the PCR protocols determined by optimization. PCR master mix (5x FIREPol Master Mix, Solis BioDYne, Estonia), a ready-to-use solution containing the necessary reagents, was added at the PCR stage. The PCR contents were mixed as follows: 0.5 μM forward and reverse primer, 5× PCR master mix 4 μL, template DNA 1 μL (approximately 50 ng), nuclease-free water 14 μL; the total volume was 20 μL. Primers and annealing temperatures used in the PCR programme for amplification of the *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3* gene regions are given in Table 1.

2.4 | PCR-RFLP and Genotyping

Casein genes (α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein) of Holstein cows were genotyped by PCR-RFLP method. Restriction enzymes, recognition sequence, incubation temperature and time of the *CSN1S1*, *CSN1S2*, *CSN2,* and *CSN3* gene regions are given in Table 2.

Electrophoresis on 2% agarose gel was used for the visualization of fragments. Fragments were examined under UV light. Genotypes of the cows were identified according to the band patterns obtained from electrophoresis.

2.5 | Statistical Analysis

Genetic diversity parameters, including expected heterozygosity (He), effective allele number (Ne) and polymorphic information content (PIC), were calculated using the methods proposed by Nei and Roychoudhury (1974) and Botstein et al. (1980). In addition, the allele and genotype frequency, chi-square tests and compatibility with the Hardy–Weinberg equilibrium (HWE) and genetic diversity parameters were computed using Popgene Ver. 1.32 (Yeh et al. 1999). The effect of casein genes' polymorphism on milk components was carried out in SPSS (Ver. 26) software using the general linear model (GLM) procedure. Dependent variables in the model were SNF, fat, protein and lactose content of milk. Besides the casein genes, farms were included as fixed factors in the model. In the model, the Duncan Multiple Comparison Test was preferred as a post hoc test to determine the difference between subgroups. The interaction between factors was calculated by GLM univariate analysis. The interaction between genotypes of casein genes and farms was considered genotype-by-environment (G × E) interaction. GraphPad Prism 8 was used to create bar charts. The variables obtained in the study were expressed as mean ± standard error and percentage and frequency values. The statistical significance level of genotype effects or interactions was determined as $p < 0.05$.

The following statistical model was used according to the milk components in the study:

$$Y_{ijklm} = \mu + a_i + b_j + c_k + d_l + (ab)_{ij} + (ac)_{ik} + (ad)_{il} + e_{ijklm}$$

TABLE 1 | Primers and PCR conditions used for amplification of the *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3* gene regions.

Gene	Primers	Region	PCR conditions	PCR product (bp)	References
CSN1S1	**Forward**: 5′-TGCATGTTCTCATAATAACC-3′ **Reverse**: 5′-GAAGAAGCAGCAAGCTGG-3′	Exon 17	5 min at 94°C; 35 cycles of 30 s at 94°C, 50 s at **59°C** and 30 s at 72°C; and 72°C for 5 min	310	Koczan et al. (1993) and Miluchova et al. (2009a)
CSN1S2	**Forward**: 5′-AAAACAAGCAGCCAAGAAGC-3′ **Reverse**: 5′-TTCCCAGTCTCCCCAGTATG-3′	Exon 8	3 min at 94°C; 35 cycles of 30 s at 94°C, 50 s at **63°C** and 40 s at 72°C; and 72°C for 5 min	356	Ibeagha-Awemu et al. (2007)
CSN2	**Forward**: 5′-CTTCTTTCCAGGATGAACTCCAGG-3′ **Reverse**: 5′-AGTAAGAGGAGGGATGTTTTGTGGGAGGCTCT-3′	Exon 7	3 min at 94°C; 35 cycles of 30 s at 94°C, 50 s at **65°C** and 30 s at 72°C; and 72°C for 5 min	121	Mclachlan (2006) and Miluchova et al. (2009b)
CSN3	**Forward**: 5′-ATCATTTATGGCCATTCCACCAAAG-3′ **Reverse**: 5′-GCCCATTTCGCCTTCTCTGTAACAGA-3′	Exon 4	3 min at 94°C; 35 cycles of 30 s at 94°C, 60 s at **65°C** and 40 s at 72°C; and 72°C for 5 min	350	Patel et al. (2007)

TABLE 2 | Restriction enzymes, recognition sequence, incubation temperature and time of the *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3* gene regions.

	CSN1S1	***CSN1S2***	***CSN2***	***CSN3***
Restriction enzymes	*Mae*III	*Mnl*I	*Dde*I	*Hinf*I
Recognition sequence	↓GTNAC CANTG↑	CCTC(N)7↓ GGAG(N)6↑	C↓TNAG GANT↑C	G↓ANT C C TNA↑G
Incubation temperature (°C)	55	37	37	37
Incubation time	1 h	15 min	15 min	15 min

where Y_{ijklm} is the value of the studied phenotypic traits (SNF, fat, protein and lactose content); μ is the population mean; a_i is the effect of the environment (farm) (i = Diyarbakır, Konya, Manisa); b_j is the effect of the *CSN1S1* gene (j = BB, BC, CC); c_k is the effect of the *CSN2* gene (k = A1A1, A1A2, A2A2); d_l is the effect of the *CSN3* gene (l = AA, AB, BB); $(ab)_{ij}$ is the genotype (*CSN1S1*) × environment interaction; $(ac)_{ik}$ is the genotype (*CSN2*) × environment interaction; $(ad)_{il}$ is the genotype (*CSN3*) × environment interaction; e_{ijklm} is the random error.

3 | Result

The casein genotypes of Holstein cows bred under three distinct environmental conditions were ascertained through the utilization of the PCR-RFLP analysis. The genotypes of the four casein genes were determined from the band patterns formed after digestion with the restriction enzymes in agarose gel electrophoresis. Following restriction digestion, *CSN1S1*/*Mae*III polymorphism revealed BB (214/96 bp), BC (310/214/96 bp) and CC (310 bp) genotypes in Diyarbakır, whereas only two genotypes

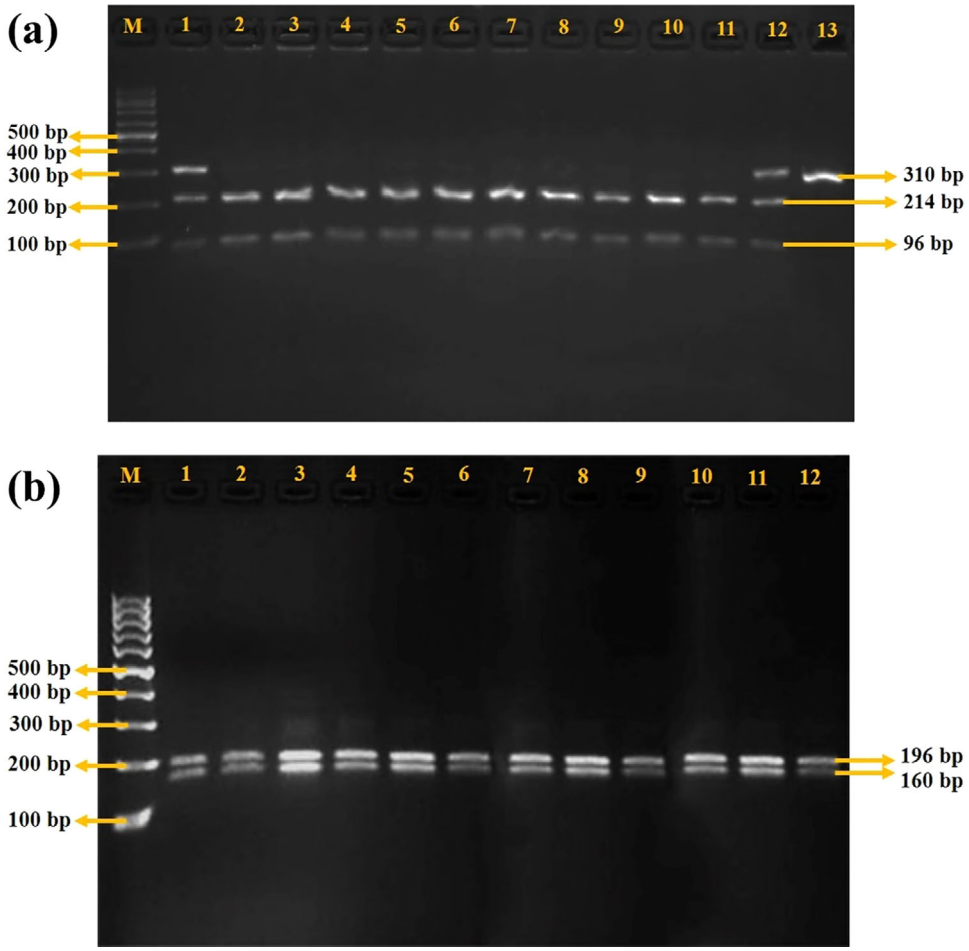


FIGURE 2 | Results of PCR-RFLP analysis for *CSN1S1* and *CSN1S2* genes digested by restriction enzymes on 2% agarose gel. (a) Bands separation in agarose gel electrophoresis of genotypes of *CSN1S1* gene digested with *Mae*III restriction enzyme. M: Marker 100 bp; BB genotype: Lines 2–11 between (two bands: 214 and 96 bp); BC genotype: Lines 1 and 12 (three bands: 310, 214 and 96 bp); CC genotype: Line 13 (uncut fragment, 310 bp); bp: base pair. (b) Bands separation in agarose gel electrophoresis of genotypes of *CSN1S2* gene digested with *Mnl*I restriction enzyme. M: Marker 100 bp; AA genotype: Lines 1–12 between (two bands: 196 and 160 bp); bp: base pair.

(BB and BC) were identified in Konya and Manisa (Figure 2a). Digestion of the PCR products of the *CSN1S2* gene with the *Mnl*I nuclease resulted in two bands (only AA genotype (196/160 bp) in all three populations (Figure 2b).

The PCR product of the *CSN2* gene exhibited three distinct genotypes, namely, A1A1, A1A2 and A2A2, following digestion with the restriction enzyme *Dde*I in all three populations. The PCR product of the *CSN2* gene exhibited three distinct genotypes, namely, A1A1, A1A2 and A2A2, following digestion with the restriction enzyme *Hind*III in all three populations. The A1A2 genotype was distinguished into three restriction patterns of 121, 86 and 35 bp, whereas the A1A1 genotype remained as a single undigested band. The A2A2 genotype was cleaved into two restriction patterns by 86 and 35 bp (Figure 3a). However, the 35 bp band, due to its very small size, could not be visualized in the agarose gel electrophoresis shown in Figure 3a.

Bands separation in agarose gel electrophoresis of genotypes of *CSN3* gene after digestion with *Hinf*I enzyme of PCR products is presented in Figure 3b. RFLP results for *CSN3*/*Hinf*I polymorphism revealed AA (134/132/84 bp), AB (266/134/132/84 bp) and BB (266/84 bp) genotypes in all three populations.

Distributions of allele and genotype frequencies, genetic diversity parameters (He, Ne, PIC) and the HWE of the *CSN1S1*, *CSN2* and *CSN3* gene in three populations are shown in Table 3. Our results indicated that the *CSN1S1*, *CSN2* and *CSN3* genes were polymorphic, whereas *CSN1S2* gene was monomorphic. The most common genotypes of the *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3* genes in overall herd were BB (88%), AA (100%), A1A2 (62%) and AA (62%) genotypes, respectively. These results demonstrated that the homozygous genotypes of all genes were the most prevalent, except for the *CSN2* gene. The highest allele frequencies of the *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3* genes in overall herd were B (93.5%), A (100%), A2 (54.6%) and A (78.3%) alleles, respectively. As with the overall population, the most prevalent alleles were observed to be consistent across the three regions (Figure 4). The analysis of *CSN3* loci revealed that the Diyarbakır, Konya, Manisa, and overall populations were consistent with HWE ($p > 0.05$). The *CSN1S1* gene was found in HWE in the Konya and Manisa populations, but it was incompatible with the Diyarbakır population ($p < 0.001$) or the overall sample ($p < 0.05$).

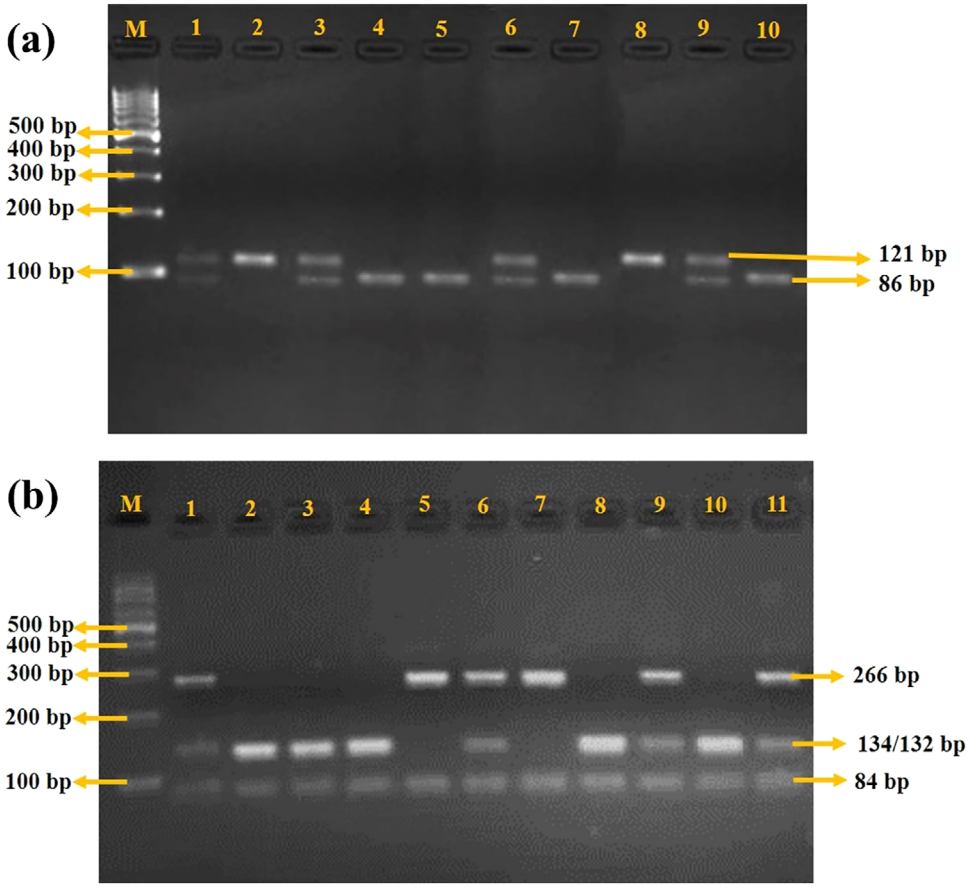


FIGURE 3 | Results of PCR-RFLP analysis for *CSN2* and *CSN3* genes digested by restriction enzymes on 2% agarose gel. (a) Bands separation in agarose gel electrophoresis of genotypes of *CSN2* gene digested with *Dde*I restriction enzyme. M: Marker 100 bp; A1A1 genotype: Lines 2 and 8 (uncut fragment, 121 bp); A1A2 genotype: Lines 1, 3, 6 and 9 (three bands: 121, 86 and 35 bp); A2A2 genotype: Line 4, 5, 7 and 10 (two bands: 86 and 35 bp); bp: base pair. After enzyme digestion, the 35 bp band was very small and, therefore, could not be visible in the agarose gel. (b) Bands separation in agarose gel electrophoresis of genotypes of *CSN3* gene digested with *Hinf*I restriction enzyme. M: Marker 100 bp; AA genotype: Lines 2, 3, 4, 8 and 10 (two bands: 132/134 and 84 bp); AB genotype: Lines 1, 6, 9 and 11 (three bands: 266, 132/134 and 84 bp); BB genotype: Line 5 and 7 (two bands: 266 and 84 bp); bp: base pair.

The *CSN2* gene was not under HWE in all populations, except for the Diyarbakır population. The assessment of genetic diversity parameters revealed that He values ranged from 0.1053 to 0.5005, Ne values from 1.1172 to 1.9946, and PIC values from 0.1001 to 0.3743.

The least squares means and multiple comparison test results for some milk components of *CSN1S1*, *CSN2* and *CSN3* genotypes are presented in Table 4. The *CSN2* gene had a significant impact on the milk SNF ($p < 0.01$), protein ($p < 0.05$) and lactose ($p < 0.01$) contents in the general population. The SNF (8.87%), protein (3.25%) and lactose (4.87%) contents of milk in the A2A2 genotype were observed to be higher than in other genotypes. The *CSN2* A2A2 genotype led to a significant increase in SNF, protein and lactose percentages by 0.14, 0.05 and 0.08 in comparison to other genotypes, respectively. Moreover, significant effects of the *CSN1S1* BC ($p < 0.05$) and *CSN3* AA ($p < 0.01$) genotypes on fat percentage were found in Konya province. Furthermore, a statistically significant G × E interaction was identified in both the *CSN1S1* ($p < 0.05$) and *CSN3* ($p < 0.01$) genes in relation to milk fat content (Figure 5).

4 | Discussion

Milk contains the nutrients required for the growth and development of neonate. It contains a colloidal dispersion of specific proteins as micelles, fats organized in emulsified globules, as well as lactose, various minerals and vitamins in solution (Kazimierska and Kalinowska-Lis 2021). The protein fraction is the most valuable of the constituents present in milk (Goulding et al. 2020). The protein composition of milk determines its technological properties as well as its nutritional value. The protein composition of bovine milk varies with season, stage of lactation, feeding and health status of the cow but is predominantly determined by genetic factors (Heck et al. 2009). Cow's milk protein consists of approximately 80% casein and 20% whey protein, both of which may elicit beneficial health outcomes and which are the source of a wide range of peptides with potential bioactive properties (Poppitt 2020).

This study identified the genetic variant of four milk protein genes and evaluated its effect on milk component traits of Holstein cows raised in three different provinces. Moreover, it was analysed

TABLE 3 | Distributions of allele and genotype frequencies, genetic diversity parameters (He, Ne, PIC) and the Hardy–Weinberg equilibrium (HWE) of the *CSN1S1*, *CSN2* and *CSN3* gene in three populations.

			CSN1S1					*CSN2*					*CSN3*				
			Genotype frequencies			Allele frequencies		Genotype frequencies			Allele frequencies		Genotype frequencies			Allele frequencies	
	N		BB	BC	CC	B	C	A1A1	A1A2	A2A2	A1	A2	AA	AB	BB	A	B
Diyarbakır	135	Frequency	0.926	0.037	0.037	0.944	0.056	0.20	0.55	0.25	0.474	0.526	0.66	0.32	0.02	0.826	0.174
		Observed	125	5	5			27	74	34			90	43	2		
		Expected	120.42	14.17	0.42			30.34	67.32	37.34			92.09	38.82	4.09		
		χ^2	56.522***					1.330					1.566				
		Ne	1.1172					1.9946					1.4036				
		He	0.1053					0.5005					0.2886				
		PIC	0.1001					0.3743					0.2461				
Konya	189	Frequency	0.85	0.15	0.0	0.926	0.074	0.11	0.67	0.22	0.444	0.556	0.624	0.323	0.054	0.786	0.214
		Observed	161	28	0.0			21	126	42			118	61	10		
		Expected	162.04	25.93	1.04			37.33	93.33	58.33			116.68	63.64	8.68		
		χ^2	1.210					23.153***					0.326				
		Ne	1.1590					1.9756					1.5077				
		He	0.1375					0.4938					0.3376				
		PIC	0.1277					0.3718					0.2798				
Manisa	195	Frequency	0.88	0.12	0.0	0.938	0.062	0.13	0.63	0.24	0.449	0.551	0.58	0.34	0.08	0.751	0.249
		Observed	171	24	0.0			26	123	46			113	67	15		
		Expected	171.74	22.52	0.74			39.26	96.47	59.26			110.06	72.87	12.06		
		χ^2	0.838					14.742**					1.267				
		Ne	1.1306					1.9792					1.5967				
		He	0.1158					0.4960					0.3747				
		PIC	0.1095					0.3724					0.3041				
Overall	519	Frequency	0.88	0.11	0.01	0.935	0.065	0.14	0.62	0.24	0.454	0.546	0.62	0.33	0.05	0.783	0.217
		Observed	457	57	5			74	323	122			321	171	27		
		Expected	454.13	62.74	2.13			106.86	257.28	154.86			318.39		24.39		
														176.23			
		χ^2		4.256*					33.865***					0.457			
		Ne		1.1373					1.9830					1.5145			
		He		0.1209					0.4962					0.3399			
		PIC		0.1142					0.3729					0.2821			

Abbreviations: He, expected heterozygosity; Ne, effective number of alleles; PIC, polymorphic information content.
*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$.

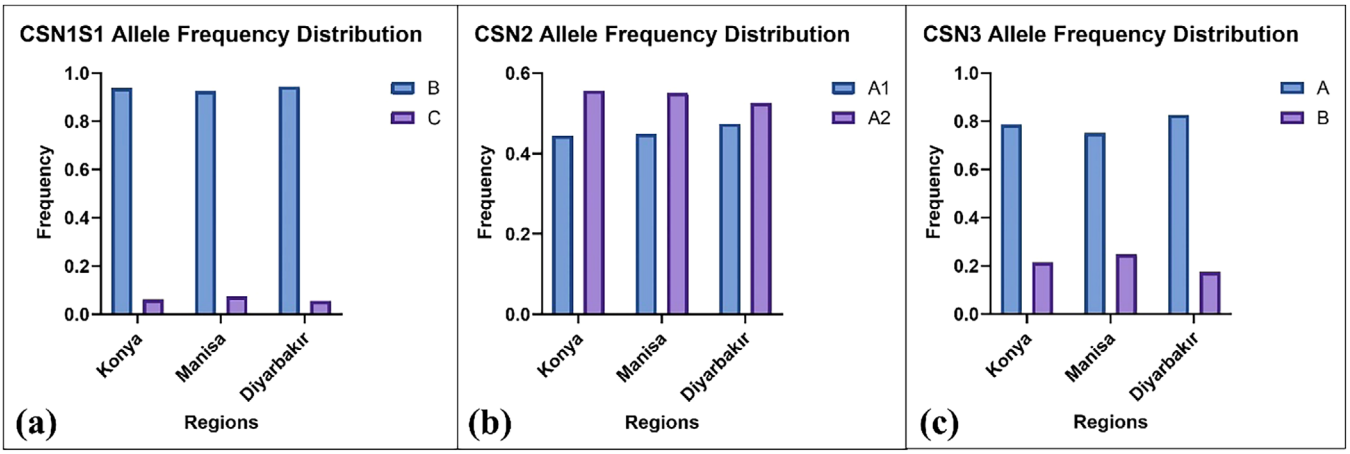


FIGURE 4 | Allele frequency distributions of *CSN1S1* (a), *CSN2* (b) and *CSN3* (c) genes across regions.

genotype × environment interaction. First, genotype and allele frequencies were obtained from the polymorphisms of the bovine *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3* genes.

In this study performed on Holstein cattle, the most common allele and genotype of the *CSN1S1* gene were B allele and BB genotype. Similar observations were reported in studies on Holstein cattle (Çardak 2005; Gürcan 2011; Zakizadeh et al. 2013; Ardıçlı et al. 2018, 2019; Čítek et al. 2023), Simmental cattle (Çardak 2005; Čítek et al. 2023), the Romanian grey steppe (Creanga et al. 2013), Slovak Pinzgau (Miluchova et al. 2014) and Slovak Spotted (Trakovická et al. 2017). But Hristov et al. (2012) reported the more common frequency of the BC genotype in Bulgarian Rhodopean Cattle.

In this study, only A allele and AA genotype were detected for the *CSN1S2* gene. Similarly, Ibeagha-Awemu et al. (2007) found only the A allele in the population of British Friesian, German Angus, Charolais and Jersey cattle. Creanga et al. (2013) found only AA genotype in Romanian grey steppe cattle. However, Ardıçlı et al. (2018, 2019) identified DD and DA genotypes. In addition, Kolenda and Sitkowska (2021) reported that the frequency of DD genotype (c.222TT genotype) to be almost at the level of 100%, and only few animals in the population were heterozygous. The detection of a single genotype for the *CSN1S2* gene suggests that it would be appropriate to identify genotyping in detail in larger populations and other gene regions of the gene.

In this study, the A1A2 genotype and A2 allele of the *CSN2* gene had higher frequency in Holstein cattle. Previous studies reported similar results for Holstein (Çardak 2005; Dinç 2009; Duifhuis-Rivera et al. 2014; Ardıçlı et al. 2019; Soyudal et al. 2019; Şahin and Boztepe 2023; Kovalyuk et al. 2024), Simmental, Brown Swiss (Şahin and Boztepe 2023), Ayrshire (Kovalyuk et al. 2024), Romanian grey steppe (Creanga et al. 2013), Slovak Pinzgau (Miluchova et al. 2014) and Turkish Grey (Dinç 2009). However, other studies have found different results. For example, Jersey (Zepeda-Batista et al. 2015; Şahin and Boztepe 2023; Kovalyuk et al. 2024), Ladakhi cattle (Sodhi et al. 2018), Anatolian Black, Eastern Anatolian Red and Southern Anatolian Red (Dinç 2009) cattle have been reported to have a higher frequency of A2A2 genotype. Moreover, Nuomin et al. (2022) identified 10 genotypes in Jersey herds across eight farms. They reported that the A2 allele and A2A2 genotype were the most prevalent. In addition, it can be stated that on perusal of the literature, researchers found that the A2A2 genotype is common in native cattle.

In the current study, the AA genotype of the *CSN3* gene had the highest genotype frequency compared to the other genotypes. Moreover, the A allele was found to have the highest allele frequency. This was compatible with other studies on a population of Holstein (Duifhuis-Rivera et al. 2014; Gürcan 2011; Gürses et al. 2018; Akyüz et al. 2013; Tanaskovska et al. 2016; Volkandari et al. 2017), Ayrshire (Kovalyuk et al. 2024) and Simmental cattle (Neamt et al. 2017; Ünal and Kopuzlu 2022). But it was not consistent with some studies on Holsteins (Çardak 2005; Soyudal et al. 2019; Ardıçlı et al. 2019; Kovalyuk et al. 2024), Simmental (Çardak 2005), Brown Swiss (Gürses et al. 2018) and Jersey (Gürses et al. 2018; Kovalyuk et al. 2024).

A synthesis of the findings from the literature discussed above for the four casein genes reveals compatibility with the results of numerous other studies. Although our results are compatible with those of other studies, there are numerical differences in terms of allele and genotype frequencies. The variations in the allele and genotype frequency reported in other studies could be due to the differences in the breed, breeding and mating systems of animals.

HWE of the analysed population was evaluated according to the chi-square test. The distributions of allele and genotype frequencies for the *CSN3* gene in three regions (Konya, Manisa and Diyarbakır) and in the whole population were consistent with HWE. However, for the *CSN2* gene, agreement with HWE was observed only in the herd in Diyarbakır. Conversely, the *CSN1S1* gene exhibited HWE balance within the herds in Konya and Manisa. A similar observation of equilibrium for the *CSN3* gene was reported in Holstein (Duifhuis-Rivera et al. 2014; Ardıçlı et al. 2019), Simmental (Ünal and Kopuzlu 2022), Brown Swiss (Doğru and Özdemir 2009), Slovak Pinzgau (Miluchova et al. 2014) and Slovak Spotted cattle (Trakovická et al. 2017). The chi-square result of the *CSN1S1* gene in the general population was unlike that reported by previous studies (Miluchova et al. 2014, Ardıçlı et al. 2018, 2019, Trakovická et al. 2017, Gürcan

TABLE 4 | Least square means and multiple comparison test results of some milk components of *CSN1S1*, *CSN2* and *CSN3* genotypes.

		Dairy farms				
Milk component	**Gene**	**Diyarbakır (135)** $\bar{x} \pm S\bar{x}$	**Konya (189)** $\bar{x} \pm S\bar{x}$	**Manisa (195)** $\bar{x} \pm S\bar{x}$	**Overall** $\bar{x} \pm S\bar{x}$	**G × E**
Solid not-fat (SNF)	*CSN1S1*	NS	NS	NS	NS	#
	BB (457)	8.99 ± 0.05	8.60 ± 0.03	8.78 ± 0.02	8.78 ± 0.02	
	BC (57)	8.75 ± 0.28	8.52 ± 0.07	8.70 ± 0.07	8.62 ± 0.05	
	CC (5)	8.90 ± 0.25	—	—	8.90 ± 0.25	
	CSN2	NS	NS	NS	**	#
	A1A1 (74)	8.87 ± 0.09	8.52 ± 0.10	8.79 ± 0.07	8.74 ± 0.05^{b}	
	A1A2 (323)	8.91 ± 0.05	8.57 ± 0.03	8.76 ± 0.03	8.72 ± 0.02^{b}	
	A2A2 (122)	9.21 ± 0.13	8.68 ± 0.06	8.80 ± 0.05	8.87 ± 0.05^{a}	
	CSN3	NS	NS	NS	NS	#
	AA (321)	8.97 ± 0.06	8.56 ± 0.04	8.76 ± 0.03	8.75 ± 0.03	
	AB (171)	9.00 ± 0.08	8.63 ± 0.05	8.77 ± 0.04	8.78 ± 0.03	
	BB (27)	9.16 ± 0.00	8.61 ± 0.16	8.89 ± 0.07	8.79 ± 0.08	
	Overall mean	8.97 ± 0.05	8.59 ± 0.03	8.77 ± 0.02	8.76 ± 0.02	
Fat	*CSN1S1*	NS	*	NS	NS	*
	BB (457)	3.50 ± 0.04	3.60 ± 0.04	3.48 ± 0.03	3.53 ± 0.02	
	BC (57)	3.60 ± 0.08	3.83 ± 0.09	3.36 ± 0.08	3.61 ± 0.06	
	CC (5)	3.72 ± 0.24	—	—	3.72 ± 0.24	
	CSN2	NS	NS	NS	NS	#
	A1A1 (74)	3.69 ± 0.08	3.73 ± 0.11	3.41 ± 0.08	3.60 ± 0.05	
	A1A2 (323)	3.44 ± 0.04	3.62 ± 0.05	3.45 ± 0.03	3.52 ± 0.03	
	A2A2 (122)	3.52 ± 0.07	3.61 ± 0.08	3.52 ± 0.05	3.55 ± 0.04	
	CSN3	NS	**	NS	NS	**
	AA (321)	3.51 ± 0.04	3.73 ± 0.05^{a}	3.42 ± 0.03	3.56 ± 0.03	
	AB (171)	3.52 ± 0.06	3.46 ± 0.07^{b}	3.54 ± 0.04	3.50 ± 0.03	
	BB (27)	3.20 ± 0.00	3.56 ± 0.12^{ab}	3.46 ± 0.11	3.49 ± 0.08	
	Overall mean	3.51 ± 0.03	3.63 ± 0.04	3.46 ± 0.03	3.54 ± 0.02	
Protein	*CSN1S1*	NS	NS	NS	NS	#
	BB (457)	3.29 ± 0.02	3.15 ± 0.01	3.22 ± 0.01	3.21 ± 0.01	
	BC (57)	3.20 ± 0.11	3.13 ± 0.03	3.18 ± 0.03	3.16 ± 0.02	
	CC (5)	3.26 ± 0.08	—	—	3.26 ± 0.08	
	CSN2	NS	NS	NS	*	#
	A1A1 (74)	3.24 ± 0.04	3.11 ± 0.04	3.22 ± 0.03	3.20 ± 0.02^{b}	
	A1A2 (323)	3.26 ± 0.02	3.14 ± 0.01	3.21 ± 0.01	3.20 ± 0.01^{b}	
	A2A2 (122)	3.38 ± 0.05	3.17 ± 0.02	3.23 ± 0.02	3.25 ± 0.02^{a}	
	CSN3	NS	NS	NS	NS	#
	AA (321)	3.28 ± 0.02	3.14 ± 0.01	3.21 ± 0.01	3.20 ± 0.01	
	AB (171)	3.30 ± 0.03	3.16 ± 0.02	3.22 ± 0.02	3.22 ± 0.01	
	BB (27)	3.40 ± 0.00	3.14 ± 0.06	3.27 ± 0.03	3.22 ± 0.03	
	Overall mean	3.28 ± 0.02	3.14 ± 0.01	3.22 ± 0.01	3.21 ± 0.01	

(Continues)

TABLE 4 | (Continued)

Milk component	Gene	Dairy farms: Diyarbakır (135) $\bar{x} \pm S\bar{x}$	Konya (189) $\bar{x} \pm S\bar{x}$	Manisa (195) $\bar{x} \pm S\bar{x}$	Overall $\bar{x} \pm S\bar{x}$	G × E
Lactose	*CSN1S1*	NS	NS	NS	NS	#
	BB (457)	4.94 ± 0.03	4.72 ± 0.02	4.82 ± 0.01	4.82 ± 0.01	
	BC (57)	4.80 ± 0.16	4.68 ± 0.04	4.78 ± 0.04	4.73 ± 0.03	
	CC (5)	4.90 ± 0.15	—	—	4.90 ± 0.15	
	CSN2	NS	NS	NS	**	#
	A1A1 (74)	4.87 ± 0.05	4.67 ± 0.06	4.82 ± 0.04	4.79 ± 0.03[b]	
	A1A2 (323)	4.90 ± 0.03	4.70 ± 0.02	4.81 ± 0.02	4.79 ± 0.01[b]	
	A2A2 (122)	5.05 ± 0.07	4.77 ± 0.03	4.83 ± 0.03	4.87 ± 0.03[a]	
	CSN3	NS	NS	NS	NS	#
	AA (321)	4.93 ± 0.04	4.70 ± 0.02	4.81 ± 0.02	4.80 ± 0.01	
	AB (171)	4.94 ± 0.04	4.74 ± 0.03	4.81 ± 0.02	4.82 ± 0.02	
	BB (27)	5.00 ± 0.00	4.73 ± 0.09	4.89 ± 0.04	4.83 ± 0.04	
	Overall mean	4.93 ± 0.03	4.71 ± 0.02	4.81 ± 0.01	4.81 ± 0.01	

Note: Different superscript (a, b) indicates statistical significant; #: genotype-by-environment interaction (G × E) not significant ($p > 0.05$), total sample numbers given in parentheses.

Abbreviation: NS, not significant.

*$p < 0.05$.

**$p < 0.01$.

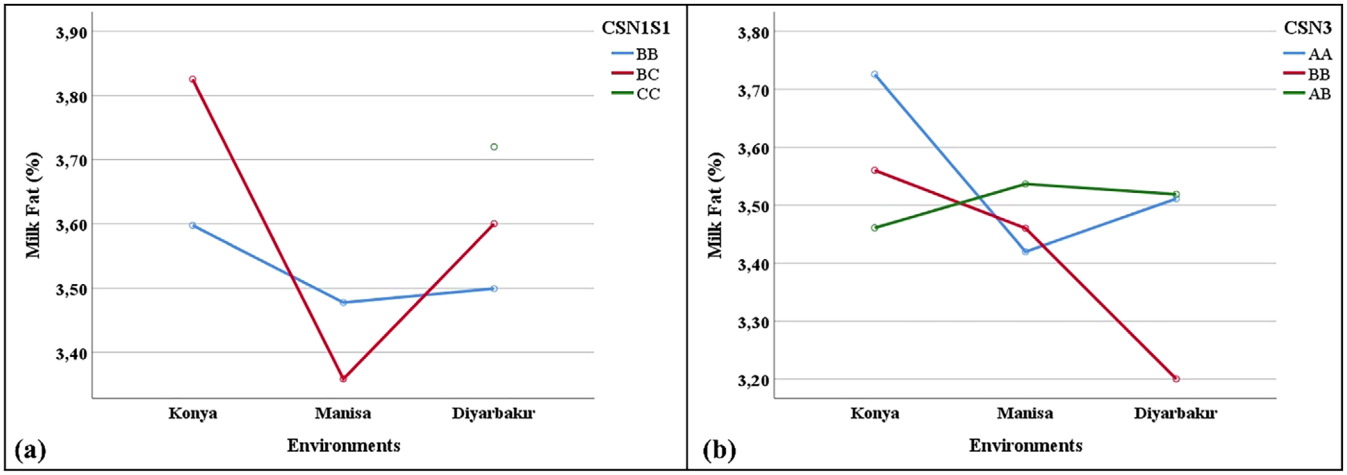


FIGURE 5 | Plots of genotype and environment interaction observed in the general linear model (GLM) for milk fat: (a) plot for the effects of *CSN1S1* genotypes on milk fat under different environmental conditions and (b) plot for the effects of *CSN3* genotypes on milk fat under different environmental conditions.

2011, Çardak 2005). Although it also was found a deviation from the equilibrium to some studies on Holstein (Çardak 2005) and Jersey cattle (Zepeda-Batista et al. 2015) for the *CSN2* gene, it was consistent with the HWE in other studies on Holstein (Duifhuis-Rivera et al. 2014; Ardıçlı et al. 2019, 2024), Simmental (Çardak 2005) and Slovak Pinzgau (Miluchova et al. 2014).

In the present study, the PIC values of casein genes ranged from 0.1001 to 0.3743. According to Botstein et al. (1980), PIC values lower than 0.25 are considered low or uninformative, whereas values between 0.25 and 0.5 are considered mildly (medium) informative. Furthermore, PIC values of 0.5 or higher are regarded as highly informative. Consequently, *CSN2* and *CSN3* genes were classified as mildly informative, whereas the *CSN1S1* gene was designated as low informative. Consistent with these findings, Ardıçlı et al. (2018, 2019) reported analogous results for the *CSN1S1* gene. Our results also showed that He and Ne values of *CSN2* gene were higher than the other two genes in

the cow population in all three regions, and this indicated a good level of genetic diversity in the *CSN2* gene.

The most popular of the casein genes is the β-casein gene. A growing body of scientific research is focusing on studying the effects of the bovine *CSN2* gene on genetic improvement and human health. Cows or bulls with the A2 variant of the *CSN2* gene are favoured in selection in some countries (the United States, New Zealand and the United Kingdom). This is due to the belief that although A2 milk is absent of any risk to human health, the A1 milk may be associated with certain diseases (e.g., Type I diabetes mellitus, sudden infant death and coronary disease) (Demirel and Çak 2018; Miluchová et al. 2023; Ardıçlı et al. 2024). Studies show that more scientific data are needed on the negative effects of A1 milk on health (Hegde 2019; Summer et al. 2020; Semwal et al. 2023). Another important aspect of β-casein is its use as a genetic marker in breeding studies. β-Casein is responsible for forming micelles that transport calcium, phosphorus and other nutrients to the mammary gland during lactation (Younis et al. 2024). In our study, *CSN2* genotypes significantly affected the SNF, protein and lactose contents of raw milk in the general herd. Cows with the A2A2 genotype had higher SNF, protein and lactose content than those with the A1A1 and A1A2 genotypes. Previous studies have revealed varying results concerning the effects of *CSN2* genotypes on milk composition. Çardak (2005) and Soyudal et al. (2019) reported that the effect of the *CSN2* gene on protein content was insignificant in Holstein cattle. But Çardak (2005) demonstrated that the A1A1 genotype of Simmental cows had high protein content (3.68%). Olenski et al. (2010) reported that the A2 variant positively affected protein yield in Holstein–Friesian dairy cows. Miluchová et al. (2023) observed positive statistical significance in favour of the A2 allele for protein yield and partly for protein content in Holstein cows. They found that the highest least squares mean for protein content occurred only in animals with the A2A2 genotype (3.32%). As reported in this study, Miluchová et al. (2023) detected that the genotypes of the *CSN2* gene had no significant effect on the milk fat content. Similarly, Ardıçlı et al. (2024) reported that the relationship between cattle *CSN2* genotypes and genetic value for protein and fat content in Holstein Friesian cattle was insignificant. In contrast, Şahin Semerci et al. (2025) reported that the fat content (4.09%) of raw milk from Turkish Holstein cows with the A1A2 genotype was significantly higher than that of other genotypes (A1A1 and A1A2) using Duncan's multiple range test. Furthermore, these researchers observed that the A1A1 genotype exhibited elevated percentage values with respect to SNF and protein contents. The variations observed in the studies may be attributable to differences in cattle breed, population size, genotype frequencies, genotypic interactions and the power of statistical models. In addition to the established association of the β-casein gene with milk composition, its association with a range of quantitative traits, including milk yield, reproductive performance and the technological characteristics of dairy products, has been investigated in previous studies (Ardıçlı et al. 2019; Soyudal et al. 2019; Kulibaba et al. 2024; Şahin Semerci et al. 2025). A synthesis of these results may lead farmers to favour the A2A2 genotype of the β-casein gene in the context of dairy cattle selection. However, it is imperative to acknowledge the complexity of quantitative traits, influenced by a multitude of genes and their pleotropic effects. Further scientific investigation is required to elucidate whether the adverse health implications associated with the A1A1 genotype dissipate following pasteurization and ultra-high temperature (UHT) treatment of milk and its subsequent processing into dairy products.

The κ-casein gene is another significant casein gene among the four milk casein proteins in dairy cattle. Variants A and B of the *CSN3* gene are associated with milk traits, such as milk components and yield, as well as technological traits, such as rennet coagulation time and thermal resistance. Therefore, it has been suggested that the *CSN3* A and B variants can be used as effective markers to assist in cattle selection for selective breeding programmes aimed at improving milk traits (Czerniawska-Piątkowska et al. 2023; Sharaz and Rabindrakumar 2024). In this study, the effect of the *CSN3* gene on fat content in the Konya population was found to be significant ($p < 0.01$). Cows with the *CSN3* AA genotype demonstrated a higher fat percentage (+0.27% and +0.17%, respectively) in comparison to those with the AB and BB genotypes. Albazi et al. (2023) indicated that genotype AA is positively associated with SNF, protein, casein and fat content of milk and milk yield in crossbred cows. The positive effect of the AA genotype in the *CSN3* gene on proteins can be explained by amino acid differences that may affect the biological properties of κ-casein in the mature protein and its interactions with other fractions in casein micelles (Albazi et al. 2023). However, some previous studies have shown that the *CSN3* genotypes did not affect milk fat content in Holstein (Çardak 2005; Gürses et al. 2018; Soyudal et al. 2019), Brown Swiss (Gürses et al. 2018), Simental (Çardak 2005), Anatolian Black (Gürses and Yuce 2012) and East Anatolian Red (Gürses and Yuce 2012) cattle. Gürses and Yuce (2012) demonstrated that genotypes of *CSN3* identified using the PCR-RFLP genotyping method did not affect SNF percentage in Anatolian Black cattle but were found significant in East Anatolian Red cattle. In contrast to this, Gürses et al. (2018) reported that the effect of the *CSN3* gene polymorphism on SNF and lactose contents is statistically important, and SNF (9.28%) and lactose (5.09%) are high in Holstein cows with the *CSN3* AB genotype. Rachagani and Gupta (2008) found that the SNF percentage (8.90%) was high in Sahiwal cattle with the *CSN3* BB genotype in India. Furthermore, another study reported that the B variant of *CSN3* had significant effect on milk protein percentage in Holsteins (Heck et al. 2009). As reported by Čítek et al. (2021), alleles B and E of the *CSN3* gene were found to be associated with a higher protein percentage. Their findings further demonstrated that allele B was associated with a higher fat percentage, whereas genotype AA was associated with increased milk yield. In addition to its substantial impact on the technological properties of milk, Czerniawska-Piątkowska et al. (2023) observed that cows possessing the BB κ-casein genotype exhibited superior reproductive indicators in comparison to other genotypes, including the shortest calving intervals. On the other hand, Ardıçlı et al. (2024) reported that the *CSN3* BE genotype exhibited the highest net merit. The *CSN3* gene is presented as a genetic marker in bull catalogues for breeders by dairy cattle insemination stations in countries such as the United States, Europe and Poland (Czerniawska-Piątkowska et al. 2023).

The present results indicated that the genotypes of the *CSN1S1* gene affected milk fat levels of cows in Konya province. In the *CSN1S1* gene, the BC genotype produced the highest fat content (3.83%), followed by the BB (3.60%) genotype. Research conducted by Ardıçlı et al. (2018) on Holstein cattle showed that the *CSN1S1*

gene was not associated with any of the milk content (milk solid, lactose, protein, fat content) and yield traits (milk solid, lactose, protein, fat yield). Çardak (2005) also found that protein content was different between the *CSN1S1* genotypes in Holstein and Simmental cows, and BC genotype (respectively, 3.43% and 3.67%) was associated with high milk protein. Čítek et al. (2023) posited that Holstein cows possessing the *CSN1S1* BB genotype during the second lactation period exhibited elevated milk, fat and protein yields in comparison to those possessing the BC genotype. However, the results of the meta-analysis study conducted by Ozdemir et al. (2018) showed that *CSN1S1* variants would not provide any advantage in improvement programmes.

In the current study, it was not possible to establish an association between the *CSN1S2* gene and milk composition because there is only one genotype of the *CSN1S2* gene in the population. However, Ardıçlı et al. (2018) found a significant effect of the *CSN1S2* gene on milk protein content in Holstein cattle. Moreover, DA genotype was associated with higher protein content (3.31%) compared with homozygous genotype. A review of the extant literature indicates a need for further research to ascertain the relationship between the bovine *CSN1S2* gene and milk composition. Moreover, the focus of future studies on other exon and intron regions of the *CSN1S2* gene, in addition to a larger population, may facilitate the identification of polymorphic regions of this gene.

G × E interaction is defined as a change in the relative performance of two or more genotypes measured in two or more environments. In general, G × E arises when the performance of different genotypes is not equally influenced by various environments (Falconer and Mackay 1996). G × E has been widely considered in animal breeding studies as it is often expected to reduce the response to selection. G × E may affect many traits of interest in breeding, such as milk and fertility. However, implementing models accounting for G × E in routine genetic improvement is scarce because they require a deep knowledge of the diverse environmental conditions in which animals are reared (Schmid et al. 2021). In this study, genotype-environment interactions were also evaluated. In this study, genotype environment interactions were also evaluated. In this study, no statistically significant interactions were found among the analysed milk components except milk fat. We observed that genotype-environment interaction in milk fat was in *CSN1S1* and *CSN3* genotypes (Figure 5). In *CSN1S1* gene, cows with BC genotypes had higher milk fat in Konya and Diyarbakır, whereas in Manisa, on the contrary, cows with BB genotypes had higher milk fat (Figure 5a). Similarly, the effect of *CSN3* gene on milk fat showed that different genotypes had superiority in different environmental conditions (Figure 5b).

In conclusion, an investigation was conducted into the association between the genotypes of *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3* genes and their effects on milk components. The PCR-RFLP method was utilized to analyse the data, and it was observed that the genes analysed in the study were polymorphic, except for the *CSN1S2* gene. For this reason, further investigation into other regions of the *CSN1S2* gene in larger populations is warranted to ascertain the relationship between these gene regions and milk composition. This will facilitate the determination of whether the *CSN1S2* gene should be included as a genetic marker in selection studies. Additionally, we identified significant differences among the genotypes belonging to the *CSN2* gene in terms of SNF, protein and lactose content in Holstein cows. The A2A2 genotype of the *CSN2* gene was obtained at a higher SNF, protein and lactose content. Consequently, dairy cattle breeders may focus on the A2A2 genotype in selection for both human health and milk quality. In addition, the *CSN1S1* BC and *CSN3* AA genotypes were significantly related to high milk fat content. We believe that the results obtained here may be useful for future studies. It is important to note that the incorporation of these markers into selection programmes has the potential to enhance not only the quality of milk but also its economic impact.

Author Contributions

Ahmet Fatih Demirel: methodology, investigation, writing – original draft, resources, conceptualization, data curation, validation, visualization. Bahattin Çak: supervision, writing – review and editing, project administration, formal analysis, conceptualization, funding acquisition, investigation, writing – original draft.

Disclosure

This study was a part of the first author's PhD thesis. It was presented as an oral presentation at the 4th International Eurasian Conference on Biological and Chemical Sciences (24 - 26 November 2021), Ankara, Türkiye and published as an abstract in the proceedings of the conference.

Ethics Statement

This study received ethics committee approval by Van Yuzuncu Yil University Animal Research Local Ethic Committee with the decision of 25.01.2018 dated number 2018/01.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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