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Effects of leptin and thyroglobulin gene polymorphisms on beef quality in Holstein breed bulls in Turkey

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Abstract: The relationship between three single-nucleotide polymorphisms (SNP) markers and the beef quality of Turkish Holstein bulls (THB) was investigated. The markers in leptin (LEP) gene (E2JW and E2FB) and thyroglobulin (TG) gene (C422T) were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in one hundred THB. Texture, pH, and marbling score (MS) are three of the most dominating determinants of meat quality. In THB, the relationship between 3 SNPs and beef quality characteristics of musculus longissimus dorsi (MLD) was investigated. The mean pH value of the raw beef samples with the TG G422T CC genotype was 5.33 ± 0.30 on day 7 and 5.31 ± 0.32 on day 14. The mean pH value of the raw beef samples with the LEP E2JW AA and AT genotypes measured on day 7 and day 14 ($P < 0.05$) was significantly different. The beef of THB with the AA and AT genotypes seemed to have different texture values compared to cooked beef samples of the TT genotype on day 7 ($P < 0.05$). There were no associations between pH value, Warner-Bratzler shear force (WBSF) scores, and MS in the E2FB genotypes. Regarding the LEP E2JW/LEP E2FB/TG C422T markers, THB with the AA/CT/CC genotypes were found to produce beef of higher quality compared to other genotypes.

Key words: Leptin gene, thyroglobulin gene, beef texture, marbling score, marker-assisted selection, Holstein cattle

1. Introduction

Consumer demand is one of the most important determinants of beef quality. The texture of beef seems to be one of the primary traits for consumers including the pH, tenderness, and marbling of the beef. The texture is defined as the degree of hardness or tenderness which is related to the fat content of the beef. The structure of muscle fibers and water content also contribute to beef tenderness. Higher fat content is associated with greater tenderness, which prevents the beef from drying and enhances the flavor/palatability. As a result, the fat distribution in the muscle influences the texture of the meat. Marbling refers to the mosaic pattern of fat within muscle fibers. In this context, the texture of cooked beef becomes important since beef is consumed after a cooking process [1]. Various methods have been developed to measure the texture including the amount of tenderness of cooked beef. Moreover, beef texture has been associated with the instrumentation used to cut muscle fibers. WBSF is one of the most widely tests used to measure the tenderness of meat closest to human eating and chewing characteristics and analyze the texture of raw/cooked meat [1,2]. The slices of beef are photographed using a digital camera and graded by image processing systems based on the rate and distribution of fat content to assess the MS [3]. One of the most important traits that determine beef quality and products is the glycogen level in muscles at the time of slaughter and the hydrogen ion concentration (Ph), which refers to the degree of acidity. Tenderness and brittleness are also related to the pH value of the beef. The ideal pH value for MLD is approximately 5–5.5 [4], and the pH values exceeding 6.2 indicate the onset of microbial deterioration in beef. The beef needs to be tender and readily broken into fragments for adequate palatability. The beef tenderness is associated with the age, nutritional status, and genotype of the animal [5]. Genetic studies conducted in cattle indicate a linear association between SNPs and beef quality and that the PCR-RFLP is a suitable method for detecting genetic polymorphisms [6]. Various studies focused on bovine genes that affect beef quality, such as tenderness and marbling traits [7–9]. TG and LEP genes play a key role in the regulation of energy balance and body weight control. The genetic variants of the TG gene were primarily associated with

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the intramuscular fat content, differentiation of bovine adipocyte, and lipid metabolism [10,11]. The E2FB (AY138588: g305C>T) marker is a SNP leading to the Arg/Cys amino acid polymorphism due to a C/T transition at position 305 within the second exon of the LEP gene. The E2JW (AY138588: g252A>T) marker is a SNP that leads to the Tyr/Phe amino acid polymorphism due to an A/T transversion at position 252 within the second exon of the LEP gene. The T allele of these two polymorphisms has been associated with greater intramuscular fat component [5,6]. The C422T marker is a SNP resulting from the C/T base change at position 422 within the 5' Promoter region of the TG gene [11,12]. This gene has been shown to be associated with differences in meat quality with respect to its effects on adipocyte growth, differentiation, and lipid metabolism [10–12]. The association of the TG gene with carcass composition and meat quality traits has been reported in Korean and Holstein crossbred cattle [11,12]. The C422T marker has been used by breeders in the USA, Japan, Canada, Argentina, and Australia [12]. The LEP and TG genes are considered as potential candidate for selection programs based on quantitative trait locus (QTL) to improve beef quality in cattle breeds [5,11–14]. In order to achieve a better understanding of the effects of SNPs at the selected candidate genes, their association with quantitative properties should be tested before using them in marker assisted selection (MAS) programs [14]. In cattle, the LEP gene (Gene ID: 280836) is located on chromosome 4 (4q32) and consists of 3 exons and 2 introns.[1] The TG gene (Gene ID: 280706) is located on the centromeric region of chromosome 14 (14q12–q15) and consists of 48 exons and 47 introns [10].[2] The most frequent TG C422T marker genotype was CC (0.92) and the TT genotype was not present in Holstein crossbred bulls in Turkey [11]. The number of cattle butchered in Turkey in 2019 was 3,633,730. The exotic and crossbreeds were 91.1% of the slaughtered cattle, whereas 8.9% of them were indigenous pure breeds. The production of red meat was 1.2 million tons in 2019 and 89.5% were obtained from cattle. The most common cattle in Turkey is pure Holstein cattle (48.4%) and crossbred with Holstein.[3] The Holstein breed is used in several countries to increase beef production. THB has been selected for both dairy qualities and meat yield due to their genetic variability [11]. Since LEP and TG are potential candidate genes concerning beef quality; their relationship with beef quality traits –WBSF, MS, pH value– have also been investigated in THB grown for butchery.

The aim of the present study is to determine the genetic variants in the TG and LEP genes of THB breed grown for beef production and to identify those with favorable effects on beef quality. Based on the results of the study, we will suggest to breeders consider the relationship between the positive quantitative traits of THBs and their genetic variants to improve breeding practices. The study further aims to inform breeding and butchery practices to improve high-quality beef production based on MAS and increase sustainable beef consumption.

2. Materials and methods

2.1. Animals and beef sample

Male Turkish Holstein calves (THC) younger than 6 months of age from various dairy cattle farms in villages in Edirne and Kırklareli provinces in Turkey were intensively fed until 17 months of age at two feedlots under similar conditions in Edirne province. Both feedlots hold about 200 head bulls [15]. In both fattening establishments, bulls are intensively fed with crushed grain, pulp, and roughage, such as wheat straw, dry clover, and corn silage in the semi-open farms. The altitude of the two farms in Edirne is 134 ft (41 m) and the time zone is UTC + 03.00 (UDAZD). One hundred beef samples were randomly selected from the abovementioned THB brought to Edirne Commodity Exchange Slaughterhouse. Bulls were slaughtered in December by the vertical cut slaughter method. Carcasses were stored at + 4 °C for 24 h following the slaughter. A 2.2-lb (about 1 kilogram) rib steak samples were collected from the MLD muscle at the 12th and 13th ribs. Phenotypic analyses –WBSF, MS, and pH value– were performed on the samples. Tissue samples for the molecular analyses of THB were also collected from the MLD [1,3,7].

2.2. Temperature and pH

At the first pH measurement, which was made 24 h after the slaughter, the pH value and temperature were measured by fixing a glass electrode and a temperature probe on each of the extracted ribs. On day 7, the measurement was made with samples that had been vacuumed and stored at + 4 °C for 7 days [3]. On day 7, they were frozen at –30 °C for 6 days after all the phenotypic measurements of the beef samples were made. The beef samples were taken out of the freezer and kept at + 4 °C for 24 h before day 14 when internal temperatures and pH were measured again.

2.3. Marbling score (MS) analysis

Cross-sections of rib steak muscles in each sample were transversely cut to the thickness of 2.5 cm for the MS analysis. MLD samples extracted from the cross-sectional area between the 12th and 13th ribs were photographed, and fat distribution within the MLDs was measured by Digimizer Image Analysis Software (5.3.4 Version, MedCalc Software Ltd., Belgium).

[1] LEP gene (2020). NCBI [online].Website https://www.ncbi.nlm.nih.gov/gene/280836 [accessed 10.02.2020].

[2] TG gene (2020). NCBI [online]. https://www.ncbi.nlm.nih.gov/gene/280706 [accessed 10.02.2020].

[3] Livestock Statistics (2020). Turkish Statistical Institute [online]. http://www.turkstat.gov.tr/Start.do;jsessionid=qwTJc4pR6251F [accessed 10.02.2020].

2.4. Texture analysis

For the analysis performed on day 7, 3 pieces of cubic MLD fragments cut to the thickness of 2.5 cm were obtained from each sample and stored at + 4 ℃ in labeled bags for 7 days. After the MLD samples were cooked, the precooling internal temperature of the cooked MLD samples was measured as 72 ℃, and the amount of force required to shear the muscle was measured by WBSF test [16].

The samples were stored at + 4 ℃ for 7 days and then they were frozen at –20 ℃. After a 6-day of storage, the samples were thawed completely on day 14 for the texture analyses. The texture of both the raw and cooked beef samples were tested on day 7 and 14. The resistance of MLD fibers during cutting was measured by TA–HD plus Texture Analyzer and Exponent 32 Software (v4.9, Stable Micro Systems Ltd., United Kingdom). The beef samples were cut at a rate of 10 mm/sec with a capacity of 250 kg.

2.5. DNA isolation and genotyping with PCR-RFLP

Genomic DNA was isolated (Exiprep Tissue Genomic DNA kit, K-3225 ver.2.0) from the resultant products using the Bioneer Exiprep 16 Plus Genomic DNA innovation robot (Bioneer Corporation, Korea). The absorbance values of the samples (260/280 nm) were measured using an Optizen NanoQ Nanodrop micro-volume spectrophotometer (K Lab Keen Innovative Solutions, K Lab Co. Ltd., Republic of Korea). The target DNA regions were amplified in 25 µL PCR amplification solution. The amplification mixture consisted of 12.5 µL (2X) PCR Master Mix (Dream Taq Hot Start Green, Thermo Scientific, UK), (10 pmol/µL) 1 µL of each primer (Sentegen Biotech, Ankara), 5 µL genomic DNA (~75 ng/µL) and 5.5 µL distilled water. The primer sequences in Table 1 were used in our study. Amplification was performed by a Thermocycler (My Genie 96 Thermal Block, Bioneer Corporation, Republic of Korea), and the Touchdown PCR method was used to amplify target DNAs containing all marker variants. The DNA samples were subjected to denaturation at 94 ℃ for 2 min, and 5 cycles for each subsequent 6 temperatures: at 94 ℃ for 20 sec / 58 ℃ for 20 sec / 72 ℃ for 60 sec / 94 ℃ for 20 sec / 54 ℃ for 20 sec / 72 ℃ for 60 sec, and 25 cycles for each subsequent 3 temperatures: at 94 ℃ for 20 sec / 52 ℃ for 20 sec / 72 ℃ for 60 sec (annealing) and then 72 ℃ for 5 min (extension) for each set of LEP E2JW primers (Table 1). The DNA samples were preheated at 94 ℃ for 2 min (denaturalization) and subsequently: 94 ℃ for 45 sec / 52 ℃ for 45 sec / 72 ℃ for 55 sec subjected to 35 cycles at each temperature (annealing) and 72 ℃ for 3 min (extension) for each set of LEP E2FB primers (Table 1). The DNA samples were preheated at 94 ℃ for 5 min (denaturalization) and subsequently: 94 ℃ for 60 sec / 55 ℃ for 60 sec / 72 ℃ for 60 sec subjected to 35 cycles at each temperature (annealing) and 72 ℃ for 7 min (extension) for each set of TG C422T primers (Table 1). The PCR products (15 µL) were digested with 1600 units of *Kpn2I* (20 U / µL, Anza™ 60 *Kpn2I,* Invitrogen Thermo Fisher Scientific, UK), 1500 units of *BSU15I* (20 U / µL, Anza 30 *BSU15I,* Invitrogen Thermo Fisher Scientific) restriction endonuclease for the LEP gene and 800 units of MBOI (5 U / µL, Anza 55 MBOI, Invitrogen Thermo Fisher Scientific) restriction endonuclease for the TG gene. The enzymatic digestion reaction was incubated in a thermal cycler at 37 ℃ for 3 h (My Genie 96 Thermal Block Bioneer Appliance, Republic of Korea). The individual PCR-RFLP products were separated by the Advanced Analytical Fragment Analyzer Capillary Electrophoresis (Agilent Technologies, Inc., USA) and the ProSize (Agilent Technologies, Inc.) software was used for imaging. PCR-RFLP values expressed for DNA size are approximate values owing to the nature of agarose gel electrophoresis. In the capillary electrophoresis, the size of DNA fragments were identified as exact values of ± 3 base pairs.

2.6. Statistical analyses

Genotypic and phenotypic characterization data of the THB were statistically compared. Allele and genotype frequencies of THB were determined using PopGene 32 software [17]. One-way and repeated measures ANOVA were used to compare the means of two or more samples using the F distribution. IBM SPSS Statistics 20.0 XLSTAT

Table 1. Primers used in this study and amplification products.

Gene	Marker	Primer sequences (5′–3′)	bp	Reference
LEP	E2JW	Forward: GATTCCGCCGCACCTCTC	467	[22]
		Reverse: CCTGTGCAAGGCTGCACAGCC		
	E2FB	Forward: ATGCGCTGTGGACCCCTGTATC	94	[10]
		Reverse: TGGTGTCATCCTGGACCTTCC		
TG	C422T	Forward: GGGGATGACTACGAGTATGACTG	545	
		Reverse: GTGAAAATCTTGTGGAGGCTGTA		

bp: Base pairs of target DNA products.

(IBM Corp., Armonk, NY, USA) demo version was used for the data analysis. Slaughter weight (SW) of bulls were taken before the slaughter. In statistical analysis, while WBFS (kg), pH and MS (%) were evaluating, effects of SW (kg) and age (mount) were considered.

3. Results

The phenotypic WBSF, MS, and pH in the THB samples were compared with respect to the LEP E2JW, LEP E2FB, and TG C422T SNP alleles, and genotype frequencies were estimated. Three different genotypes –AA, AT, TT– in LEP E2JW (Figure 1) and two different genotypes –CT and TT– in LEP E2FB (Figure 2) were observed in the THB samples. Individual PCR-RFLP products; DNA fragments of 252 and 215 bp for the A allele and 467 bp for the T allele of LEP E2JW marker genotypes were identified. In case of the TG gene, fragments of 72, 178, and 295 bp were identified for the C allele of TG C422T marker genotype (Figure 3). The population was monomorphic for the C allele at the TG C422T locus. Genotype frequencies were 0.54 for AA, 0.42 for AT, and 0.04 for TT in LEP E2JW, 0.94 for CT, and 0.06 for TT in LEP E2FB. In addition, among the LEP E2JW/LEP E2FB genotypes, the frequencies were 0.06 for AA/TT, 0.48 for AA/CT, 0.42 for AT/CT, and 0.04 for TT/CT. The frequencies of the alleles and genotypes of the LEP E2FB and E2JW loci, and their combinations were shown in Table 2. The A and T allele frequencies in LEP E2JW were 0.75 and 0.25, respectively, whereas C and T allele frequencies in LEP E2FB were 0.47 and 0.53. Four different genotype combinations —AA/TT, AA/CT, AT/CT, and TT/CT— were observed out of 9 different genotypic combinations expected in the LEP gene E2JW and E2FB marker loci in the THB. The results revealed that the frequency of the LEP E2JW AA/E2FB CT genotype was the highest (0.48), while those in the LEP E2JW TT/E2FB CT genotypes were the lowest (0.04) in the THB. The population was unbalanced as it did not fit the Hardy-Weinberg (HW) equilibrium ($P < 0.05$).

The THB carcasses were stored at + 4 °C for 24 h after slaughter. All beef samples –with AA, AT, and TT genotypes for LEP E2JW; CT and TT genotypes for LEP E2FB and, CC genotype for TG C422T– of the four genotypes completed the maturation process at an internal temperature ranging between 8.27 and 10.77 °C during the first 24 h. The pH value of the beef stored + 4 °C for 7 days was measured both 24 h after the slaughter and on day 7. The pH value of the beef was the lowest with the AA and the highest with AT genotypes for the LEP E2JW locus in all three measurements (24 h after the slaughter, on day 7 and 14). The mean pH value of the raw beef samples with the LEP E2JW genotypes measured on days 7 and 14 differed significantly ($P < 0.05$). The effects of the LEP E2JW, LEP E2FB, and TG C422T markers on the beef quality traits of the THB in Turkey were demonstrated in Table 3.

The mean MS was 11.07 ± 4.91% for all cattle regardless of the genotype. Regarding the LEP E2JW locus, the highest MS value was found in the AT, whereas the lowest MS was observed in the TT genotype. The difference between the genotypes was not statistically significant ($P > 0.05$). Toughening was detected in the raw slices of beef with the LEP E2JW AA genotype on day 14 due to the effect of freezing. The same beef –with E2JW AA genotype– had a higher tenderness when cooked on day 14 compared to those cooked on day 7. For cattle with the LEP E2JW AT genotype, freezing was observed to increase tenderness leading to a softer beef texture both in the raw and cooked beef. In raw MLD samples with the LEP E2JW TT genotype, the softening was noted with the effect of freezing and the beef was more tender after cooking. For the THB with the LEP E2JW AA and AT genotypes, the beef cooked on day 7 was

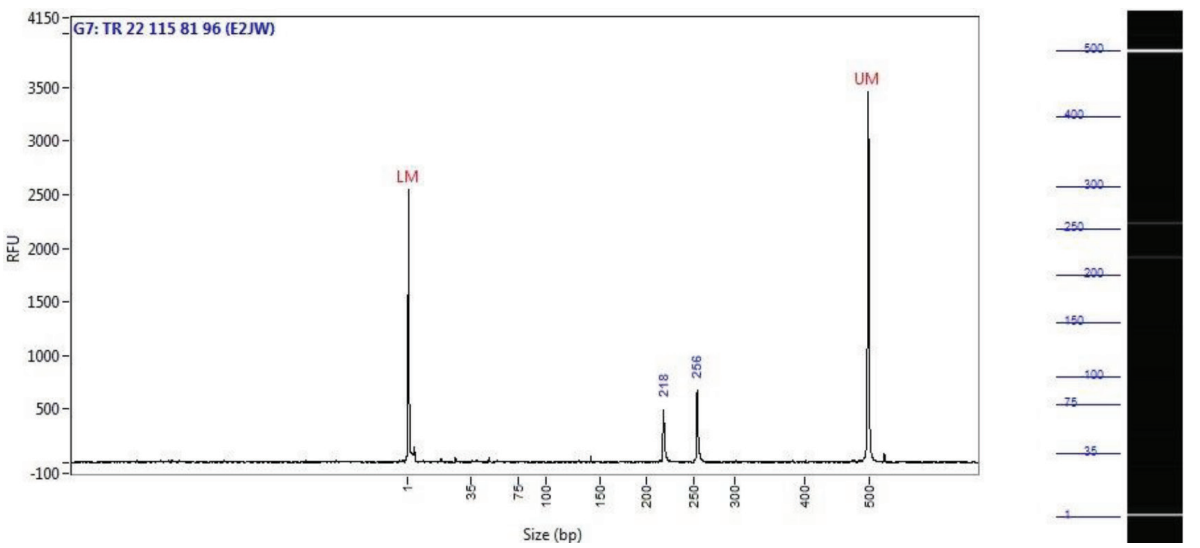

Figure 1. Electropherogram of LEP E2JW locus AA genotype.

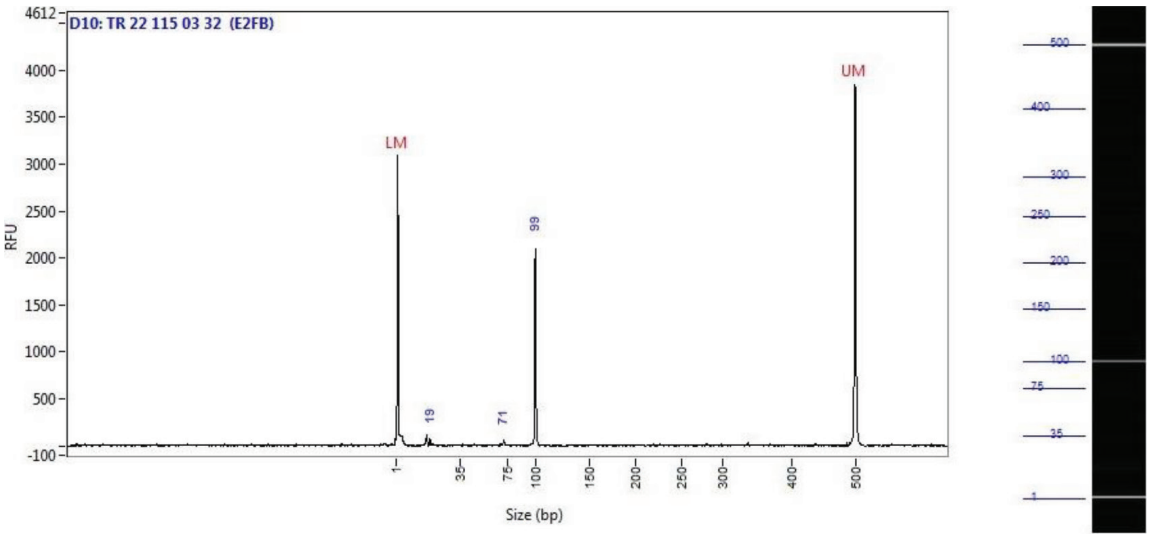

Figure 2. Electropherogram of LEP E2FB locus CT genotype.

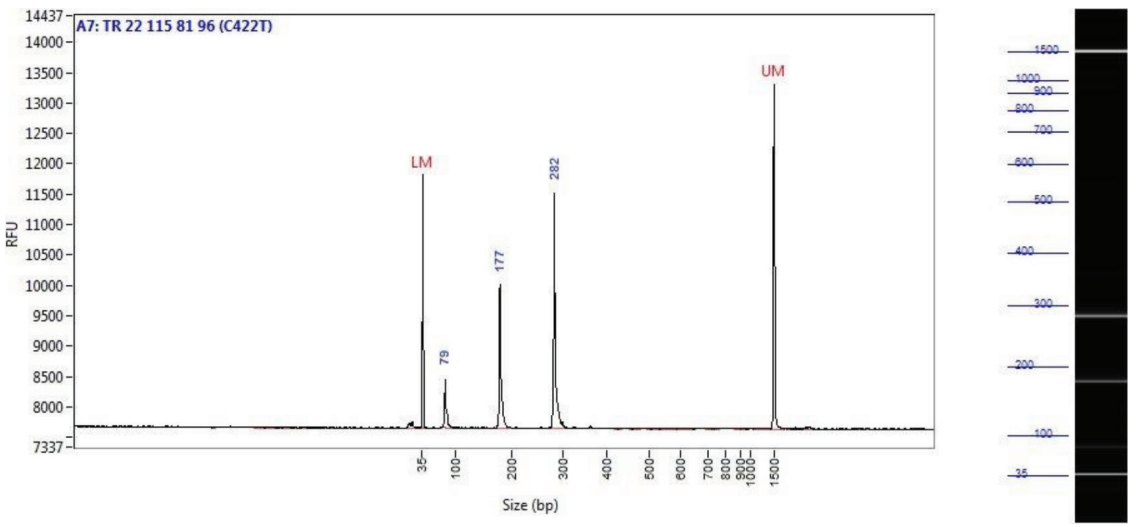

Figure 3. Electropherogram of TG C422T locus CC genotype.

more tender and had significantly different WBSF values ($P < 0.05$) compared to those with the LEP E2JW TT genotype (Table 3).

There was no significant difference between the MS of the LEP E2FB CT genotype (10.92 ± 4.75%) and the LEP E2FB TT genotype (13.43 ± 7.12%) ($P > 0.05$). The freezing procedure performed for 6 days for the raw beef samples with LEP E2FB CT and TT genotypes resulted in the hardening of beef texture. However, beef of both genotypes was found to be more tender after cooking on day 14. The softening of cooked beef was favorable in terms of texture. Beef samples with the LEP E2FB CT genotype cooked on day 7 were more tender compared to those with the LEP E2FB TT genotype; however, the difference was statistically insignificant ($P > 0.05$) (Table 3).

The mean MS was 11.07 ± 4.91% in the MLD of the THB with the TG C422T locus CC genotype. On day 7, the raw beef samples with the TG C422T CC genotype had a WBSF value of 7.58 ± 2.19 kg/cm², which was higher in cooked beef of the same samples (7.89 ± 3.83 kg/cm²). On day 14, the resistance of the raw MLD (7.69 ± 2.42 kg/cm²) was noted to be lower after cooking (6.98 ± 3.23 kg/cm²). The beef samples with the TG C422T CC genotype cooked on day 14 had higher tenderness values than those cooked on day 7, but the difference was not statistically significant ($P > 0.05$) (Table 3). The relationship between the SW averages of THBs and the variants of the LEP E2JW locus (AA, AT, TT) and the variants of the LEP E2FB locus (CT, TT) was statistically insignificant ($P > 0.05$).

Table 2. The frequencies of alleles and genotypes of the LEP E2FB, E2JW loci and their combinations.

Cattle breed	Allele frequency LEP E2FB		Genotype frequency *LEP E2FB			Chi Square (χ^2)
Turkish Holstein Bull (n :100)	C	T	CT (n)	TT (n)		78.64 (P < 0.05)
	0.47	0.53	0.94 (94)	0.06 (6)		
	*LEP E2JW		LEP E2JW			
	A	T	AA (n)	AT (n)	TT (n)	1.44 (P < 0.05)
	0.75	0.25	0.54 (54)	0.42 (42)	0.04 (4)	
	LEP E2JW / LEP E2FB haplotype frequency					
	A/C		A/T	T/C	T/T	
	0.35		0.40	0.12	0.13	
	*LEP E2JW / LEP E2FB genotype frequency					
	AA/TT (n)		AA/CT (n)	AT/CT (n)	TT/CT (n)	83.48 (P < 0.05)
	0.06 (6)		0.48 (48)	0.42 (42)	0.04 (4)	

*The population is unbalanced according to the HW equilibrium (P < 0.05). LEP E2JW: Leptin gene E2JW locus, LEP E2FB: Leptin gene E2FB locus.

Table 3. The effects of LEP E2JW, LEP E2FB, and TG C422T markers on the beef quality traits of Holstein cattle population in Turkey.

Phenotypic Traits ($\bar{x}$±sd)	LEP E2JW			LEP E2FB		TG C422T
	AA	AT	TT	CT	TT	CC
Temperature, °C (at 24 h)	9.48 ± 3.76	9.02 ± 3.78	9.25 ± 5.82	9.34 ± 3.82	8.27 ± 3.88	9.28 ± 3.82
pH (at 24 h)	5.25 ± 0.22	5.35 ± 0.44	5.31 ± 0.17	5.28 ± 0.33	5.52 ± 0.31	5.29 ± 0.33
Temperature, °C (on d 7)	10.28 ± 3.87	9.50 ± 3.27	10.65 ± 3.19	9.91 ± 3.65	10.77 ± 2.67	9.97 ± 3.60
*pH (day 7)	5.28 ± 0.25[a]	5.42 ± 0.36[b]	5.30 ± 0.21	5.33 ± 0.29	5.48 ± 0.43	5.33 ± 0.30
Temperature, °C (on d 14)	8.65 ± 4.52	8.83 ± 5.69	9.72 ± 3.63	8.87 ± 5.05	7.15 ± 3.61	8.77 ± 4.92
*pH (day 14)	5.23 ± 0.24[a]	5.41 ± 0.40[b]	5.33 ± 0.15	5.31 ± 0.32	5.41 ± 0.41	5.31 ± 0.32
WBSF of raw beef, on d 7 (kg/cm^2)	7.30 ± 2.07	7.81 ± 2.33	9.01 ± 1.62	7.65 ± 2.20	6.42 ± 1.71	7.58 ± 2.19
*WBSF of cooked beef, on d 7 (kg/cm^2)	7.85 ± 3.49[a]	7.60 ± 4.12[a]	11.53 ± 4.18[b]	7.83 ± 3.91	8.81 ± 2.28	7.89 ± 3.83
WBSF of raw beef, on d 14 (kg/cm^2)	7.60 ± 1.94	7.78 ± 2.99	7.93 ± 2.16	7.68 ± 2.45	7.86 ± 2.06	7.69 ± 2.42
WBSF of cooked beef, on d 14 (kg/cm^2)	7.02 ± 2.88	6.79 ± 3.52	8.64 ± 4.78	6.99 ± 3.28	6.96 ± 2.36	6.98 ± 3.23
Marbling score (%)	10.64 ± 4.48	11.75 ± 5.60	9.77 ± 2.15	10.92 ± 4.75	13.43 ± 7.12	11.07 ± 4.91
Slaughter weight (kg)	499.76 ± 6.9	527.55 ± 1.2	509.00 ± 71.8	512.14 ± 79.8	506.50 ± 85.9	511.8 ± 79.7

*According to ANOVA test, genotypic differences are statistically significant (P < 0.05). According to LSD's test, genotypic differences between a and b are significant (P < 0.05). $\bar{x}$: Mean, sd: standard deviation; LEP E2JW: Leptin gene E2JW locus; LEP E2FB: Leptin gene E2FB locus, WBSF: Warner Bratzler shear force texture.

In the THB, 4 different genotypes were observed from combinations of LEP gene E2JW/E2FB and TG gene C422T marker loci. The effect formed of LEP E2JW/LEP E2FB/TG C422T marker haplotypes on bovine quality traits of the four observed genotypes –AA/CT/CC, AT/CT/CC, AA/TT/CC, and TT/CT/CC– in the THB were demonstrated in Table 4. In the beef samples, the mean pH value differences between the AA/CT/CC and the AT/CT/CC, AA/TT/CC genotypes were significant (P < 0.05) at 24 h and on days 7 and 14, but there was no significant difference in other genotypes. The mean WBSF (kg/cm^2) values of the raw and cooked beef of the MLD were examined in the THB groups. The mean WBSF (kg/cm^2) differences of the cooked beef samples between the AA/CT/CC, AT/CT/CC, AA/TT/CC and the TT/CT/CC genotypes were significant (P < 0.05) on day 7. Similarly, the differences

Table 4. The effects of form of LEP E2JW/ LEP E2FB/ TG C422T marker haplotypes on the beef quality traits of the Holstein bull samples in Turkey.

Phenotypic traits ($\bar{x}$±sd)	LEP E2JW/ LEP E2FB/ TG C422T genotypes			
	AA/CT/CC	AT/CT/CC	AA/TT/CC	TT/CT/CC
Temperature ℃ (at 24 h)	9.63 ± 3.76	9.02 ± 3.78	8.27 ± 3.88	9.25 ± 5.82
*pH (at 24 h)	5.22 ± 0.18[a]	5.35 ± 0.44[b]	5.52 ± 0.31[b]	5.31 ± 0.17
Temperature, ℃ (on d 7)	10.21 ± 4.01	9.50 ± 3.27	10.77 ± 2.67	10.65 ± 3.19
*pH (day 7)	5.25 ± 0.21[a]	5.42 ± 0.36[b]	5.48 ± 0.44[b]	5.30 ± 0.21
Temperature, ℃ (on d 14)	8.83 ± 4.62	8.84 ± 5.69	7.16 ± 3.62	9.73 ± 3.63
*pH (day 14)	5.21 ± 0.21[a]	5.41 ± 0.40[b]	5.42 ± 0.41[b]	5.33 ± 0.15
*WBSF of raw beef, on d 7 (kg/cm^2)	7.41 ± 2.10[ab]	7.81 ± 2.34[ab]	6.42 ± 1.71[a]	9.01 ± 1.63[b]
*WBSF of cooked beef, on d 7 (kg/cm^2)	7.73 ± 3.62[a]	7.60 ± 4.12[a]	8.81 ± 2.28[a]	11.53 ± 4.10[b]
WBSF of raw beef, on d 14 (kg/cm^2)	7.58 ± 1.94	7.78 ± 2.99	7.86 ± 2.07	7.93 ± 2.16
WBSF of cooked beef, on d 14 (kg/cm^2)	7.03 ± 2.96	6.79 ± 3.52	6.96 ± 2.36	8.64 ± 4.79
Marbling score (%)	10.29 ± 3.98	11.74 ± 5.60	13.4 ± 7.12	9.77 ± 2.15
Slaughter weight (kg)	498.92 ± 67.9	527.54 ± 91.1	506.50 ± 85.9	509.00 ± 71.8

*According to RM-ANOVA test, genotypic differences are statistically significant ($P < 0.05$). According to LSD's test, genotypic differences between a, b, ab are significant ($P < 0.05$), $\bar{x}$: Mean; sd: standard deviation; LEP E2JW: Leptin gene E2JW locus; LEP E2FB: Leptin gene E2FB locus; WBSF: Warner Bratzler shear force texture.

of the raw beef samples among four different genotypes were significant ($P < 0.05$) on day 7 with the AA/TT/CC (6.42 ± 1.71 kg/cm^2) having the most tender beef and the TT/CT/CC (9.01 ± 1.63 kg/cm^2) having the toughest beef while the AA/CT/CC (7.41 ± 2.10 kg/cm^2) and AT/CT/CC (7.81 ± 2.34 kg/cm^2) genotypes were the mild ones ($P < 0.05$). The most tender beef in the THB was the AA/TT/CC genotypes, which was supported by the MS (13.4 ± 7.12%) indicating a high fat content. Furthermore, the TT/CT/CC bovine genotype had the lowest MS (9.77 ± 2.15%) and the toughest beef. A total of four different WBSF values of raw and cooked beef were demonstrated in Figure 4 according to the genotypes. The SW averages of THBs were 511.8 ± 79.7 kg. The relationship between the SW averages of THBs and the observed marker genotypes –AA/CT/CC, AT/CT/CC, AA/TT/CC, and TT/CT/CC– of LEP E2JW / LEP E2FB / TG C422T was statistically insignificant ($P > 0.05$).

4. Discussion

Nellore and British Holstein cattle with the LEP E2JW AT genotype were associated with greater fat content in carcasses compared to those with the AA genotype [18,19]. The LEP E2JW marker was shown to have no independent effects on fat deposition; however, it was associated with increased carcass fat in certain haplotypes [18]. In another study [20], a significant association was found between the E2JW marker genotypes, MS, and WBSF ($P < 0.05$). The E2JW AA genotype (10.1 ± 0.46%) beef had higher MS compared to the E2JW AT genotype (8.9 ± 0.59%) ($P < 0.05$), which suggests that the E2JW A allele has a positive effect on tenderness and MS of the beef.

In the present study, the highest MS (11.75 ± 5.60%) was observed in the THB with the E2JW AT genotype. However, no association was found between MS and E2JW marker genotypes ($P > 0.05$). With regard to the WBSF values of the beef samples cooked on day 7, a significant difference was found between the E2JW TT genotype and the AA and AT genotypes ($P < 0.05$). The E2JW TT genotype had a higher WBSF value and lower MS, which differs from the findings of previous studies [18,20]. A favorable contribution of the E2JW A allele to the beef texture of the THB was observed.

The mean WBSF values of the raw and cooked beef of the THB with the LEP E2FB CT genotypes were higher than the mean WBSF values for Bos Taurus and Bos Indicus hybrids (3.70 ± 0.88 kg/cm^2) [21]. The differences between LEP E2FB CC and CT genotypes concerning the MS and WBSF values for Bos Taurus and Bos Indicus hybrids were shown to be statistically insignificant ($P > 0.05$), as was the case with the THB. The relationship between LEP E2FB marker genotype and carcass fat distribution, pH, and MS were also statistically insignificant in Nellore cattle ($P > 0.05$) [19]. In Brazilian hybrid cattle, the difference in WBSF values between the beef samples with the LEP E2FB CC (5.22 ± 0.32 kg/cm^2) genotype and CT and TT genotypes was found to be statistically significant ($P <$

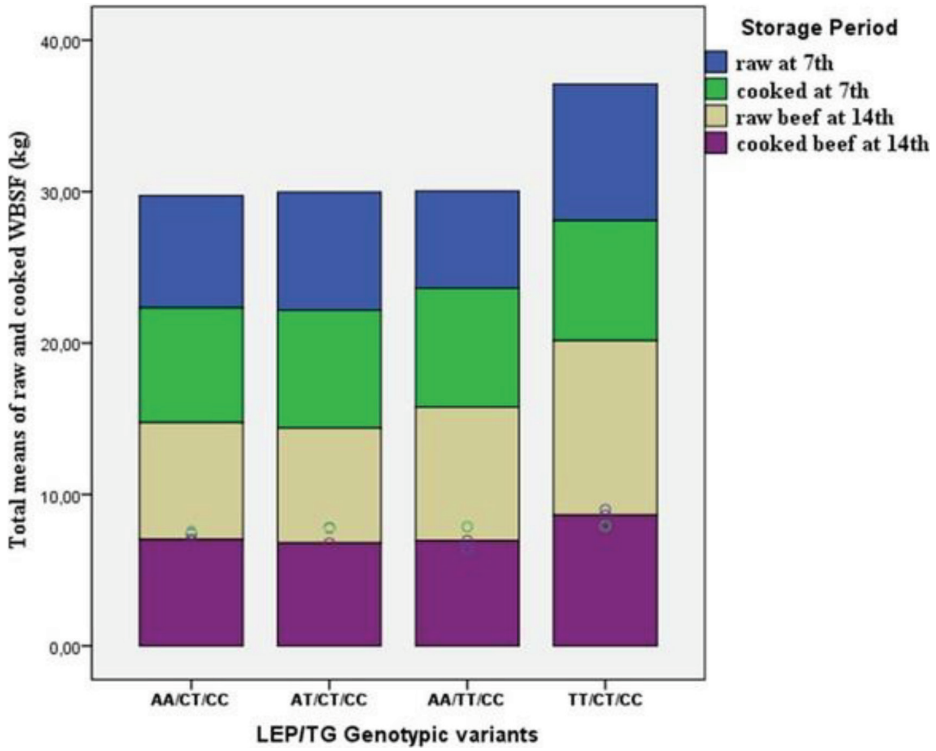

Figure 4. A total of four different WBSF values of raw and cooked beef was demonstrated according to genotypes.

0.05) [22]. However, the difference between the LEP E2FB CT genotype (6.02 ± 0.26 kg/cm^2) and the LEP E2FB TT genotype (5.75 ± 0.37 kg/cm^2) was insignificant, with values comparable to WBSF of raw beef on day 7 in the THB. In Aberdeen Angus hybrids, no association was observed between LEP E2FB genotypes and phenotypic traits –WBSF, pH_{24}– [23]. In a study conducted in Brazilian crosses [22], no significant difference was reported between LEP E2FB marker genotypes –CC, CT, TT– and MS. Similarly, there was no significant difference in MS between the LEP E2FB CT and TT genotypes in the THB ($P > 0.05$). In Bos Taurus hybrids, the LEP E2FB CT (10.2 ± 0.54%) had the highest MS, whereas the LEP E2FB CC genotype (8.7 ± 0.69%) had the lowest MS [20]. The MS of the THB with the LEP E2FB CT genotype (10.92 ± 4.75%) was lower compared to the LEP E2FB TT genotype (13.43 ± 7.12%). The mean WBSF values measured in beef of THB with the T allele of the LEP E2FB marker were lower while MS was greater, indicating a favorable effect of the T allele on beef texture.

A significant association between the C allele of the TG C422T marker and MS ($P < 0.05$) was observed in Korean cattle [12] and in Brahman cattle [24]. In Nellore cattle, the monomorphism in the TG C422T locus was associated with the CC genotype only [21], which is consistent with the findings in THB. The mean WBSF value (3.93 ± 0.3 kg/cm^2) of Mexican cattle with CC genotype [21] was lower than the mean WBSF value of the THB with CC genotype. The mean MS for the THB sample with the C422T CC genotype was 11 ± 4%, which is similar to the MS of the Hungary cattle breed with the C422T CC genotype [25]. However, Anton et al. [26] concluded that the TT genotype in the TG gene had the highest fat percentage in the MLD of Angus bulls, which was significant ($P < 0.05$), which contradicts our results.

In conclusion, in THB, the most tender beef was produced by the cattle with the LEP E2JW AA, LEP E2FB TT, TG C422T CC genotypes (13.4 ± 7.12% MS, 6.42 ± 1.71 kg/cm^2 WBSF). The breeders are recommended to select THB by the MAS method at the calf stage and use it in cattle breeding to produce high-quality beef. THB with the genotypes providing an advantage for high-quality beef production are expected to be preferable and sold at higher prices compared to those bearing other genotypes in Turkey. The LEP E2JW A allele is very likely to make a favorable contribution to the tenderness of THB beef in MAS studies. In stock farms, THB should be herded in different paddocks based on their marker genotypes to increase high-quality beef production. Further investigation of the associations between different gene markers, beef quality, and phenotypic traits of THB might extend the findings of the existing literature.

Acknowledgement/Disclaimers/Conflict of interest

The original data are available upon request to the corresponding authors.

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GV contributed as an investigator in the study, collected samples and conducted experiments. SK guided the experiments and aided in the statistical analysis of the data. Both authors contributed to writing of the manuscript.

The authors declare that they have no conflict of interest.

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