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The effect of ellagic acid on performance, digestibility, egg quality, cecal bacterial flora, antioxidant activity, and some blood parameters in laying quails reared at different temperatures

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Abstract: In this study, the effect of ellagic acid on the performance, egg quality, digestibility, cecal microflora, serum malondialdehyde (MDA) level, and biochemical parameters in quails reared under normal conditions and at high environmental temperatures was investigated. The animals (n = 240; 5-weeks old) reared at 2 environmental temperatures [thermoneutral (TN) and heat stress (HS)] and in 4 diet groups containing different ellagic acid doses (0, 100, 200, and 400 mg/kg) were randomly assigned to 8 groups according to the 2 × 4 factorial design. Feed intake, egg weight, and egg production were higher ($P < 0.001$) and feed conversion ($P < 0.01$) was better in the TN groups compared to the HS groups. Egg ($P < 0.01$) and shell weights ($P < 0.001$) were higher in the TN groups compared to the HS groups. The effect of ellagic acid supplementation on shell weight was statistically significant in the TN and HS groups ($P < 0.05$). The effects of environmental temperature and the supplementation of ellagic acid on digestibility of dry matter ($P < 0.05$), ether extract ($P < 0.01$), and crude protein ($P < 0.01$) were statistically significant in the TN and HS groups. Supplementation of ellagic acid reduced the total coliform bacteria count ($P < 0.01$) and increased the total lactic acid bacteria count ($P < 0.001$) in the cecum. Serum MDA levels increased with heat stress ($P < 0.001$) and decreased with the supplementation of ellagic acid ($P < 0.001$). The effect of environmental temperature on serum glucose ($P < 0.001$), cholesterol ($P < 0.05$), total protein ($P < 0.05$), alanine aminotransferase (ALT) ($P < 0.001$), sodium ($P < 0.05$), and magnesium was significant. In conclusion, the supplementation of ellagic acid decreased oxidative stress and total coliform bacteria count and improved the feed conversion and egg quality in quails under heat stress. Moreover, ellagic acid increased the digestibility of nutrients and had a positive effect on lactic acid bacteria. It was observed in this study that these beneficial effects of ellagic acid increased with an increasing dose, and 400 mg/kg was the most effective dose.

Key words: Ellagic acid, performance, egg quality, digestibility, microflora, malondialdehyde

1. Introduction

An increasing number of products have tried to be obtained from animals in order to meet the ever-growing food need in the world, thus leading to problems such as crowdedness, noise, inadequate hygiene, inadequate feeding, unfavorable care, and poor feeding conditions. These problems cause animals, especially poultry, to experience stress and, as a result, their yield decreases and their health is negatively affected [1].

High environmental temperature, one of the most important stress factors in poultry, causes oxidative stress in animals, weakens the antioxidant defence system, and results in yield decrease in poultry [2–4]. Also, physical activity, feed intake, feed conversion, egg production, egg weight, and eggshell quality have been observed to decrease in animals under heat stress [2–4]. Natural or synthetic antioxidant substances are added into rations in order to protect animals from oxidative stress and also eliminate these problems [5]. Numerous studies conducted to protect quail under heat stress from oxidative stress have reported that free oxygen radicals generated due to oxidative stress are inactivated upon addition of antioxidant substance into rations [2–4,6].

Pomegranate and its by-products contain flavonoids (anthocyanins, catechins, and other complex flavonoids), hydrolysable tannins (punicalin, pedunculgin, punicalagin, gallic, and oleic acid esters of glucose), polyphenols, fatty acids (conjugated and unconjugated), tocopherols, sterols, terpenoids, alkaloids, and phenolic compounds that make up 92% of antioxidant activity [7]. Ellagitannins belonging to the hydrolysable tannin class of polyphenols are complex derivatives of ellagic acid. Hydrolysis of the ellagitannins with acids or bases yields hexahydroxydiphenic acid, from which ellagic acid is obtained [8]. Ellagic acid, the

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most important bioactive component in the pomegranate fruit, has many beneficial effects on human health [9]. It has also been reported to have antioxidant [10], anticancerogenic [11], and antimutagenic [12] properties, as well as antiatherosclerotic [13], antimicrobial [14], and antiinflammatory [15] effects.

No studies have been found that determine the effects of ellagic acid on performance, production, and digestion in animals, although numerous studies have been conducted to determine its biological effects. For this reason, the aim of this study was to investigate the effect of ellagic acid on performance, egg quality, digestibility of nutrients, total coliform, and total lactic acid bacteria counts in the cecum, serum malondialdehyde (MDA) level, and biochemical parameters of laying quails reared at different temperatures.

2. Materials and methods

2.1. Animals, diets, and experimental design

Japanese quails (*Coturnix coturnix Japonica*; n = 240; 5-weeks old) were obtained from a commercial company. Ethical approval was approved by the Local Ethics Committee (Decree no: 27.01.2016/12). The experiment was conducted in Elazığ (38°40”S, 39°13” W), Turkey.

Maize and a soybean meal-based diet containing 18% crude protein and 2800 kcal/kg metabolizable energy was used and is presented in Table 1 [16]. The chemical composition of feed ingredients (dry matter, crude protein, crude ash, and ether extract) was analyzed according to AOAC [17] procedures, and crude fibre was determined by the methods of Crampton and Maynard [18]. Carpenter and Clegg's [19] equation was applied to calculate metabolizable energy. In the study, ellagic acid at 90% purity (PureBulk Inc., Roseburg, OR, USA) was used.

Eight groups consisting of 30 female quails at 35 days of age were assigned into 6 replicates, and each replicate included 5 quails. The research was conducted in a 2 × 4 (heat, dose) factorial trial. Thermoneutral (TN) groups were kept in cages in temperature-controlled rooms, and heat stress (HS) groups were kept at 34 °C for 9 h (08:00–17:00 h). The night-time temperature for all groups was the same (18–22 °C). The quails received either a basal diet or a basal diet supplemented with ellagic acid in 0, 100, 200, or 400 mg/kg of the diet. The animals had unlimited access to feed and fresh water, and the lighting was implemented as 16L:8D h/day. The experiment lasted 75 days.

2.2. Performance and egg quality

The amount of feed intake was determined weekly and weighed and given daily in front of the quails. Feed intake (g/quail/day) and feed conversion ratios were determined weekly. The feed conversion ratio was calculated by dividing total feed intake (g) by total egg mass. The hen-day

Table 1. Ingredient and nutrient composition of the basal diet (%)[1].

Ingredient	%	Nutrient composition	
Maize	51.40	Dry matter, %	90.40
Wheat bran	9.00	Crude protein, %	18.00
Soybean meal (44% CP)	22.00	Crude cellulose, %	4.40
Corn meal	2.00	Ether extract, %	5.35
Sunflower meal (45% CP)	4.30	Crude ash, %	10.19
Vegetable oil	3.50	Calcium[3]	2.50
Calcium phosphate	0.88	Phosphorus[3]	0.35
Calcium carbonate	4.50	Sodium[3]	0.18
Limestone	1.43	Lysine[3]	1.00
L-lysine hydrochloride	0.16	Methionine+cystine[3]	0.59
L-treonine	0.12	Threonine[3]	0.76
Sodium bicarbonate	0.16	Tryptophan[3]	0.25
Salt	0.20	ME, kcal/kg[3]	2800
Vitamin-mineral premix[2]	0.35		

[1]Ellagic acid (0.1, 0.2, and 0.4 g ellagic acid per kg diet) was added to the basal diet.
[2]Vitamin-mineral premix (per 1 kg): vitamin A 15.500 IU; vitamin D3 3.500 IU; manganese 120 mg; iron 40 mg; zinc 100 mg; copper 16 mg; cobalt 200 mg; iodine 1.25 mg; selenium 0.30 mg.
[3]Calculated values.

egg production was calculated in percentage by dividing the number of weekly collected eggs by the number of quail. For egg quality parameters, eggs were collected on the 15th, 30th, 45th, 60th, and 75th day of the study. The eggs were kept under room conditions for 1 day. Shells were washed under tap water, left to dry in the air for 24 h, and then weighed. Shell thickness was determined using a digital micrometre (IP54, Qinghai, China). Albumen width, albumen length, albumen height, yolk height, and yolk width were measured using a digital calliper (Tresna, 0–300 mm, Guilin Guanglu Measuring Instrument Co., Ltd., Guilin, China). Haugh units were calculated using the following formula: Haugh unit = 100 log (H + 7.57 – $1.7W^{0.37}$), where H is albumen height in millimetres and W is the observed weight of the egg in grams [20].

2.3. Nutrient digestibility

In the last 7 days of the experiment, 10 animals from each group were taken individually to cages. The lignin indicator method was used for digestibility analysis. Excreta were collected daily during the last 7 days. The excreta samples were dried in an oven at 60 °C for 36–48 h and were then ground for chemical analysis. Lignin (ADL) in the feed and excreta was measured according to the method of Van Soest [21]. The chemical compositions of the feed and excreta samples (dry matter, crude protein, and ether extract) were determined according to the AOAC [17] procedures. A part of nitrogen in excreta originates from uric acid. The fecal nitrogen should be corrected for uric acid nitrogen in order to estimate protein digestibility. The protein content of the excreta samples was corrected based on uric acid as [22]: (% nitrogen of excreta sample–% nitrogen of uric acid in excreta sample) × 6.25. At the end of the experiment, 96 quails (2 per replicate) were randomly slaughtered by decapitation.

2.4. Cecal microbial flora

The ceca of the animals slaughtered at the end of the experiment were cut using a sterile scalpel and forceps, removed, and put into sterile stomacher bags [23]. Violet red bile agar (VRB-A) (Merck KGaA, Darmstadt, Germany) medium was used for total coliform analysis. One gram of cecal content was put into a sterile test tube and 9-mL sterile 0.1% peptone water was added so that a 1/10 dilution could be prepared. After preparing the other decimal dilutions from this predilution, they were inoculated on plates, and VRB-A medium was added to them. After the medium was hardened, a second layer VRB-A medium was added to the plates, and the plates were incubated at 35–37 °C for 24 h. At the end of the incubation process, the red colonies with a 1–2 mm diameter and a precipitate zone around them were evaluated as coliform group bacteria [24]. In the intestine samples obtained from the cecum region, inoculations were performed on the plates from the dilutions (prepared as above mentioned), and de Man Ragosa Sharp agar (MRS) (Merck KGaA) medium was added to the plates for lactic acid bacteria counting. After the inoculated plates were incubated under anaerobic conditions at 37 °C for 48 h, all of the cream colonies of fusiform shape (both ends are spiked) were evaluated and calculated as lactic acid bacteria [25].

2.5. Biochemical parameters

HPLC analysis of MDA was made according to Karatepe [26]. Serum glucose, total protein, cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), calcium, phosphorus, magnesium, and sodium were measured using an autoanalyzer (Olympus AU-600, Olympus Corporation, Tokyo, Japan).

2.6. Statistical analysis

The 2 × 4 factorial design was conducted based on general linear model and 2 environmental temperatures (TN and HS) and 4 diets (0, 100, 200, and 400 mg/kg) were the main determinants. This procedure was applied using the IBM SPSS22 (IBM Corporation, Armonk, NY, USA) package program. The data were presented as the mean and standard error of the mean (SEM) [27].

3. Results

Feed intake was not affected by the ellagic acid level ($P > 0.05$) but decreased in HS groups ($P < 0.001$). The interaction effects of dietary ellagic acid level and environmental temperatures were not significant for feed intake ($P > 0.05$, Table 2).

The egg weight of the HS groups decreased by about 4% compared to the TN groups. The highest egg weight was obtained in the TN groups compared to HS groups ($P < 0.001$, Table 2). The egg weight was not affected by the ellagic acid level or its interaction with environmental temperature ($P > 0.05$). However, egg weight significantly decreased in the HS groups ($P < 0.001$).

Feed conversion improved in quail feeding diets supplemented with ellagic acid (3.11–2.80, Table 2). However, this improvement was statistically insignificant. Feed conversion significantly reduced at high environmental temperature ($P < 0.01$). The interaction between factors (ellagic acid level and environmental temperature) had no effect on feed conversion ($P > 0.05$).

High environmental temperature significantly influenced egg production ($P < 0.001$), which was not affected by ellagic acid level or by the interaction between environmental temperature and ellagic acid level ($P > 0.05$), as shown in Table 2.

There was a significant effect ($P > 0.05$) of dietary ellagic acid levels ($P < 0.05$) and environmental temperature ($P < 0.001$) on eggshell weight, and no significant interaction between these 2 factors ($P > 0.05$, Table 3). The ellagic acid level and environmental temperature or their interaction

Table 2. Effect of ellagic acid on performance of laying quail reared at different temperatures.

		Feed intake (g/quail/day)	Egg weight (g)	Feed conversion ratio (g:g)	Egg production %
TN		29.39	12.06	2.85	85.75
HS		27.38	11.58	3.08	77.66
TN	0	29.19	11.76	3.00	83.08
	100	29.34	12.06	2.84	85.89
	200	29.48	12.19	2.79	86.94
	400	29.57	12.23	2.78	87.08
HS	0	27.12	11.54	3.22	73.35
	100	27.16	11.56	3.14	75.76
	200	27.41	11.60	3.14	76.82
	400	27.83	11.63	2.83	84.70
SEM		0.75	0.12	0.09	0.05
ANOVA		P			
ET		0.001	0.001	0.004	0.001
ELA		0.908	0.123	0.053	0.095
ET×ELA		0.993	0.385	0.516	0.495

TN: thermoneutral; HS: heat stress; ET: environmental temperature; SEM: standard error of mean; ELA: ellagic acid, $P < 0.05$. Feed conversion ratio = (g feed intake/egg production × egg weight). Data are presented as mean and SEM.

Table 3. Effect of ellagic acid on egg quality of laying quail reared at different temperatures.

		Eggshell weight, g	Shell thickness, mm	Yolk index	Albumen index	Haugh units
TN		0.93	0.204	38.52	9.50	86.43
HS		0.90	0.203	38.38	9.42	86.43
TN	0	0.92	0.203	38.42	9.37	86.28
	100	0.92	0.203	38.50	9.49	86.30
	200	0.94	0.204	38.56	9.52	86.54
	400	0.94	0.206	38.58	9.64	86.62
HS	0	0.89	0.202	38.26	9.29	86.23
	100	0.89	0.202	38.32	9.31	86.46
	200	0.90	0.203	38.37	9.46	86.50
	400	0.91	0.205	38.55	9.58	86.52
SEM		0.01	0.13	0.28	0.13	0.27
ANOVA		P				
ET		0.001	0.401	0.497	0.328	0.970
ELA		0.018	0.069	0.888	0.166	0.669
ET×ELA		0.850	0.964	0.993	0.965	0.971

TN: thermoneutral; HS: heat stress; ET: environmental temperature; SEM: standard error of mean; ELA: ellagic acid, $P < 0.05$. Haugh units = $100 \times \log (H + 7.57–1.7 \times W^{0.37})$; H = albumen height, mm; W = egg weight, g. Data are presented as mean and SEM.

did not affect the shell thickness, yolk index, albumen index, or Haugh units (P > 0.05, Table 3).

While the effects of heat stress on digestibility of dry matter (P < 0.05), ether extract (P < 0.01), and crude protein (P < 0.01) were statistically significant, there was no significant interaction between the ellagic acid level and environmental temperature on these parameters (P > 0.05). Ellagic acid level had a significant effect on the digestibility of dry matter (P < 0.05), ether extract (P < 0.01), and crude protein (P < 0.01, Table 4).

As shown in Table 5, the effect of ellagic acid on total coliform and lactic acid bacteria counts in the cecum was statistically significant. There was no significant effect of ellagic acid level and environmental temperature or their interaction on total coliform and lactic acid bacteria counts in the cecum (P > 0.05). The TN groups had lower total coliform bacteria count compared to the HS groups.

The highest serum MDA level was observed in the HS groups (P < 0.001). Supplementation of ellagic acid significantly decreased MDA concentrations in the serum (P < 0.001). The interaction between dietary ellagic acid level and environmental temperature had no significant effect on serum MDA level (P > 0.05, Table 6).

According to Tables 7 and 8, serum concentrations of glucose (P < 0.001), cholesterol (P < 0.05), total protein (P < 0.05), and ALT (P < 0.001) were greater; those of sodium (P < 0.01) and magnesium (P < 0.001) were lower in the HS groups. Ellagic acid level and environmental temperature or their interaction had no significant effect on any of the studied parameters (P > 0.05).

Table 4. Effect of ellagic acid on nutrient digestibility of laying quail reared at different temperatures.

		Dry matter (%)	Ether extract (%)	Crude protein (%)
TN		63.70	88.04	83.47
HS		60.77	84.84	82.16
TN	0	60.37	87.16	82.53
	100	62.01	87.12	82.59
	200	66.11	87.96	83.96
	400	66.32	89.94	84.85
HS	0	59.13	79.56	81.17
	100	61.21	86.34	82.03
	200	61.37	86.36	82.58
	400	61.39	87.08	82.87
SEM		1.55	1.25	0.60
ANOVA		P		
ET		0.015	0.006	0.004
ELA		0.049	0.014	0.009
ET×ELA		0.454	0.145	0.731

TN: thermoneutral; HS: heat stress; ET: environmental temperature; SEM: standard error of mean; ELA: ellagic acid, P < 0.05. Data are presented as mean and SEM.

4. Discussion

When environmental temperature exceeds the normal limits, heat stress develops [28] and negatively affects the health and yield of animals [2–4]. Feed intake, body weight gain, feed conversion [2,3,28], and feed digestibility decrease in poultry under heat stress [28].

When the mean feed intake values of the experimental groups were examined (Table 2), it was found that feed intake in the TN groups was similar to each other and that heat stress significantly decreased feed intake. When the HS groups were compared with the TN groups, a decrease of approximately 7% was observed in their mean feed intake values. The effect of ellagic acid on feed intake was not statistically significant in both the TN and HS groups. The first physiological reaction observed in poultry under heat stress is the reduced feed intake. The metabolic changes observed due to reduced feed intake and stress lead performance parameters to decrease. This may be associated with the fact that the feed intake was lower in the HS groups than in the TN groups. Many studies have revealed that when the environmental temperature exceeds the optimum limits (18–22 °C), feed intake, feed conversion, and performance are negatively affected [2,3].

It was observed in the present study that the average egg weight was lower in the HS groups compared to the TN groups. Lower average egg weight in heat stress groups was caused by low feed intake. However, improvements depending on dose were observed in average egg weight with the supplementation of ellagic acid in both the TN and HS groups, although it was not statistically significant. These improvements were due to the fact that the antioxidant properties of ellagic acid protected cells from oxidative stress and had a positive effect on the yield and performance of the animals and the digestibility of raw nutrients (Table 4). There was a decrease of 10% in egg yield in the HS groups compared to the TN groups. This decrease was considered to be associated with the decrease of feed intake based on heat stress and due to the fact that poultry have problem in balancing their body temperatures. In the TN and HS groups, higher feed conversion ratios were obtained, based on doses related to the supplementation of ellagic acid even if it was not statistically significant. An improvement of 4% was determined in feed conversion with the supplementation of ellagic acid in the TN groups. This improvement, based on the supplementation of ellagic acid, was approximately 12% in the HS groups. The effect of heat stress on the feed conversion ratio was

Table 5. Effect of ellagic acid on cecal bacteria populations of laying quail reared at different temperatures.

		Coliform (log_{10} CFU/g)	Lactobacilli spp. (log_{10} CFU/g)
TN		2.69	7.98
HS		2.86	7.92
TN	0	3.48	7.49
	100	2.36	7.95
	200	2.28	8.18
	400	2.41	8.28
HS	0	3.27	7.29
	100	2.77	8.12
	200	2.75	8.14
	400	2.69	8.26
SEM		0.23	0.16
ANOVA		P	
ET		0.235	0.858
ELA		0.007	0.001
ET×ELA		0.600	0.801

TN: thermoneutral; HS: heat stress; ET: environmental temperature; SEM: standard error of mean; ELA: ellagic acid, $P < 0.05$. Data are presented as mean and SEM.

Table 6. Effect of ellagic acid on serum concentration of malondialdehyde (MDA) of laying quail reared at different temperatures.

		MDA (µmol/L)
TN		1.131
HS		2.030
TN	0	1.270
	100	1.186
	200	1.071
	400	0.998
HS	0	2.547
	100	2.113
	200	1.810
	400	1.648
SEM		0.404
ANOVA		P
ET		0.001
ELA		0.001
ET×ELA		0.060

TN: thermoneutral; HS: heat stress; ET: environmental temperature; SEM: standard error of mean; ELA: ellagic acid; MDA: malondialdehyde, $P < 0.05$. Data are presented as mean and SEM.

statistically significant. When compared to the HS groups, there was an improvement of 8% in feed conversion ratio in the TN groups. These improvements, obtained with the supplementation of ellagic acid, may be associated with the fact that pomegranate-based products increase the activity of digestion enzymes in the intestines, the digestibility of nutrients, and the absorption levels of small intestine [29,30]. Similarly, Atılgan [29] reported that the supplementation of pomegranate peel extract of 100 and 200 ppm, as the source of proanthocyanidin to ration improved the feed conversion ratio of broilers, significantly compared to the ones fed by control ration. The results of the present study are compatible with the study findings reporting that high environmental temperatures decrease feed conversion significantly in laying quails under heat stress [1]. This effect may be caused by the fact that metabolic changes occur due to heat stress. Heat stress decreases feed intake in poultry in order to control the temperature increase caused by the metabolism. Accordingly, as noted earlier, high environmental temperature causes oxidative stress in animals. As a result, stress affects yield negatively, resulting in causes performance loss [2,31]. Also, high environmental temperature leads to an increase in glucocorticoid concentrations, mainly in cortisol and thus an increase in catabolic effect, a decrease in synthesis activities and, finally impaired yield and performance [32,33].

It was found that the effect of both heat stress and ellagic acid on eggshell weight was statistically significant, and their effects on shell thickness, yolk-albumen index, and Haugh units were not statistically significant (Table 3). Eggshell weight decreased with increasing environmental temperature and supplementation of ellagic acid increased eggshell weight based on dose. This positive effect was associated with feed intake differences between the groups as well as the positive effect of ellagic acid on digestion (Table 4) and metabolism. Similarly, Yassein et al. [34] reported that supplementation of 10 and 15 g/kg pomegranate peel powder into ration increased eggshell weight significantly in experimental groups, and Saki et al. [35] reported that supplementation of pomegranate seed pulp into ration in different doses (0, 5, 10, and 15%) did not affect egg weight, eggshell weight, and Haugh units in laying hens, and this may be associated with the fact that the test rations were balanced and the total calcium, phosphorus, and vitamin D3 contents were similar.

It was observed in the study that heat stress decreased the digestibility of nutrients and that the supplementation

Table 7. Effect of ellagic acid on serum concentrations of glucose, cholesterol, total protein, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) of laying quail reared at different temperatures.

		Glucose (mg/dL)	Cholesterol (mg/dL)	Total protein (g/dL)	AST (U/L)	ALT (U/L)
TN		257.75	141.19	3.33	219.11	5.41
HS		300.88	155.26	3.56	229.20	7.05
TN	0	269.67	142.18	3.40	228.50	5.67
	100	267.58	141.58	3.32	223.17	5.40
	200	249.92	141.44	3.29	209.92	5.42
	400	243.83	139.40	3.30	215.90	5.20
HS	0	317.08	160.91	3.65	245.88	7.55
	100	310.83	154.55	3.57	237.00	7.30
	200	295.00	153.55	3.51	221.82	7.00
	400	280.58	151.70	3.52	219.00	6.22
SEM		12.12	8.70	0.14	10.05	0.37
ANOVA		P				
ET		0.001	0.030	0.024	0.128	0.001
ELA		0.058	0.925	0.809	0.153	0.151
ET×ELA		0.979	0.979	0.999	0.921	0.659

TN: thermoneutral; HS: heat stress; ET: environmental temperature; SEM: standard error of mean; ELA: ellagic acid, $P < 0.05$. Data are presented as mean and SEM.

of ellagic acid provided improvements of different levels in both groups based on dose (Table 4). An improvement of 3% was observed in the digestibility of dry matter in the TN100 group compared to the TN0 group, in which no ellagic acid was added, and this improvement reached 9%–10% in the TN200 and TN400 groups. Similarly, improvements of 3.52%, 3.79%, and 3.82% were observed in the digestibility of dry matter in the HS100, HS200, and HS400 groups, respectively, compared to the SS0 group. An improvement of about 3% was observed in the digestibility of ether extract in the TN400 group, compared to TN0 group; on the other hand, improvements of 8.5%, 8.5%, and 9.4% were observed in the digestibility of ether extract in the HS100, HS200, and HS400 groups, compared to the HS0 group. There was an improvement of 1.59% in the digestibility of crude protein in the TN groups compared to the HS groups. As was seen, the digestibility of nutrients decreased in heat stress, but this decrease remained limited based on dose upon the supplementation of ellagic acid. Under normal environmental conditions, the supplementation of ellagic acid increased the digestibility of nutrients based on dose. The low digestibility of nutrients in heat stress may be caused by the decrease in feed intake. Also, improvements and increases in both groups may be associated with the positive biological effects of ellagic acid. As is known, high environmental temperature causes reactive oxygen species to generate in high amounts by resulting in oxidative stress. Reactive oxygen species kill the biological molecules in cells, cause oxidation in biomacromolecules, and lead to various impairments in tissues. These factors may be the cause of intestinal function disorder and, therefore, decrease the digestibility of nutrients. Moreover, the decreased digestibility of nutrients is one of the negative effects of high environmental temperature. In a previous study, it was reported that ellagic acid had a strong in vitro oxidant capacity in oxidative stress-induced mice and a protective effect against in vivo oxidative damage, especially intestine damage. It was also effective in protecting intestine mucosal morphology and inhibiting the expression of intestinal proinflammatory factors [10]. This may be associated with the high accumulation level of ellagic acid in intestines and its superior skill of recovering intestine damage. In addition, it was stated that the accumulation of ellagic acid in the intestine epithelium may generate a first antioxidant barrier against harmful oxidation products and, thus, the entrance of harmful oxidation products into the blood circulatory system; other tissues may also be

Table 8. Effect of ellagic acid on serum concentrations of calcium, phosphorus, sodium, and magnesium of laying quail reared at different temperatures.

		Calcium (mg/dL)	Phosphorus (mg/dL)	Sodium (mEq/dL)	Magnesium (mg/dL)
TN		18.55	8.31	150.19	5.30
HS		17.39	7.31	146.40	4.53
TN	0	18.88	8.45	148.25	5.48
	100	18.06	8.74	149.58	5.37
	200	18.84	7.62	151.58	5.20
	400	18.41	8.48	151.33	5.15
HS	0	16.42	6.52	145.92	4.14
	100	17.81	6.69	147.83	4.72
	200	18.22	7.65	145.08	4.58
	400	17.11	8.34	146.75	4.69
SEM		1.42	0.91	1.67	0.28
ANOVA		P			
ET		0.271	0.128	0.002	0.001
ELA		0.933	0.760	0.685	0.882
ET×ELA		0.884	0.551	0.494	0.472

TN: thermoneutral; HS: heat stress; ET: environmental temperature; SEM: standard error of mean; ELA: ellagic acid, $P < 0.05$. Data are presented as mean and SEM.

protected [10]. In the present study, it was thought that supplementation of ellagic acid into ration may contribute to the increase of the digestibility of nutrients due to its antioxidant properties (Table 6). Accordingly, numerous studies have reported that antioxidant substances affect the digestibility of nutrients positively [36–38]. In many other studies conducted using similar approaches, it has been stated that herbal active compounds stimulate the digestive enzymes released by the pancreas and intestine mucosa [39], increase the pancreatic digestive enzymes such as amylase, lipase, trypsin, and chymotrypsin [40], and increase the digestibility of ether extract by increasing the secretion of the gall bladder [41]. The improvements in the digestibility of nutrients determined in the present study may be associated with the similar effects of ellagic acid that have not been determined. As is seen in Table 5, ellagic acid had an antibacterial effect on the intestines. Ellagic acid may contribute positively to the digestibility of nutrients by decreasing the count of pathogenic coliform bacteria and increasing the count of lactic acid bacteria that provide a positive contribution to digestion.

When the effects of environmental temperature and ellagic acid on the total coliform bacteria count in the cecum were examined (Table 5), it was observed that the supplementation of ellagic acid decreased significantly the coliform bacteria count of cecum in both groups based on doses. Many studies have reported that pomegranate byproducts (extract or powder) have an antibacterial activity against both gram-positive and gram-negative bacteria [29,42,43], which supports the results of the present study. The antimicrobial effect of pomegranate byproducts is believed to be caused by the high amounts of polyphenols, flavonoids, and hydrolysable tannins they contain [44]. It has been stated that polyphenolic compounds may have bactericidal or bacteriostatic properties because they prevent the adherence of pathogenic bacteria to small intestine mucosa [45]. Also, as seen in Table 5, in both groups, ellagic acid decreased the coliform bacteria count and increased the lactic acid bacteria count in the cecum, both of which are beneficial for intestine health and digestion. In the study conducted by Atılgan [29], by adding pomegranate peel extract as a proanthocyanidin source in different doses (0, 100, and 200 ppm) into broiler rations, it was reported that 100 and 200 ppm of pomegranate peel extract significantly increased the ileum lactobacillus count of broilers. In another study, it was stated that pomegranate extract and pomegranate juice prevented the development of *Bacteroides fragilis*, *Clostridia*, and *Enterobacteriaceae* bacteria and increased the development of *bifidobacteria* and *lactobacillus* after their use in vitro stool cultures. Pomegranate may be considered to be prebiotic due to

its effect on the beneficial bacteria [14]. It was stated that the positive effects of polyphenolic compounds on the growth of beneficial bacteria were associated with the fact that these polyphenolics are used by beneficial bacteria as nutrients and especially as source of energy due to their being metabolized [46].

When the serum MDA levels were examined in the experimental groups (Table 6), they were found to be higher in the HS groups (approximately 30%). The high MDA level in the HS groups may be associated with high lipid peroxidation. However, this increase decreased significantly depending on the dose of ellagic acid. The fact that an increasing ellagic acid dose and serum MDA level had a significant tendency to decrease may be due to the strong antioxidant characteristic of ellagic acid [10]. It was stated in a previous study on quail that oxidative stress increased the MDA amount [47], which supports the results of the present study. In the study conducted by Özkaya [48], it was reported that ellagic acid decreased lipid peroxidation. Also, in the study conducted by Yüce et al. [49] on rats, the authors determined that the supplementation of 10 mg/kg ellagic acid significantly decreased the MDA level in liver and heart tissues compared to the control group; these researchers suggested that ellagic acid prevented the formation of free radicals and decreased lipid peroxidation by preventing the damage of oxidative DNA. It was stated in another study that ellagic acid had a strong in vitro oxidant capacity in mice in which oxidative stress was induced with oxidized fish oil. It also produced a protective effect against in vivo oxidative damage, especially intestinal damage [34]. In their study, Zeweil et al. [50] reported that supplementation of pomegranate peel at different levels (1.5, 3, and 4.5%) into ration decreased the lipid peroxide level significantly. The fact that pomegranate peel decreased MDA level was considered to be associated with ellagitannins, which are the primary substances of ellagic acid found in pomegranate peel that contain antioxidant properties [11]. Althunibat et al. [51] revealed that pomegranate peel extract caused a significant decrease in MDA levels and had a protective role against oxidative damage. Similarly, Saki et al. [35] indicated that pomegranate seed pulp caused a significant decrease in MDA levels and had a protective role against oxidative damage.

It was observed that serum glucose levels were within normal limits in the TN and HS groups, but they were higher in the HS groups. In both the TN and HS groups, serum glucose levels had a tendency to decrease, depending on the dose of ellagic acid. When compared to organism stress factors, in the alarm period, catecholamines are released in the sympathetic nervous system, and the relevant hormones are released by the adrenal medulla. As a result of these changes, glucose is activated by the body reserves, and animals try to eliminate the effect of stress by means of the rapid energy provided in this way. After the alarm period, blood glucose, one of the body reserves, is regulated through a gluconeogenesis event by releasing adrenocortical hormones. The increased blood glucose level seen in poultry in the heat stress situations may be associated with this mechanism [52]. The increased blood glucose level is accepted as an indicator of the formation of heat stress [53]. This mechanism may have been the cause of the high serum glucose level in the HS groups. While Ciftci et al. [54] determined that serum glucose level was statistically significantly high in quails under heat stress, Arslan [55] found that the serum glucose level was statistically significantly high due to the stress caused by stocking density. Yassein et al. [34] reported that pomegranate peel extract decreased blood glucose level. On the other hand, in a previous study conducted in dairy cattle, it was reported that the blood glucose level was not affected after addition of 0, 400, 800, and 1200 mL/day pomegranate peel extract into ration for 28 days [56].

It was observed that supplementation of ellagic acid had a hypocholesterolemic effect in the TN and HS groups, and this effect increased depending on its dose, even though it was not statistically significant. Previous studies revealed that ellagic acid and pomegranate [34,57] products had a hypocholesterolemic effect. The hypocholesterolemic effect of pomegranate products may be associated with the polyphenols they contain because the polyphenolic compounds decrease the absorption of cholesterol and increase the amount of cholesterol removed via stool. Also, polyphenolic compounds have a preventive effect for the synthesis of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase and sterol-O-acyltransferase, which have an important role in cholesterol mechanism [29].

Heat stress significantly increased serum total protein level, and the highest value was found in the HS group, in which no ellagic acid was added. Supplementation of ellagic acid into ration did not statistically affect serum total protein level but decreased the total protein value numerically. It has been reported that the level of serum total protein, occurring as a result of protein metabolism, is important in protecting the immune system, and serum total protein level increases under stress [51]. This explains the high serum total protein level in the HS groups.

When Table 7 is examined, it can be determined that heat stress increased ALT levels significantly and did not affect the AST level. The effect of ellagic acid supplementation on ALT and AST levels was not statistically significant. However, serum ALT and serum AST values decreased, depending on the dose of ellagic acid, even though they were not statistically significant. Stress increases plasma ALT and AST levels. It has been reported that the level of these enzymes in the blood increases as a result of

the damage occurring in the liver due to oxidative stress [58]. It was determined in previous studies that different pomegranate extracts significantly decreased the increases in ALT and AST levels during liver damage [34,59].

When the mineral substance levels in the groups were examined (Table 8), it was observed that the serum calcium, phosphorus, sodium, and magnesium levels decreased due to heat stress. This decrease in calcium and phosphorus was not statistically significant. On the other hand, the decreases in sodium and magnesium were statistically significant. Supplementation of ellagic acid into ration did not affect serum mineral levels in a statistically significant manner. Furthermore, serum mineral levels were not affected by the interaction between environmental temperature and supplementation of ellagic acid. The decreased mineral substance level in the blood of animals under heat stress may be caused by the decrease of feed intake (Table 2), and the decreased digestibility of nutrients (Table 4) could be due to heat stress. Accordingly, it has been emphasised that the decrease of the absorption components in the duodenum in animals under heat stress is effective in the decrease of the blood ionized calcium level [60].

5. Conclusion

Consequently, it was determined that supplementation of ellagic acid did not affect feed intake but that it increased nutrient digestibility, decreased total coliform bacteria count in the cecum, had a positive effect on lactic acid bacteria (which are among the beneficial bacteria) and decreased serum MDA levels due to its antioxidant properties. Also, ellagic acid did not have a negative effect on quails in terms of the doses used. The positive effects of ellagic acid increased depending on dose, and the 400-mg/kg dose was the most effective one. Moreover, when balanced rations and optimum hygienic conditions were provided, ellagic acid revealed its other biological effects, mainly its performance parameters.

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