The effects of *Pinus pinaster* extract supplementation in low protein broiler diets on performance, some blood and antioxidant parameters, and intestinal histomorphology

Gonca ÖZTAP[1,a,✉], Seher KÜÇÜKERSAN[2,b]

[1]Ministry of Agriculture and Forestry, Ankara, Türkiye; [2]Ankara University, Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Diseases, Ankara, Türkiye

[a]ORCID: 0000-0003-2982-7055; [b]ORCID: 0000-0002-2882-9203

ARTICLE INFO

Article History
Received : 10.08.2021
Accepted : 05.05.2022
DOI: 10.33988/auvfd.981159

Keywords
Broiler
Encapsulation
Low protein
Pinus pinaster extract
Performance

✉Corresponding author
goncaoztap@gmail.com

How to cite this article: Öztap G, Küçükersan S (2023): The effects of *Pinus pinaster* extract supplementation in low protein broiler diets on performance, some blood and antioxidant parameters, and intestinal histomorphology. Ankara Univ Vet Fak Derg, 70 (3), 267-276. DOI: 10.33988/auvfd.981159.

ABSTRACT

This study was conducted to investigate the effects of *Pinus pinaster* extract (PPE) and encapsulated *Pinus pinaster* extract (EPPE) supplementation in normal and low protein broiler diets on performance, some blood and antioxidant parameters, and intestinal histomorphology. In the present study, PPE was covered with alginate in order to obtain EPPE. The present research was conducted during 41-days with 288 one-day-old male broiler chicks. Chicks were classified into two groups that had different protein levels, one of with normal, the other one with 10% low protein. Also, normal and low protein level groups were divided into one control and two trial groups. The amount of 100 mg/kg PPE was added to each trial group diet; and the same amount of EPPE was added to other trial group diets. Consequently, compared to PPE and EPPE groups with control, statistically significant differences were observed for body weight and body weight gain in terms of protein on 41-day results ($P<0.05$). For feed conversation ratio, better results were detected in PPE groups at 41 days ($P<0.05$). Besides that, statistically significant differences were found in breast meat thiobarbituric acid reactive substances (TBARS) values and intestinal histomorphology in PPE and EPPE groups compared to the control groups ($P<0.05$). In this study, the findings suggest that 100 mg/kg PPE and EPPE can be supplemented in normal and low protein broiler diets without any adverse effects and considered alternative aromatic substances for broiler rations.

Introduction

A standardised extract of *Pinus pinaster* is called Pycnogenol® most common commercially extract composed of a mixture of flavonoids (26). The chemical composition of Pycnogenol® mainly contains procyanidins and phenolic acids. The phenolic acids are derivatives of benzoic and cinnamic acids. Procyanidins, biopolymers of catechin and epicatechin, are composed of 70 -/+5 % of the content of Pycnogenol® (25, 32). It has been reported that Pycnogenol® shows antioxidant activity thanks to its rich natural polyphenols composition. Also, it has stated antimutagenic, anticarcinogenic effects, cardiovascular benefits, and enhancing microcirculation. Thus, it has been set out the protective role of Pycnogenol® against many diseases associated with oxidative stress (9, 18).

The proteins are one of the basic nutrients in the diet and deficiency, excess, or lack of a certain ratio with other basic nutrients in the ration of proteins are among the factors that may cause stress for poultry. In the case of low protein in the diets, the energy-protein balance is disrupted and this causes stress in poultry. Oxidative stress may lead to degenerative disorders, loss of performance and decreases in product quality. However, it is possible to reduce or eliminate these negativities with the addition of natural or synthetic antioxidant substances into the poultry rations for economic poultry nutrition (8, 19, 34). In addition, PPE with its antioxidant properties can be

supplemented as one of the antioxidant substances in this case.

Encapsulation is a technique preferred more commonly in recent times to preserve or stabilize of contents of active compounds for coating them with one or more other substances (17). For encapsulation, various types of polysaccharide-protein hydrogel carriers are used. One of the carriers is alginate obtained from brown algae (6). Coating with alginate is a commonly used encapsulation of microorganisms, enzymes, drugs, oils, and aromatic substances (7). Encapsulation of aromatic extracts allows to maintain the stability of phenolic contents, increase the bioavailability of compounds, and mask the strong odour and taste of aromatic extracts (20).

This study was conducted to investigate the effects of Turkish PPE and EPPE supplementation on broiler diets and present an alternative natural product to synthetic ones in animal nutrition. Additionally, in this study, Turkish PPE was coated with alginate for the first time in Türkiye to be used in animal feeding and evaluate the encapsulation of PPE.

Materials and Methods

Birds husbandry and diets: The duration of the present research was 41 days with 288 one-day-old male Ross 308 broiler chicks (initial weight 41.4 ± 0.05 g). Chicks were divided into six groups and then each group was divided into six subgroups that contained eight chicks. Besides, normal (23; 21.5; 19.5%) and low protein (20.7; 19.4; 17.5%) level groups were divided into one control and two trial groups. To conduct this study, ethics committee approval was taken, which was 2017-22-178 number from Ankara University Animal Experimental Local Ethics Committee. Ad-libitum feeding was applied to control and trial groups' diets during the trial, and the water requirements of chicks were met as ad-libitum.

The basal diets were formulated in normal and low protein groups at starter, grower and finisher periods, as shown in Table 1. While the control groups were fed only a basal diet, the amount of 100 mg/kg PPE, one of as normal and one of as coated, was added to the basal diets of the trial groups. EPPE was coated form of PPE, was obtained by encapsulation of PPE with alginate.

Table 1. Ingredients, chemical composition, and metabolic energy value of basal diets in normal and low protein groups at starter, grower and finisher periods.

Ingredients (%)	Normal Protein Group			Low Protein Group		
	Starter (0-10 days)	Grower (11-24 days)	Finisher (25-41 days)	Starter (0-10 days)	Grower (11-24 days)	Finisher (25-41 days)
Corn	51.02	47	50	56.40	50	52
Full Fat Soybean	18	16	15	13	12.2	12
Vegetable Oil	1	2	3	1	2.2	2.5
Soybean Meal	24.97	22.7	18.5	24.4	19	14
Wheat	0	8	9.3	0	12.05	15.1
Monocalcium Phosphate	2	1.7	1.6	2	1.7	1.6
Limestone	1.8	1.55	1.6	1.8	1.55	1.5
Sodium bicarbonate	0.1	0.1	0.1	0.1	0.1	0.1
Salt	0.3	0.3	0.3	0.3	0.3	0.3
DL-Methionine	0.3	0.2	0.2	0.34	0.25	0.25
L-Lysine	0.2	0.15	0.1	0.35	0.35	0.35
Vitamin Premix*	0.15	0.20	0.2	0.15	0.20	0.2
Mineral Premix**	0.1	0.10	0.1	0.1	0.10	0.1
Anticoccidial	0.06	0.0	0.0	0.06	0.0	0.0
Total	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Chemical Composition (Analyzed)						
Crude Protein, (%)	22.65	21.21	19.83	20.65	19.52	17.50
Metabolizable Energy***(kcal/kg)	3018	3101	3218	3000	3127	3211
Crude Fiber,(%)	7.19	6.59	6.07	6.33	5.45	5.25
Calcium, (%)	1.40	0.98	0.97	1.12	0.96	0.92
Phosphorus, (%)	0.65	0.50	0.47	0.57	0.50	0.46

*Vitamin premix in per kg of diets: Vitamin A: 11.000 IU, Vitamin D_3: 3.500 IU, Vitamin E: 100 mg, Vitamin K_3: 3 mg, Vitamin B_1: 3 mg, B_2: 6 mg, B_6: 4 mg, B_{12}: 0.02 mg, Niacin: 35 mg, Folic acid:1.5 mg, Vitamin H: 0.2 mg, Vitamin B_5: 15 mg.
**Mineral premix in per kg of diets: Manganese: 120 mg, Zinc:110 mg, Copper: 30 mg, Iron: 50 mg, Cobalt:0.5 mg, Iodine: 2 mg, Selenium:0.3 mg.
*** Metabolizable energy content of diets was calculated according to the equation of Carpenter and Clegg (5).

DOI: 10.33988/auvfd.981159

For the experimental design, it is used a completely randomized design with 2x3 factorial arrangement of dietary protein level (normal and low) and PPE (no supplementation, PPE and EPPE).

***Encapsulation of pinus pinaster extract*:** PPE was encapsulated using Encapsulator Device (BUCHI B-390). After determining the best conditions for the encapsulation process, 1.5% powder of PPE was mixed and homogenized with 1% alginate solution. $CaCl_2$ was prepared as 1.7 M. After it was determined that the best option of the nozzle, frequency, bar pressure, and volt flow for this device, the mixture was passed through the encapsulator device, and sprayed into the $CaCl_2$ solution. When $CaCl_2$ solution was filtered, PPE microcapsules were obtained. These microcapsules were washed with deionized water and then dried.

Traits measured: During the trial body weight (BW) and feed intake (FI) for every chick were measured on 0, 10, 21, 31, and 41 days of the trial. Feed conservation ratio (FCR) was calculated as the amount of feed intake per kg body weight gain (BWG). At the end of the trial, immediately after slaughtering, carcass weight was measured. Carcass yield (%) was determined at the end of the trial with this formula. Carcass yield (%)= (body weight (g) on 41st day/carcass weight (g) on 41st day) X100. European Production Efficiency Factor (EPEF) was calculated per pen at 41 days with this formula. EPEF, %= [(Body weight (kg))* (Number of total alive chick/number of total chick at initial)*100)]/[(Trial period (day))*FCR)*100)].

***Sampling*:** On the 41st day of the trial, two birds per pen were randomly selected and slaughtered. Meanwhile, the slaughtering 12 blood samples were collected from each of the groups.

Measurement of biochemical parameters in serum samples: HDL (high-density lipoproteins), LDL (low-density lipoproteins), triglyceride, and total cholesterol levels were measured in serum samples collected at the end of the trial by an auto-analyzer (BT 3000, Biotechnica Instruments, Italy) using Commercial kits of Randox RX series (Randox Laboratories Ltd., London, United Kingdom).

Determination of breast meat malondialdehyde (MDA) value: Two pieces of breast meat samples were taken at slaughtering from each animal. Then TBARS method was used to determine the lipid oxidation level. In the beginning of MDA analysis, 10 gr of breast meats were taken and then 97.5 ml distilled water was added on each of them and mixtures were homogenized with ultra turrax. The mixtures were poured into Kjeldahl tubes then 2 ml (6N) HCl added and distilled. It was taken 5 ml from distillate and 0.02 M, 2-thiobarbütirik asit (TBA) added equal amount on distillate. The mixture was incubated in boiling water for 35 minutes.The absorbance was measured at 530 nm with a spectrophotometer (Shimadzu UV-1208). For calculation of TBARS values, the absorbance values were multiplied by K=7.8 and calculated in mg malondialdehyde/kg. It was calculated that TBARS values for samples stored at different time intervals were stored at + 4 ^{o}C for one day and three days after slaughtering (29).

Intestinal histomorphology: On the 21st and 41st day of trial, while slaughtering was carried out, intestinal samples were taken for the groups and preparations were prepared. In the preparation, villus height (VH) from the villus tip to bottom and crypt deep (CD) from villus bottom to the crypt were measured, then the ratio of villus height to crypt deep (VH/CD) was calculated. For each intestine section, 10 villi and crypts were measured using the camera system and Cellsens CS-ST-V1.8 standard software (3, 16).

Statistical analysis: All data were analyzed with Shapiro-Wilk and Levene Statistical tests. According to the results of these tests, a two-way analysis of Variance (ANOVA) statistical test was performed to detect the differences between average values of groups. In addition, to distinguish the significance of the differences between groups, the Tukey multiple range test was carried out. The statistical results were evaluated on the 95% confidence interval. The SPSS 22.0 (SPSS Inc., Armonk, NY) software was used.

Results

The growth performance values of groups are presented in Table 2. As shown in Table 2 for BWG, statistically significant differences were found between the groups in terms of protein at 11-21, 0-21, 22-41, and 0-41 days. In terms of PPE, it was determined that the increases between the 11-21 and 0-21 days also in these periods, the protein and PPE interaction values (Protein X PPE) were statistically significant ($P<0.05$). For feed intake values of groups, there were statistically significant differences in terms of protein between 22-31 and 0-41 days ($P<0.05$), no statistically significant differences were observed in terms of PPE for all the periods ($P>0.05$). Additionally, Protein X PPE values were not statistically significant either ($P>0.05$). For feed conversion rates of the groups, the differences were statistically significant in terms of protein for days 22-41 and 0-41 ($P<0.05$) and also it was determined significant differences in terms of PPE for 11-21, 0-21, and 0-41 days ($P<0.05$). However, no statistically significant values were observed in terms of the protein and PPE interaction for all the periods ($P>0.05$).

DOI: 10.33988/auvfd.981159

DOI: 10.33988/auvfd.981159

Table 2. Body weight gains, feed intakes, and feed conversion rate values of the group.

Dietary Treatments		Body Weight Gain (g)					Feed Intake (g)					Feed Conversation Ratio (kg feed/kg weight gain)				
		Days														
		0-10	11-21	22-41	0-21	0-41	0-10	11-21	22-41	0-21	0-41	0-10	11-21	22-41	0-21	0-41
NP-C		185.48	591.60^{bc}	2155.13	777.08^{bc}	2932.21	261.86	1033.67	3434.80	1295.53	4730.34	1.41	1.75	1.60	1.67	1.62
NP-PPE		192.46	632.39^{b}	2183.40	824.85^{ab}	3008.25	278.12	1026.52	3295.98	1304.64	4600.62	1.45	1.63	1.51	1.58	1.53
NP-EPPE		189.89	675.35^{a}	2096.43	865.24^{a}	2961.67	285.39	1028.12	3304.85	1313.51	4618.36	1.51	1.52	1.59	1.52	1.56
LP-C		184.67	574.23^{c}	1802.41	758.89^{c}	2561.31	276.28	1004.76	3156.95	1281.05	4438.01	1.50	1.76	1.77	1.69	1.74
LP-PPE		187.92	611.41^{bc}	2015.78	799.33^{bc}	2815.11	269.88	1001.14	3235.77	1271.02	4506.78	1.44	1.64	1.61	1.59	1.60
LP-EPPE		186.40	588.83^{bc}	2010.21	775.23^{bc}	2785.44	268.21	954.79	3265.27	1223.00	4488.27	1.44	1.62	1.63	1.58	1.61
Protein	**Normal**	189.27	2144.99^{A}	2144.9^{A}	822.39^{A}	2967.38^{A}	275.46	1029.43	3345.22	1304.56	4649.7^{A}	1.45	1.63	1.56^{A}	1.59	1.57^{A}
	Low	186.33	1942.80^{B}	1942.80^{B}	777.82^{B}	2720.62^{B}	271.46	986.90	3219.33	1258.36	4477.6^{B}	1.46	1.67	1.67^{B}	1.62	1.65^{B}
PPE	**NI**	185.07	582.91^{y}	1978.77	767.98^{y}	2746.76	269.08	1019.21	3295.88	1288.29	4584.17	1.46	1.75^{x}	1.68	1.68^{x}	1.68^{x}
	PPE	190.19	621.90^{x}	2099.59	812.09^{x}	2911.68	274.00	1013.83	3265.88	1287.83	4553.70	1.44	1.63^{xy}	1.56	1.59^{xy}	1.57^{y}
	EPPE	188.15	632.09^{x}	2053.31	820.23^{x}	2873.56	276.80	991.45	3285.06	1268.26	4553.32	1.47	1.57^{y}	1.61	1.55^{y}	1.59^{xy}
SEM		2.29	4.83	28.07	6.082	29.97	3.02	11.64	35.45	12.72	42.10	0.01	0.02	0.02	0.02	0.02
		Significance (P-value)														
Protein		0.52	< 0.001	0.001	0.001	0.001	0.548	0.078	0.086	0.079	0.049	0.87	0.351	0.03	0.36	0.02
PPE		0.66	0.001	0.224	0.003	0.079	0.577	0.592	0.941	0.766	0.943	0.71	0.004	0.09	0.008	0.03
Protein X PPE		0.94	0.010	0.157	0.042	0.353	0.106	0.649	0.328	0.456	0.597	0.16	0.59	0.49	0.81	0.63

NP-C: Normal Protein Control, NP-PPE: Normal Protein *Pinus pinaster* extract, NP-EPPE: Normal Protein Encapsulated *Pinus pinaster* extract, LP-C: Low Protein Control, LP-PPE: Low Protein *Pinus pinaster* extract, LP-EPPE: Low Protein Encapsulated *Pinus pinaster* extract, NI: Not included
SEM: Standard error of the mean.
a,b,c: Mean values within the same column carrying different superscripts are significantly different at P<0.05.
x,y: Mean values within the same column carrying different superscripts are significantly different at P<0.05.
A,B: Mean values within the same column carrying different superscripts are significantly different at P<0.05.

For carcass yield and EPEF, it was shown numerically better values PPE and EPPE groups than control both normal and low protein groups, respectively (Table 3). However, there were no statistically important differences in PPE and EPPE groups (P>0.05).

For biochemical parameters in serum samples, the findings showed that there were no significant differences between the groups' values of HDL, LDL, total cholesterol, and triglycerides (P>0.05) in terms of PPE. However, it was shown a certain significant decrease (P <0.05) in LDL levels in low protein groups (Table 4). But there were no statistically significant values were shown in terms of the protein and PPE interaction (P>0.05).

Table 3. Carcass yield and European Production Efficiency Factor (EPEF) values of the groups.

Dietary Treatments		Carcass yield (%)	EPEF
NP-C		72.49	444.62
NP-PPE		72.96	482.79
NP-EPPE		73.20	467.87
LP-C		71.84	361.05
LP-PPE		73.53	433.93
LP-EPPE		74.24	426.78
Protein	**Normal**	73.20	465.09
	Low	72.82	407.25
PPE	**NI**	72.16	402.83
	PPE	73.25	458.36
	EPPE	73.62	447.32
SEM		0.25	0.001
		Significance (P-value)	
Protein		0.442	1.0
PPE		0.063	1.0
Protein X PPE		0.31	1.0

NP-C: Normal Protein Control, NP-PPE: Normal Protein *Pinus pinaster* extract, NP-EPPE: Normal Protein Encapsulated *Pinus pinaster* extract, LP-C: Low Protein Control, LP-PPE: Low Protein *Pinus pinaster* extract, LP-EPPE: Low Protein Encapsulated *Pinus pinaster* extract, NI: Not included, SEM: Standard error of the mean.
The differences between the mean values of the groups are not statistically significant (P>0.05).

Table 4. Levels of some biochemical parameters in blood serum and plasma samples of the groups.

Dietary Treatments		HDL (mg/dl)	LDL (mg/dl)	Triglyceride (mg/dl)	Total cholesterol (mg/dl)
NP-C		69.80	65.26	44.25	60.67
NP-PPE		86.52	74.25	52.25	69.17
NP-EPPE		75.07	61.11	50.67	59.58
LP-C		79.40	54.19	50.17	65.92
LP-PPE		71.45	40.06	48.83	58.75
LP-EPPE		71.58	41.05	51.08	59.92
Protein	**Normal**	77.12	66.88^{A}	49.06	63.14
	Low	74.14	45.10^{B}	50.03	61.53
PPE	**NI**	74.58	59.73	47.21	63.29
	PPE	78.99	57.16	50.54	63.96
	EPPE	73.32	51.08	50.87	59.75
SEM		2.15	2.22	0.94	1.7
			Significance (P-value)		
Protein		0.49	0.001	0.61	0.64
PPE		0.53	0.28	0.23	0.56
Protein X PPE		0.08	0.12	0.14	0.17

NP-C: Normal Protein Control. NP-PPE: Normal Protein *Pinus pinaster* extract, NP-EPPE: Normal Protein Encapsulated *Pinus pinaster* extract, LP-C: Low Protein Control, LP-PPE: Low Protein *Pinus pinaster* extract, LP-EPPE: Low Protein Encapsulated *Pinus pinaster* extract, NI: Not included, SEM: Standard error of the mean.
The differences between the mean values of the groups are not statistically significant (P>0.05).
A,B: Mean values within the same column carrying different superscripts are significantly different at P<0.05.

As shown in Table 5, there were no significant differences in breast meat MDA level on the 1st-day results, but on the 3rd day's results, statistically significant decreases were found in terms of protein, PPE, and interaction value (Protein X PPE) (P<0.05). Besides this, groups of EPPE levels were determined numerically lower than PPE groups (Table 5). Also, it was detected the lowest MDA level on the 3rd day's results in normal protein encapsulated *Pinus pinaster* extract group (NP-EPPE).

As shown in Table 6, on the 21st day, while it was found statistically significant differences for jejunum VH and CD in terms of protein and PPE (P<0.05), there were no statistically significant differences in ileum values (P>0.05). Additionally, Protein X PPE values were not statistically significant (P>0.05) except the value of jejunum CD (P<0.05).

As shown in Table 7, on the 41st day, while it was observed statistically significant differences for jejunum VH in terms of PPE (P<0.05), there were detected statistically significant differences in terms of both PPE and protein for jejunum CD (P<0.05). However, no statistically significant differences were shown in the ratio of VH/CD for both jejunum and ileum (P>0.05). In addition, for ileum CD values, statistical significance was found in terms of PPE (P<0.05).

Table 5. Levels of MDA values on the first and third day in breast meat of the groups.

Dietary Treatments		TBARS Values (mg/kg)	
		1st day	3rd day
NP-C		0.46	1.02^{a}
NP-PPE		0.31	0.68^{c}
NP-EPPE		0.25	0.63^{c}
LP-C		0.49	1.09^{a}
LP-PPE		0.40	0.86^{b}
LP-EPPE		0.33	0.67^{b}
Protein	**Normal**	0.34	0.77^{A}
	Low	0.41	0.90^{B}
PPE	**NI**	0.47	1.05^{x}
	PPE	0.35	0.74^{y}
	EPPE	0.29	0.60^{y}
SEM		0.07	0.10
		Significance (P-value)	
Protein		0.21	0.02
PPE		0.3	0.05
Protein X PPE		0.25	0.01

NP-C: Normal Protein Control, NP-PPE: Normal Protein *Pinus pinaster* extract, NP-EPPE: Normal Protein Encapsulated *Pinus pinaster* extract, LP-C: Low Protein Control, LP-PPE: Low Protein *Pinus pinaster* extract, LP-EPPE: Low Protein Encapsulated *Pinus pinaster* extract, NI: Not included, SEM: Standard error of the mean.
a,b,c: Mean values within the same column carrying different superscripts are significantly different at P<0.05.
x,y: Mean values within the same column carrying different superscripts are significantly different at P<0.05.
A,B: Mean values within the same column carrying different superscripts are significantly different at P<0.05.

Table 6. Effects of intestinal histomorphology of jejunum and ileum on the 21st day in groups.

Dietary Treatments		Jejunum			Ileum		
		Villus Height (µm)	Crypt Depth (µm)	Villus Height/ Crypt Depth Ratio	Villus Height (µm)	Crypt Depth (µm)	Villus Height/ Crypt Depth Ratio
NP-C		1174.69	156.33^{ab}	7.57	726.42	126.05	6.30
NP-PPE		1181.70	146.94^{abc}	8.05	754.83	127.61	5.72
NP-EPPE		1288.67	162.5^{a}	7.94	779.22	134.00	5.64
LP-C		1071.83	135.08^{c}	7.58	710.00	120.19	6.15
LP-PPE		1164.11	141.61^{bc}	7.96	736.33	134.97	5.68
LP-EPPE		1186.69	162.36^{a}	7.33	748.91	137.08	5.21
Protein	**Normal**	1215.02	155.26^{A}	7.86	753.49	129.22	5.89
	Low	1140.88	146.35^{B}	7.62	731.75	130.75	5.68
PPE	**NI**	1123.26^{y}	145.71^{y}	7.58	718.21	123.12	6.23
	PPE	1172.90^{xy}	144.28^{y}	8.00	745.58	131.29	5.70
	EPPE	1237.68^{x}	162.43^{x}	7.63	764.07	135.54	5.43
SEM		12.40	1.72	0.11	14.02	2.26	0.15
				Significance (P-value)			
Protein		0.006	0.015	0.312	0.44	0.73	0.45
PPE		0.003	0.001	0.251	0.42	0.09	2.28
Protein X PPE		0.29	0.047	0.5	0.98	0.49	0.14

NP-C: Normal Protein Control, NP-PPE: Normal Protein *Pinus pinaster* extract, NP-EPPE: Normal Protein Encapsulated *Pinus pinaster* extract, LP-C: Low Protein Control, LP-PPE: Low Protein *Pinus pinaster* extract, LP-EPPE: Low Protein Encapsulated *Pinus pinaster* extract, NI: Not included, SEM: Standard error of the mean.
a,b,c: Mean values within the same column carrying different superscripts are significantly different at P<0.05.
x,y: Mean values within the same column carrying different superscripts are significantly different at P<0.05. A,B: Mean values within the same column carrying different superscripts are significantly different at P<0.05.

Table 7. Effects of intestinal histomorphology of jejunum and ileum on the 41st day in groups.

Dietary Treatments		Jejunum Villus Height (µm)	Jejunum Crypt Depth (µm)	Jejunum Villus Height/ Crypt Depth Ratio	Ileum Villus Height (µm)	Ileum Crypt Depth (µm)	Ileum Villus Height/ Crypt Depth Ratio
NP-C		1267.30	154.41^{b}	8.26	1117.24	143.83	6.05
NP-PPE		1334.47	171.77^{a}	7.79	1151.60	153.55	7.63
NP-EPPE		1392.16	190.11^{a}	7.33	1150.50	146.19	7.57
LP-C		1256.33	154.19^{b}	8.16	877.82	135.64	8.26
LP-PPE		1320.08	168.83^{b}	7.83	1159.50	147.30	7.83
LP-EPPE		1322.13	160.17^{b}	8.26	1101.99	159.11	7.23
Protein	**Normal**	1331.31	172.10^{A}	7.79	1139.78	147.86	7.08
	Low	1299.51	161.17^{B}	8.08	1046.44	147.35	7.77
PPE	**NI**	1261.82^{y}	154.31^{y}	8.21	997.53	139.73^{y}	7.15
	PPE	1327.27^{x}	170.31^{x}	7.82	1140.89	150.43^{xy}	7.72
	EPPE	1357.15^{x}	175.29^{x}	7.80	1126.25	152.65^{x}	7.40
SEM		10.77	1.79	0.10	31.30	2.03	0.23
			Significance (P-value)				
Protein		0.15	0.005	0.15	0.15	0.90	0.14
PPE		0.04	0.001	0.16	0.22	0.032	0.6
Protein X PPE		0.46	0.003	0.08	0.12	0.08	0.07

NP-C: Normal Protein Control, NP-PPE: Normal Protein *Pinus pinaster* extract, NP-EPPE: Normal Protein Encapsulated *Pinus pinaster* extract, LP-C: Low Protein Control, LP-PPE: Low Protein *Pinus pinaster* extract, LP-EPPE: Low Protein Encapsulated *Pinus pinaster* extract, NI: Not included, SEM: Standard error of the mean.
a,b: Mean values within the same column carrying different superscripts are significantly different at P<0.05.
x,y: Mean values within the same column carrying different superscripts are significantly different at P<0.05.
A,B: Mean values within the same column carrying different superscripts are significantly different at P<0.05.

Discussion and Conclusion

In this study, statistically significant differences between the groups of BWG in terms of protein at 0-41 days (P <0.05) were observed. For all periods except 0-10th days interval, higher BWG in normal protein groups than low protein ones (P<0.05) (Table 2) were determined. In agreement with our study, Sigolo et al. (28) stated that growth performance was negatively affected in their study, conducted by reducing the protein level in broilers by 2.5% from the recommended levels. Aftab et al. (2) investigated the effects observed when protein levels indicated in NRC (1994) were reduced by 10% using balanced rations of amino acids at 0-21, 21-42, and 42-56 days of broiler chickens. For this purpose, they gave rations containing 20.7, 18.0, and 16.2% crude protein at 0-21, 21-42 and 42-56 days, respectively. However, they stated that there was a decrease in BW, BWG and carcass yield in all low protein rations compared to the control group. Likewise, in our study, when the protein level decreased 10% in low protein groups, a decrease was found compared to the normal ones for BWG and carcass yield. Cardinal et al. (4), in their studies, comparing growth performance and intestinal health in broiler groups with standard protein, the protein level reduced by 6% and protease added standard and low protein groups; reported that in the low protein group, BW, BWG and FCR were significantly adversely affected in 1-42 days compared to the standard protein group. These findings were consistent with the findings we obtained in our study that were determined in terms of protein.

In another study, Hilliar et al. (13) conducted to evaluate the effects of low protein (LP) diets supplemented with approximately 3% of glycine, serine and threonine amino acids in broilers. It was stated that the results showed LP group, also LP and supplemented with amino acids groups had lower final BW than the standard protein group. It was notified that the standard protein group FCR is better than LP; moreover, it was better than low protein supplemented with amino acids groups during the trial. Zhou et al. (37) was designed a study to evaluate the effects of dietary serine supplementation on performance in laying hens fed low protein (LP) diets. The trial included a control diet with standard protein (16.49% CP) and 4 low protein diets (14.05% CP) supplemented with 0, 0.114, 0.306, and 0.498% L-serine, respectively. At the end of the study, it was notified the supplementation of serine to LP diets improved performance and led to an optimal egg production with serine level of 0.498%. In addition, total protein and globulin contents were significantly increased (P<0.05) with serin supplementation at the levels of 0.306% and 0.498%. These results are in line with our findings about reducing protein level in broiler diets. While it was stated the supplementation of amino acids was used for compensation of the adverse effect of reducing protein level in broiler diets in above-mentioned studies, PPE and EPPE aromatic substances

were used to fortify compensation of this effect in our study.

Also, it was notified in a study when plant extracts from rosemary, olive leaves, pine bark concentration of 2.5 and 5.00 g/kg, and polyphenolic compound quercetin 0.25 and 0.50 mg/kg concentration were added in broiler diets, no effect was observed on BWG and FCR for the olive leaves, pine bark extracts as well as quercetin (27). However, it was reported to be observed better BWG, FI and FCR when added 600 and 2400 mg/kg PPE in broiler diets during 21 days before slaughtering by Herranen et al. (12). In our study at 0-41 days period, the numerically highest body weight gain was found respectively in NP-PPE and then NP-EPPE groups. In terms of PPE, statistically significant increases on days 11-21 and 0-21 for BWG and better FCR that showed significant effects on days 11-21 and 0-41($P<0.05$) were found.

In another study to investigate the effects of chitosan nano-encapsulating mint, thyme, and cinnamon essential oils in broiler diets added at 0.025, 0.04, and 0.055%, respectively, starter, grower, finisher period notified that encapsulated forms of essential oils were significantly improved ($P<0.05$) for BWG and FCR compared to free forms (23). Also, it was reported that in the comparison of powder and encapsulated form of garlic and *Phyllanthusniruri L.* mixture, the encapsulated form had more powerful effects on BWG and FCR than powder form (22). Additionally, Haafez et al. (11) reported that encapsulation of aromatic substances affected FCR positively compared to powder form. Mourtzinos et al. (21) and Zhang et al. (36) stated that the encapsulation process increases the bioavailability of the product. Also, in our study between 11-21 and 0-21 days of age, better BWG and FCR values were observed in EPPE groups ($P<0.05$) than PPE and EPPE values. In these periods, the protein and PPE interaction values were statistically significant also ($P<0.05$). It was observed that the addition of coated extract in 0-21 days provided statistically significant positive effects for BWG in normal protein groups and better FCR values in both normal and low protein groups. These findings were in harmony with the general approach of increase in the bioavailability using encapsulation of aromatic substances.

It was stated that conducted a study to evaluate the effects of supplementation of 5-10% Moringa olifera leaves meal (MLM) and 50-100 ml Moringa olifera extracts (MLEx) in standard and low protein (LP) diets in broilers. It was notified in this study the lowest BW was detected in the LP control group, whereas the best BW value was in the LP+100 ml MLEx group. Also, it was reported the best FCR was found in the LP+50 ml MLEx group and the best TBARS value in the LP+100 ml MLEx group (1). The above mentioned results of adding aromatic substances (MLM and MLEx) to LP diets to improve growth performance are in line with the findings of our study.

Guo et al. (10) reported that with the supplementation of 0, 1, 3, 5% pine needles powder in broiler diets, decreases were found in triglyceride levels for the groups of 3 and 5% and in cholesterol levels for groups 1 and 5% in blood serum when compared to control. Meanwhile, in the present study, there were no significant differences compared to control when it was used 100 mg/kg of PPE and EPPE.

MDA is one of the most important and toxic substances that occurred by lipid peroxidation of unsaturated fatty acids with oxygen. Due to based on detection of MDA levels in blood plasma and poultry meat, the TBARS method is commonly used as an indicator of lipid oxidation level (14). Guo et al. (10) also declared to detection a decrease in blood TBARS level compared to control when 5% of pine needles powder was added into the broiler diets. It was reported that when fermented pine needles powder was used, 0.3% for the starter period and 0.6% for the grower-finisher period in broiler diets, it was observed better MDA level and antioxidant activity (35). Ramay and Yalçın (24) explained to observe a linear decrease in MDA levels in breast meat stored for 1 and 10 days when broilers were fed a linseed oil-based basal diet supplemented with 0.25, 0.5, 0.75, 1% pine needles powder. In parallel to earlier studies, it was found statistically significant decreases in the 3rd-day breast meat MDA values in PPE and EPPE groups than control ($P <0.05$). When compared MDA values in PPE and EPPE groups, a numerical decrease was observed in EPPE groups in our study ($P>0.05$).

Villus height (VH) and crypt depth (CD) are related to the digestive capacity of the small intestine. Laudadio et al. (15) conducted a study with equal energy and three different protein levels that were high 22.5%, medium 20.5%, low 18.5% HP for comparison of intestinal VH and CD values; they found significant numerical increases in values of medium and high protein groups than low protein one. Similar to our study, higher VH and CD values were found in normal protein groups than low protein ones. In another study, it was reported that conducted to evaluate the effects of low protein (LP) diets supplemented with arginine, glutamine, methionine, and threonine in Eimeria-infected chickens. The results revealed that the intestinal health of chickens challenged with a mild coccidia infection can be improved when fortified in 0.75% of arginine, glutamine, methionine, and threonine to LP diets (30). Van Nevel et al. (33) reported that the low VH/CD ratio indicates that the intestinal turnover rate decreases. Thus, it may lead to an increase in growth rate by consuming less energy for vital activities.

Also, Tufarelli et al. (31) emphasized that the increases of villi height improve total absorption area in the intestine. This situation provides a positive effect on digestive enzymes and transporting nutrients on the villi surface. In our study, it was found a lower VH/CD ratio in the normal protein groups than in the low protein groups. Moreover, in terms of PPE, the jejunum VH/CD ratio was found lower in the PPE and EPPE added groups on the 41st-day results. When compared to control, addition to PPE and EPPE provide higher VH and deeper CD in jejunum at 21 and 42 days age. It was thought that the increasing VH-CD and improvement of performance could be caused by growing so that it could be a positive increase, but no data were found about the effects of PPE on intestinal histomorphology in the broiler, so it was thought to be investigated with further studies.

In light of the findings obtained in this study, it was observed that with supplementation of 100 mg/kg PPE or EPPE obtained from Türkiye in normal and 10% low protein broiler rations; no adverse effect was observed in the groups. Also, positive effects on performance, intestinal histomorphology, and better TBARS levels with antioxidant properties were detected. In conclusion, it was estimated that 100 mg/kg PPE or EPPE would be used as an alternative aromatic substance for broiler diets. Also, encapsulation can be preferred to obtain better antioxidant activity and intestinal capacity on stress conditions in the field. Besides that, further studies are needed to determine the range of usage amount to provide maximum effects for dietary supplementations in broiler diets.

Acknowledgements

This manuscript was prepared based on a part of Gonca ÖZTAP's doctoral thesis.

Financial Support

This research was supported by Ankara University, Scientific Research Projects Unit Under project number 18L0239003.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Author Contributions

GÖ and SK conceived, planned and carried out the experiments. GÖ took the lead in writing the manuscript. GÖ and SK provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by the Ankara University Animal Experiments Local Ethics Committee (2017-22-178).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

References

1. **Abousekken MSM** (2015):*Performance, immun response and carcass quality of broilers fed low protein diets contained either Moringa Oleifera Leaves meal or its Extract*. J Am Sci, **11**,153-164.
2. **Aftab U, Ashraf M, Jlang Z** (2006): *Low protein diets for broilers*. Poult Sci, **62**, 688-698.
3. **Applegate TJ, Dibner JJ, Kitchell ML, et al** (1999): *Effect of turkey (Meleagridis gallopavo) breeder hen age and egg size on poult development. 2. Intestinal villus growth, enterocyte migration, and proliferation of the turkey poult.* Comp Biochem Physiol, **124**, 381-389.
4. **Cardinal KM, Moraes Ml, Andretta I, et al** (2019): *Growth performance and intestinal health of broilers fed a standard or low-protein diet with the addition of a protease.* R Bras Zootec, **48**, e20180232.
5. **Carpenter K, Clegg K** (1956): *The metabolizable energy of poultry feeding stuffs in relation to their chemical composition.* J Sci Food Agr, **7**, 45-51.
6. **Chan ES** (2011): *Preparation of Ca-alginate beads containing high oil content: Influence of process variables on encapsulation efficiency and bead properties.* Carbohydr Polym, **84**, 1267-1275.
7. **Chan ES, Lee BB, Ravindra P, et al** (2009): *Prediction models for shape and size of ca-alginate macrobeads produced through extrusion-dripping method*. J Colloid Interf Sci, **338**, 63-72.
8. **Ferrari R, Ceconi C, Curello S, et al** (1985): *Role of oxygen in myocardial ischemic and reperfusion damage: Effect of alpha-tocopherol.* Acta Vitaminol Enzymol, **7**, 61-70.
9. **Gulati OP** (2015): *Pycnogenol in metabolic syndrome and related disorders*. Phytother Res, **29**, 949-968.
10. **Guo A, Cheng L, Al-Mamun M, et al** (2018): *Effect of dietary pine needles powder supplementation on growth, organ weight and blood biochemical profiles in broilers*. J Appl Anim Res, **46**, 518-522.
11. **Hafeez A, Manner K, Schieder C, et al** (2016): *Effect of supplementation of phytogenic feed additives (powdered vs. encapsulated) on performance and nutrient digestibility in broiler chickens.* Poult Sci, **95**, 622-629.
12. **Herranen K, Pietarinen S, Luukko K, et al** (2011): Method for modulating animal digestive tract microbiota and feed composition comprising bark extract. US Patent Application Publication Sep.1, Sheet 1 of 2.
13. **Hilliar M, Huyen N, Girish CK, et al** (2019): *Supplementing glycine, serine and threonine in low protein diets for meat type chickens.* Poult Sci, **98**, 6857-6865.

14. **Jo C, Ahn DU** (1998): *Fluorometric analysis of 2-thiobarbituric acid reactive substances in turkey*. Poult Sci, **77**, 475-480.
15. **Laudadio V, Passantino L, Perillo A, et al** (2012): *Productive performance and histological features of intestinal mucosa of broiler chickens fed different dietary protein levels*. Poult Sci, **91**, 265-270.
16. **Li P, Lin JE, Chervoneva I, et al** (2007): *Homeostatic control of the crypt-villus axis by the bacterial enterotoxin receptor guanylyl cyclase c restricts the proliferating compartment in ıntestine*. Am J Pathol, **171**, 1847-1858.
17. **Madene A, Jacquot M, Scher J, et al** (2006): *Flavour encapsulation and controlled release*. Int J Food Sci Tech, **41**, 1-21.
18. **Maimoona A, Naeem I, Saddiqe Z, et al** (2011): *Review on biological, nutraceutical and clinical aspects of French maritime pine bark extract.* J Ethnopharmacol, **133**, 261-277.
19. **Mccord JM** (1985): *Oxygen-derived free radicals and tissue injury.* N Engl J Med, **312**, 159.
20. **Mohammedi A, Jafari SM, Assadpour F, et al** (2016): *Nano-encapsulation of olive leaf phenolic compounds through wpc–pectin complexes and evaluating their release rate.* Int J Biol Macromol, **82**, 816-822.
21. **Mourtzinos I, Salta F, Yannakopoulou K, et al** (2007): *Encapsulation of olive leaf extract in β-cyclodextrin.* J Agri Food Chem, **55**, 8088-8094.
22. **Natsir MH, Hartutik OS, Widodo E** (2013): *Effect of either powder or encapsulated form of garlic and Phyllanthus niruri L. mixture on broiler performances, intestinal characteristics, and intestinal microflora.* Int J Poult Sci, **12**, 676.
23. **Nouri A** (2019): *Chitosan nano-encapsulation improves the effects of mint, thyme, and cinnamon essential oils in broiler chickens*. Br Poult Sci, **60**, 530-538.
24. **Ramay MS, Yalçın S** (2020): *Effects of supplemental pine needles powder (Pinus brutia) on growth performance, breast meat composition, and antioxidant status in broilers fed linseed oil-based diets.* Poult Sci, **99**, 479-486.
25. **Rohdewald PJ** (2002): *A review of the French maritime pine bark extract (Pycnogenol®), a herbal medication with a diverse clinical pharmacology.* Int J Clin Pharmacol Ther, **40**, 158-168.
26. **Rohdewald PJ** (2005): *Pycnogenol®, French maritime pine bark extract. Encyclopedia of dietary supplements*, **1**, 545-553.
27. **Sierżant K, Orda J, Korzeniowska M, et al** (2019): *Effect of dietary supplementation with extracts of rosemary, olive leaves, pine bark and quercetin on selected performance indices of broiler chickens and microbiological status of their ileum.* Med Weter, **75**, 247-252.
28. **Sigolo S, Zohrabi Z, Gallo A, et al** (2017): *Effect of low crude protein diet supplemented with different levels of threonine on growth performance, carcass traits, blood parameters, and immune responses of growing broilers.* Poult Sci, **96**, 2751-2760.
29. **Tarladgis BG, Watts BM, Younathan MT, et al** (1960): *A distillation method for the quantitative determination of malonaldehyde in rancid foods.* J Am Oil Chem Soc, **37**, 44-48.
30. **Teng PY, Choi J, Yadav S, et al** (2021): *Effects of low-crude protein diets supplemented with arginine, glutamine, threonine, and methionine on regulating nutritient absorption, intestinal health, and growth performance of Eimeria-infected chickens*. Poult Sci, **100**, 101427.
31. **Tufarelli V, Desantis S, Zizza S, et al** (2010): *Performance, gut morphology, and carcass characteristics of fattening rabbits as affected by particle size of pelleted diets.* Arch Anim Nutr, **64**, 373-382.
32. **United States Pharmacopeia (USP)** (2005): Maritime Pine Extract. Rockville, United States of Pharmacopeial Convention, **28**, 2115-2116.
33. **Van Nevel CJ, Decuypere JA, Dieric NA, et al** (2005): *Incorporation of galactomannans in the diet of newly weaned piglets: Effect on bacteriological and some morphological characteristics of the small intestine.* Arch Anim Nutr, **59**, 123-138.
34. **Wolf SP, Garner A, Dean RT** (1986): *Free radicals, lipids, and protein degradation.* TIBS, **11**, 27-31.
35. **Wu QJ, Wang ZB, Wang GY, et al** (2015): *Effects of feed supplemented with fermented pine needles (Pinus ponderosa) on growth performance and antioxidant status in broilers.* Poult Sci, **94**, 1138-1144.
36. **Zhang L, Li J, Yun TT, et al** (2015): *Effects of pre-encapsulated and pro-encapsulated Enterococcus faecalis on growth performance, blood characteristics, and cecal microflora in broiler chickens.* Poult Sci, **94**, 2821-2830.
37. **Zhou JM, Qiu K, Wang J, et al** (2021): *Effect of dietary serine supplementation on performance, egg quality, serum indices, and ileal mucosal immunity in laying hens fed a low crude protein diet.* Poult Sci, **100**, 101465.