Combined use of essential oils with organic acids in modifying performance, intestinal health, caecal microflora, and selected blood and bone parameters in broilers

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ABSTRACT

Essential oils (EOs) and organic acids (OAs) are promising feed additives with crucial roles in promoting animal health and performance. This study aimed to assess the combined effects of phytobiotics with organic acids (EOAs) in broiler diets for 39 days. A total of daily 300 male chicks were assigned to three groups, each containing 100 chicks with 5 replicates. The basal diet was supplemented with 0, 0.1, and 0.2% EOAs, respectively. EOAs supplementation did not significantly affect performance values. The villus height/crypt depth ratio in the jejunum and villus dimensions in the ileum showed improvement with EOAs. Dietary EOAs supplementation led to a reduction in the count of *Enterobacteriaceae* and an increase in *Lactobacillus* in the caecum. Serum IgA and IgG levels increased with EOAs. Ultimate load, yield load, and the levels of ash, calcium, phosphorus, zinc, and manganese in tibia and femur were higher at high levels of EOAs than in the other groups. In conclusion, the study suggests that 0.1% EOAs usage in diets could be a viable option for enhancing intestinal health, immunity, and bone mineralization as an alternative growth promoter, especially in commercial broiler production.

Introduction

Feed additives such as phytobiotics (Ps) and organic acids (OAs), have gained increasing recognition as natural growth promoters in poultry diets. Ps can be defined as natural compounds derived from plants, which encompass whole plants, plant components, extracts, or essential oils (EOs). The positive impacts of Ps can be attributed to various factors such as the stimulation of feed

consumption, improved nutrient digestion and absorption, modulation of gut microbiota, decreased colonization of harmful pathogens in the gut, and reinforcement of the birds' immune status. They also exhibit antibacterial, antiviral, antioxidant, and anti-inflammatory properties. In addition to Ps, OAs have found widespread use in poultry nutrition due to having vital roles in lowering pH, decreasing the survival of pathogens, enhancing the activity of digestive enzymes, improving intestinal morphology, and consequently fostering a healthier gut microflora. This, in turn, contributes to enhanced performance and increased profitability in poultry production (7, 13, 19, 37).

Researchers (1, 5, 30, 40, 43, 49, 50) have studied the usage of the combined use of OAs with EOs (EOAs) in broilers. The synergistic effects of EOs and OAs can be attributed to the improved efficiency of digestive enzymes in acidic conditions (43). Yang et al. (49) indicated that the addition of the blends of sorbic acid, fumaric acid, and thymol during the grower phase increased efficiency, possibly by improving intestinal morphology and increasing digestive enzyme activities of broiler chickens.

Leg weakness, lameness and various bone abnormalities linked to metabolic disorders continue to pose challenges in rapidly growing meat-type chickens, resulting in significant production losses and adverse effects on the welfare of birds (26, 42). Modern broiler lines often exhibit poor bone calcification and high porosity, which can increase the susceptibility to bone damage (44). Nutrition plays a crucial role in the development of these bone disorders, and optimizing dietary factors may help reduce the severity of leg lesions in broilers. Liu et al. (29) reported that supplementation with a mixture of essential oils and organic acids (citric acid, sorbic acid, thymol, and vanillin) had no significant effect on leg bone growth or bone length in broilers. Notably, there is a lack of published research specifically examining the detailed effects of dietary EOAs on broilers, particularly concerning bone parameters. We hypothesize that the combined use of essential oils and organic acids in broiler diets will enhance growth performance, improve intestinal health, and positively modulate caecal microflora. Additionally, this supplementation is expected to influence selected blood parameters and support bone development, potentially mitigating bone disorders associated with rapid growth in broilers. Building on this hypothesis, the objective of this study is to evaluate the effects of dietary supplementation with a combination of essential oils and organic acids on growth performance, caecal fermentation, intestinal health, and selected blood and bone parameters in broilers.

Materials and Methods

***Experimental Design and Diets*:** A total of 300 daily Ross 308 male broiler chicks were divided into three groups, each consisting of 100 chicks. Within each group, there were five replicates, each containing 20 chicks. The chicks were housed in pens (2m x 1m) with wood shavings as litter. The experimental period lasted for 39 days, during which the broilers were fed with different diets as follows: starter diets from day 0 to 13, grower 1 diets from day 14 to 24, grower 2 diets from day 25 to 36, and finisher diets from day 37 to 39. Basal diets were formulated according to the commercial management guide (6) and supplemented with EOAs. The EOAs included thyme oil, orange oil, garlic oil, sorbic acid, acetic acid, malic acid, lactic acid, citric acid, tri-sodium citrate, tartaric acid, salicylic acid, ascorbic acid (Nafoil A Plus, Biotem Ltd Company, İstanbul, Türkiye) at three levels: 0% (EOA0), 0.1% (EOA1), and 0.2% (EOA2). The ingredients and chemical composition of the basal diets are presented in Table 1. The diets were provided in mash form, and feed and water were available *ad libitum* throughout the experiment. Room temperature was 32±2°C during the first week and gradually reduced to an average of 24 to 26°C, which was maintained until slaughter age.

***Traits Measured*:** The nutrient composition of the diets was determined using the methods described in AOAC (4), and metabolisable energy values were calculated using the equation of Carpenter and Clegg as reported by Yalçın et al. (45). The volatile oil profile of the EOAs was determined by the GC-MS (Agilent:6890 MS:5973, New Jersey, USA) with an HP-5 MS column (30 meters).

Individual bird weights were recorded at the beginning of the experiment and on the 13th, 24th, 36th, and 39th days to calculate live weight gains. Daily monitoring of the birds was conducted, and feed intake was measured and expressed in g per bird per period. The feed conversion ratio (FCR) was determined as kg feed consumed per kg weight gain. Percentage of livability {(number of broilers at the end of the study x 100)/number of chicks at the beginning} and European Production Efficiency Factor {EPEF, % = [(livability, % x live weight, kg x 100)/(age, day x FCR, kg feed/kg gain)]} values were calculated (24).

At the end of the experiment (day 39), 10 broilers from each group were weighed, and slaughtered by severing the jugular vein, and their hot carcass weights and carcass yields were determined. The absolute and proportional weights of abdominal fat, liver, gizzard, heart, bursa Fabricius, and spleen were recorded. Duodenal, jejunal, and ileal samples were collected after slaughtering to evaluate morphological changes as reported by Onbaşılar et al. (32). Samples were stained with Mallory's trichrome, and sections were analysed under a light microscope (Olympus BX-40). Measurements were done using Cellsens CS-ST-V1.8 (Standard) software program. For measurement 10 well-oriented crypt-villus units were selected for each intestinal cross-section. Villus height (VH)

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Table 1. The ingredients and chemical composition of the basal diets (as-fed basis)

Items (%)	Broiler starter (0-13 d)	Broiler grower-1 (14-24 d)	Broiler grower-2 (25-36 d)	Broiler finisher (37-39 d)
Ingredients (%)				
Corn	36.55	33.79	35.54	37.71
Soyabean meal, 46% CP	18.83	11.32	4.65	10.47
Fullfat soya	18.00	18.00	19.00	12.50
Wheat	13.50	13.00	14.50	15.50
Sunflower seed meal, 34% CP	4.00	7.00	8.00	5.00
Red dog	2.50	4.50	5.00	5.00
Rice	0.00	3.00	3.00	3.00
Meat and bone meal	3.00	1.75	2.14	1.01
Poultry rendering meal	0.00	3.50	4.00	5.00
Soyabean oil	1.13	1.88	2.19	2.78
Limestone	0.80	0.81	0.74	0.79
Lysine sulphate	0.40	0.41	0.39	0.39
Methionine	0.35	0.29	0.23	0.23
Monocalcium phosphate	0.30	0.20	0.10	0.10
Salt	0.18	0.19	0.18	0.18
Treonine	0.13	0.08	0.06	0.06
Vitamin premix[1]	0.10	0.10	0.10	0.10
Mineral premix[2]	0.10	0.10	0.10	0.10
Choline chloride	0.08	0.08	0.08	0.08
Anticoccidial[3]	0.05	0.00	0.00	0.00
Chemical composition (Analysed values)				
ME[4] (kcal/kg)	3030	3124	3160	3223
Crude protein (%)	23.27	22.16	20.99	20.12
Calcium (%)	0.98	0.93	0.90	0.87
Total phosphorus (%)	0.68	0.66	0.63	0.63

[1]: Supplied per kg: 11 000 000 IU vitamin A, 3 500 000 IU vitamin D3, 100 g vitamin E, 3 g vitamin K3, 3 g vitamin B1, 6 g vitamin B2, 15 g calcium D-pantothenate, 1 g vitamin B6, 20 mg vitamin B12, 35 g niacin, 1.5 g folic acid, 200 mg D-biotin
[2]: Supplied per kg: 30 g Cu, 120 g Mn, 110 g Zn, 2 g I, 300 mg Se, 50 g Fe
[3]: Salinomycin
[4]: Metabolisable energy content of diets was estimated as stated in Yalçın et al (45)

was measured from the tip of the villi to the villus crypt junction, and crypt depth (CD) was defined as the depth of the invagination between adjacent villi. The ratio of villus height to crypt depth (VH/CD) was calculated.

Blood samples were collected from vena brachialis under the wing from 10 broilers from each group (two from each replicate) at day 39 and centrifuged at 3220 x g for 5 min for collection serum. Levels of triglyceride, total cholesterol, aspartate aminotransferase (AST), alkaline phosphatase (ALP), and alanine aminotransferase (ALT) were determined using an autoanalyzer (Olympus AU400) using their accompanying commercial kits. ELISA kits were used to determine the level of Immunoglobulins (Ig) G (BT-E0019Ch, Bioassay Technology Laboratory, Shanghai, China) and IgA (ab157691, Abcam, Shanghai, China) in blood serum according to the instructions.

Caecal digesta was collected immediately after slaughtering for the determination of total aerobic bacteria (17), *Enterobacteriaceae* (23), and *Lactobacillus* spp. (22). Data were expressed as $\log_{10}$ colony-forming units/g caecal digesta.

The right tibia and femur of broilers were removed, cleaned from all tissue, and then weighed. The lengths of the bones at the distance between the distal and proximal ends and the outer diameter at the narrowest point of the bone shaft were measured by a digital caliper. The breaking strength was determined by 3-point bending with Material Testing Machines (Instron 5944 testing frame, Instron, USA) using Instron Plus software and a standard 2 kN load cell. Distances of 50 mm were established between the two fixed points supporting the bone. The weight load was applied to the midpoint of the shaft under a crosshead speed of 5 mm/min until failure. The Load-Displacement curve was obtained by using the load and displacement values recorded throughout the test. By using the curve obtained, values of stiffness, yield point, elongation at yield point, and maximum load were obtained. The stiffness value was obtained by calculating

the slope of the load-displacement graph. For yield point and yield point elongation values, the load and displacement values at the point that the linear region ends and bone deformation begins were determined. The ultimate load was determined as the maximum load point resisted by the bone sample (33, 38). Bones were prepared for mineral analysis as explained by Yalçın et al. (47). Calcium, phosphorus, magnesium, zinc, and manganese concentrations were determined (9) using ICP-OES (Perkin Elmer OptimaTM DV 2100 Model, Dual View, Perkin Elmer Life and Analytical Sciences).

***Statistical Analysis*:** Data distribution was checked for normality using the Kolmogorov-Smirnov test. The effects of different levels of the EOAs were analysed using One-way ANOVA with five replicates for each dietary treatment. The significance of mean differences between groups was tested by Tukey. Linear effects were determined using polynomial contrasts. Statistical significance was considered at $P \leq 0.05$ (12).

Results

The volatile oil profile of EOAs is detailed in Table 2. It is noteworthy that the volatile oil was particularly rich in carvacrol (40.23%) and linalool (17.59%). The effects of EOAs on various performance parameters are presented in Table 3. Body weight, weight gain, feed intake, FCR, and EPEF values remained largely unaffected by the inclusion of EOAs. However, it is important to note that as the levels of EOAs increased, there was a significant improvement in livability ($P < 0.001$). There were no significant effects of EOAs on carcass yield and the relative weights of various organs (Table 4). Nonetheless, a linear increase ($P = 0.039$) in carcass yield was observed with increasing levels of EOAs.

Table 2. Volatile oil profile of the mixture of essential oils and organic acids (% of volatile oil)

Components	% of volatile oil
Carvacrol	40.23
Linalool	17.59
Diallyl disulphide	10.40
Limonene	10.25
Thymol	7.52
Diallyl trisulphide	5.32
Allyl methyl trisulphide	3.91
p-Cymene	2.41
Allyl methyl disulphide	2.37

Table 3. Effects of combined use of essential oils with organic acids on performance

Parameters	Mixture of essential oils and organic acids (%)			P-value	
	0	0.1	0.2	Combined	Linear
			Body weight (g)		
d 0	43.05±0.52	42.90±0.35	42.95±0.49	0.973	0.880
d 39	2929.20±12.13	2921.72±17.72	2927.82±18.36	0.979	0.972
			Body weight gain (g/bird)		
d 0-13	399.95±5.63	388.44±5.92	388.85±6.29	0.334	0.212
d 14-24	923.12±10.74	881.43±14.99	903.31±12.75	0.116	0.068
d 25-36	1187.30±17.48	1207.90±26.83	1224.71±18.08	0.481	0.237
d 37-39	375.79±10.82	401.06±22.85	368.01±12.29	0.354	0.168
d 0-39	2886.15±12.64	2878.82±20.13	2884.87±19.78	0.953	0.765
			Feed intake (g/bird)		
d 0-13	440.55±12.52	413.53±13.12	422.35±12.27	0.338	0.329
d 14-24	1214.01±16.60	1169.11±13.65	1174.03±10.44	0.078	0.063
d 25-36	2002.76±25.86	2043.21±27.22	2031.52±16.89	0.594	0.485
d 37-39	751.78±11.26	759.87±13.79	754.15±14.68	0.908	0.902
d 0-39	4409.11±46.65	4385.71±56.47	4382.05±31.06	0.915	0.703
			Feed conversion ratio (kg feed intake/kg weight gain)		
d 0-13	1.10±0.03	1.06±0.02	1.09±0.04	0.714	0.767
d 14-24	1.32±0.02	1.33±0.03	1.30±0.01	0.502	0.511
d 25-36	1.69±0.02	1.69±0.03	1.66±0.03	0.637	0.484
d 37-39	2.01±0.06	1.90±0.05	2.06±0.07	0.210	0.548
d 0-39	1.53±0.02	1.52±0.01	1.52±0.01	0.927	0.703
Livability, %	94.64±0.25^{b}	99.00±1.00^{a}	100.00±0.00^{a}	<0.001	<0.001
EPEF, %	465.55±6.75	486.93±4.95	494.49±7.54	0.081	0.033

n: 5, EPEF: European production efficiency factor

^{a,b}: Means within a row with different superscripts differ significantly at $P < 0.05$.

Table 4. Effects of combined use of essential oils with organic acids on carcass yield (%) and relative organ weights (%)

Parameters (%)	Mixture of essential oils and organic acids (%)			P-value	
	0	0.1	0.2	Combined	Linear
Carcass yield	74.52±0.44	74.87±0.31	75.51±0.17	0.108	0.039
Liver	1.93±0.07	2.01±0.05	2.00±0.06	0.552	0.375
Heart	0.54±0.03	0.52±0.02	0.53±0.02	0.823	0.781
Spleen	0.10±0.01	0.11±0.01	0.11±0.01	0.365	0.176
Bursa Fabricius	0.06±0.01	0.07±0.01	0.08±0.01	0.320	0.135
Gizzard	0.99±0.07	1.00±0.06	0.98±0.05	0.965	0.897
Abdominal fat	1.10±0.07	1.08±0.05	1.10±0.06	0.981	0.977

n:10

Table 5. Effects of combined use of essential oils with organic acids on intestinal histomorphology of broilers

Parameters	Mixture of essential oils and organic acids (%)			P-value	
	0	0.1	0.2	Combined	Linear
		Duodenum			
Villus height (µm)	1881.01±48.90	1855.95±39.42	1855.31±38.30	0.931	0.742
Crypth depth (µm)	131.31±5.00	145.69±7.89	150.88±7.17	0.128	0.052
Villus width (µm)	224.11±6.99	201.47±7.00	220.80±7.85	0.127	0.778
Villus height/crypt depth	14.54±0.71	13.14±0.86	12.59±0.71	0.234	0.101
		Jejunum			
Villus height (µm)	1169.42±70.81	1192.38±68.13	1280.13±71.89	0.510	0.275
Crypth depth (µm)	137.44±7.33	115.08±6.62	116.03±8.64	0.079	0.056
Villus width (µm)	275.61±15.44	290.29±20.45	269.26±15.02	0.677	0.796
Villus height/crypt depth	8.65±0.56^{b}	10.60±0.71^{ab}	11.53±0.93^{a}	0.033	0.011
		Ileum			
Villus height (µm)	821.30±18.83^{b}	952.93±21.56^{a}	899.10±23.06^{b}	0.004	0.038
Crypth depth (µm)	91.51±4.37	102.48±3.56	100.50±4.78	0.173	0.148
Villus width (µm)	163.94±7.72^{b}	212.25±10.11^{a}	209.96±9.94^{a}	0.018	0.015
Villus height/crypt depth	9.14±0.44	9.34±0.23	9.19±0.63	0.953	0.931

n:10
^{a,b}: Means within a row with different superscripts differ significantly at $P < 0.05$.

The influence of EOAs on intestinal morphology is summarized in Table 5. VH/CD ratio in the jejunum, villus height, and villus width in the ileum exhibited improvements with the addition of EOAs ($P < 0.05$). In contrast, the values of villus height, crypt depth, villus width, and the VH/CD ratio in the duodenum remained unaffected by the addition of EOAs.

Table 6 provides data on the microbial populations in the caecum and blood serum parameters. No significant differences were observed in the total aerobic bacteria in the caecum among the groups. However, the inclusion of EOAs in the diets led to an increase in *Lactobacillus* spp. count ($P = 0.048$) and a decrease in *Enterobacteriaceae* count ($P = 0.009$) in the caecum. Blood serum levels of triglycerides, total cholesterol, and the activities of ALT, ALP, and AST were not significantly affected by the inclusion of EOAs. Notably, there were significant increases in serum IgA levels ($P < 0.001$) and IgG levels ($P = 0.005$) with increasing levels of EOAs in the diets.

Table 7 presents data on the tibia and femur characteristics. Wet weight, length and diameter, and yield load displacement values of the tibia and femur were not significantly influenced by the use of EOAs. However, it was observed that ultimate load ($P = 0.002$), yield load ($P = 0.001$), and stiffness ($P = 0.001$) values were significantly higher with the inclusion of 0.2% EOAs in the diets compared to the other groups in the tibia. In the femur, the ultimate load and yield load values were significantly increased with both levels of inclusion in the diets compared to the control group ($P < 0.001$). Moreover, linear increases ($P < 0.05$) were observed in ash content and the levels of calcium, phosphorus, zinc, and manganese in both tibia and femur bones due to the inclusion of EOAs. Magnesium levels in bones, however, were not affected by the inclusion of EOAs.

Table 6. Effects of combined use of essential oils with organic acids on caecal microorganisms and some blood biochemical indices in broilers

	Mixture of essential oils and organic acids (%)			P-value	
	0	0.1	0.2	Combined	Linear
Caecal microorganisms (log_{10}cfu/g)					
Total aerobic bacteria	6.83±0.09	6.82±0.08	6.83±0.09	0.992	0.955
Lactobacillus	6.32±0.15^{b}	6.75±0.15^{a}	6.81±0.14^{a}	0.048	0.023
Enterobacteriaceae	6.47±0.09^{a}	6.03±0.14^{b}	5.98±0.10^{b}	0.009	0.005
Blood serum biochemical indices					
Total cholesterol (mg/dl)	215.40±10.43	203.10±6.82	193.80±8.61	0.234	0.092
Triglyceride (g/l)	10.80±0.84	9.50±0.83	9.40±0.78	0.414	0.236
ALT (U/l)	18.90±1.19	18.40±1.30	18.80±0.77	0.945	0.950
ALP (U/l)	258.40±17.91	270.00±16.83	259.30±16.45	0.868	0.971
AST (U/l)	121.20±9.16	129.10±8.50	123.30±9.75	0.820	0.872
IgA (mg/dl)	56.00±2.10^{b}	70.30±3.19^{a}	73.10±2.90^{a}	<0.001	<0.001
IgG (mg/dl)	135.30±7.20^{b}	160.40±5.53^{a}	164.90±5.90^{a}	0.005	0.002

n:10
^{a,b}: Means within a row with different superscripts differ significantly at $P < 0.05$.

Table 7. Effects of combined use of essential oils with organic acids on bone parameters in broilers

	Mixture of essential oils and organic acids (%)			P-value	
	0	0.1	0.2	Combined	Linear
Tibia					
Wet weight (g)	15.23±0.37	14.50±0.34	14.81±0.39	0.377	0.429
Length (mm)	99.40±0.97	98.26±0.81	97.41±0.51	0.217	0.084
Diameter (mm)	9.17±0.16	9.17±0.14	9.45±0.29	0.553	0.354
Ultimate load (N)	307.91±14.54^{b}	332.68±12.60^{b}	387.15±15.82^{a}	0.002	0.001
Yield load (N)	261.15±15.84^{b}	294.64±12.83^{b}	343.27±12.33^{a}	0.001	<0.001
Yield load displacement (mm)	2.09±0.14	2.32±0.13	2.37±0.12	0.269	0.135
Stiffness (N/mm)	124.80±5.40^{b}	125.27±5.45^{b}	157.79±7.48^{a}	0.001	0.001
Ash (%DM)	48.29±0.22^{b}	49.12±0.23^{a}	49.45±0.24^{a}	0.005	0.002
Calcium (g/kg DM)	215.10±3.29	220.63±3.21	225.07±2.53	0.084	0.028
Phosphorus (g/kg DM)	106.63±1.04^{c}	110.20±1.18^{b}	113.83±0.99^{a}	<0.001	<0.001
Magnesium (g/kg DM)	4.94±0.08	5.07±0.07	5.05±0.10	0.505	0.375
Zinc (mg/kg DM)	153.91±4.89^{b}	175.81±4.86^{a}	189.44±4.99^{a}	<0.001	<0.001
Manganese (mg/kg DM)	4.36±0.11^{c}	5.06±0.13^{b}	5.76±0.15^{a}	<0.001	<0.001
Femur					
Wet weight (g)	11.39±0.23	11.14±0.24	11.50±0.31	0.619	0.764
Length (mm)	73.98±0.71	72.75±0.25	72.55±0.87	0.277	0.140
Diameter (mm)	10.00±0.15	9.88±0.10	9.71±0.22	0.458	0.218
Ultimate load (N)	255.52±9.04^{b}	296.63±5.99^{a}	320.83±8.18^{a}	<0.001	<0.001
Yield load (N)	231.69±9.04^{b}	264.21±7.42^{a}	293.63±8.77^{a}	<0.001	<0.001
Yield load displacement (mm)	2.83±0.17	2.97±0.08	2.99±0.07	0.567	0.324
Stiffness (N/mm)	81.79±3.67^{b}	89.65±2.25^{ab}	97.02±3.51^{a}	0.009	0.002
Ash (%DM)	47.12±0.28^{b}	48.22±0.25^{a}	48.25±0.27^{a}	0.008	0.006
Calcium (g/kg DM)	210.85±2.12^{b}	215.28±2.31^{ab}	220.22±1.99^{a}	0.017	0.005
Phosphorus (g/kg DM)	104.43±1.36^{b}	108.00±1.36^{ab}	111.63±1.30^{a}	0.003	0.001
Magnesium (g/kg DM)	4.82±0.05	4.87±0.06	4.84±0.09	0.878	0.917
Zinc (mg/kg DM)	160.46±4.49^{c}	181.66±3.46^{b}	195.29±3.52^{a}	<0.001	<0.001
Manganese (mg/kg DM)	4.16±0.12^{c}	4.71±0.15^{b}	5.51±0.13^{a}	<0.001	<0.001

n:10, DM: dry matter ^{a,b,c}: Means within a row with different superscripts differ significantly at $P < 0.05$.

Discussion and Conclusion

Numerous studies have investigated the effects of OAs and EOs when used as feed additives in poultry diets, and these additives have shown positive effects on various aspects of poultry production (1, 25, 40, 41). However, in the present study, no statistically significant differences were observed in body weight, weight gain, feed intake, and FCR among groups that received different levels of EOAs. A noteworthy finding was the significant increase in livability percentages in the groups that received EOAs at 0.1% and 0.2% levels compared to the control group (EOA0), with ($P < 0.001$). This suggests that the inclusion of EOAs in the diets improved livability, although it may not have been at a sufficient level to affect body weight gain and feed efficiency. Similar findings were reported by Kaya and Tuncer (28) and Fascina et al. (16), where growth performance and FCR were not affected by EOAs supplementation. Liu et al. (30) revealed that there were no significant differences in feed intake, weight gain, and FCR among the three groups during the first stage (days 0-21) using the protected EOAs, however, reduced feed intake and improved FCR at 22-42 days of age. Basmacıoğlu-Malayoğlu et al. (8) indicated that EOAs (formic acid, propionic acid, oregano, clove, cumin) did not affect feed intake but had positive effects on body weight gain and feed efficiency in broilers. In another study (21) body weight and weight gain were not affected, while FCR was significantly worsened when the diet was supplemented with a combination of plant extracts and organic acid salts compared to a control diet.

Consistent with our findings, Fascina et al. (16) indicated that EOAs (lactic acid, benzoic acid, formic acid, acetic acid, citric acid, citrus extract, turmeric extracts, grape seed extract + Chinese cinnamon essential oil, fenugreek seeds, Chile Boldo leaves) supplementation did not affect productive efficiency index. EOAs (benzoic acid, thymol, eugenol, piperine) supplementation (5) and different Ps (thymol and cinnamaldehyde, cumin, mint, cloves, and anise or thymol) inclusion (24) significantly increased EPEF. The variability in these findings may be attributed to differences in the chemical composition and dosages of EOs, OAs, and EOAs, as well as differences in diet formulation, diet composition, poultry breed, age, health status, and environmental conditions (8, 18, 34).

In the present experiment, the effects of EOAs on carcass yield showed no significant differences among groups. However, a significant linear increment ($P = 0.039$) in carcass yield was observed with increasing levels of EOAs. The relative weight percentages of abdominal fat, liver, heart, spleen, bursa Fabricius, and gizzard were not significantly affected by the inclusion of EOAs. These results are in line with previous studies (8, 15, 28) where carcass yield and relative organ weight percentages were not significantly affected by EOAs supplementation. Similarly, Dong et al. (14) reported that the percentages of liver, bursa of Fabricius, and abdominal fat were not affected but spleen index value was increased with the usage of EOA. The immune system, in conjunction with lymphoid tissue and immune cells, is largely composed of immune organs. These immune organs, including the thymus, spleen, and bursa Fabricius, are typically responsible for the generation, proliferation, differentiation, and maturation of immune cells (10). In line with the findings of the present study, Liu et al. (30) similarly reported that the usage of protected EOAs did not yield significant differences in the immune organ indexes, specifically the spleen and bursa indexes.

In the present study, the improved VH/CD ratio in the jejunum and enhanced VH in the ileum with the addition of EOAs suggest an improvement in the digestion and absorption efficiency of the diet. The increased counts of *Lactobacillus* spp. ($P = 0.048$) and decreased counts of *Enterobacteriaceae* ($P = 0.009$) in the caecum indicate that the inclusion of EOAs positively affected the intestinal microbiota. These changes in intestinal morphology and microbial populations may have contributed to better digestibility and a reduction in pathogenic coliforms.

Yang et al. (49) found that EOA supplementation during the finisher period increased VH and muscular layer thickness in the duodenum, as well as improved VH and the VH/CD ratio in the jejunum. Similarly, Basmacıoğlu-Malayoğlu et al. (8) reported a significant increase in VH in both the jejunum and ileum following EOA supplementation. Pham et al. (34) also demonstrated that adding different levels of EOAs to broiler diets significantly reduced CD and improved the VH/CD ratio, particularly in the context of a necrotic enteritis challenge. Additionally, Liu et al (29) noted that EOAs enhanced intestinal VH due to an increase in goblet cell content. However, Liu et al. (30) reported that dietary EOA supplementation had no effect on duodenal and ileal morphology at 21 and 42 days, as well as jejunal morphology at 21 days, but significantly increased VH and CD in the jejunum at 42 days of age. In contrast to the present study, Dong et al. (14) observed that EOA supplementation had no influence on small intestinal morphology.

Consistent with the present study, Yang et al. (48) demonstrated that diets supplemented with EOAs enhanced the regenerative capacity of epithelial cells, leading to improved intestinal absorptive capacity, primarily due to a reduction in *E.coli* populations in the ileal contents at 42 d of age. Furthermore, EOAs were found to be effective in protecting the intestinal mucosa, which plays a critical role in safeguarding animals against microbial infections, as noted by Stefanello et al. (40). Giannenas et al (18) reported that a combination of benzoic acid and essential oils improved growth performance, reduced the pH levels in caecal contents, increased lactic acid bacteria populations, and decreased coliform bacteria in the caecum of turkey poults. Several

authors (2, 11, 30) have also suggested that supplementation of EOs, OAs, or EOAs can increase the proportion of *Lactobacillus* spp. in chickens. Similarly, Dong et al. (14) reported that EOA supplementation significantly decreased *E. coli* counts while increasing *Lactobacillus* in excreta. The antibacterial effects of organic acids and essential oils were shown to be synergistic. Essential oils, due to their high hydrophobicity, increase bacterial membrane permeability, allowing more organic acids in their undissociated form to penetrate the bacterial cytoplasm. This process ultimately leads to the death of pH-sensitive bacteria such as *E.coli* (14). In contrast, Pham et al. (34) reported that dietary EOAs did not affect *E.coli* and *Lactobacillus* counts in the caecum.

The antibacterial effects of EOAs against pathogenic bacteria have been explained by various researchers (8, 27) through three main hypotheses: a) the membrane-damaging effect of EOs may render bacteria more susceptible to acidic environments, b) EOs exhibit increased hydrophobicity or antilisterial activity at low pH, making them more soluble in the lipids of bacterial cell membranes, and c) OAs appear to be particularly effective in the feed, crop, and gizzard, while EOs seem to work more efficiently in the lower segments of the intestinal tract.

Another significant finding in the present study is the increase in serum IgA ($P < 0.001$) and IgG ($P=0.005$) levels with higher levels of EOAs in the diet. This suggests that EOAs may have immunomodulatory effects. Both IgA and IgG play crucial roles in the immune system, with IgG being responsible for neutralizing pathogens, toxins, and viruses (39), while IgA serves as a reliable serum biomarker for assessing intestinal inflammation (35). Dong et al. (14) reported that EOA supplementation increased IgA and IgM levels, contributing to enhanced immune status. The rise in serum IgA may be linked to the potential stimulation of B and T lymphocytes (14). Additionally, supplementation with a high level of mixed OAs (including formic acid, ammonium formate, propionic acid, acetic acid, lactic acid, malic acid, citric acid) increased serum IgA levels but had no effect on IgG in broilers aged 42 days of age (31).

In the current study blood serum levels of total cholesterol, triglycerides, and the enzyme activities of ALT, ALP, and AST were not affected by the inclusion of EOAs in the diet. The activities of ALT, AST, and ALP in the blood serve as indicators of liver integrity (3). The findings suggest that EOAs did not have any detrimental effect on liver function. Similarly, Iqbal et al. (20) reported that serum cholesterol levels in the EOA-supplemented group (containing oregano, rosemary, cinnamon, chili pepper extract, and sodium diformate) were comparable to those in the negative control group. Kaya and Tuncer (28) also found that serum triglyceride and cholesterol levels remained unchanged with EOA supplementation. However, Liu et al. (29) observed a significant reduction in triglyceride concentration, while cholesterol levels were unaffected by EOA inclusion. Dong et al (14) reported that EOA treatment significantly decreased the serum total cholesterol levels but had no effect on serum triglyceride levels. Ajibaiye et al. (3) found that different levels of EOAs supplementation had no impact on serum ALT and ALP levels but led to a reduction in serum AST levels at certain supplementation levels. Additionally, Yalçın et al. (46) found that serum cholesterol concentration increased with different levels of lactic acid supplementation in quail fattening, while triglyceride levels remained unchanged.

Ultimate load, yield load, and stiffness values were found to be significantly higher at the usage of 0.2% EOA in the diets than those of other groups in the tibia. The ultimate load and yield load values in femur were significantly increased when EOA was used at both levels in diets compared to the control group. With increasing doses of EOAs usage, linear increases were reported in levels of ash, calcium, phosphorus, zinc, and manganese in the tibia and femur bones. In a study conducted by Sevim and Çufadar (36), it was observed that the tibia Ca, Mg, and P contents were higher in the group that was fed the diet supplemented with thyme essential oil as compared to the control group due to the stimulating effect of thyme essential oil on osteoblast proliferation, and thereby potentially influencing tibia mineral concentrations positively. However, it is worth noting that despite these effects on mineral content, thyme essential oil did not have a significant effect on tibia-breaking strength in broilers (36). In another study by Ruff et al. (35), tibia-breaking strength and total ash content were increased significantly with supplementation of EOs (*Lippia origanoides*, *Rosmarinus officinalis*) or EOs+betaine compared to the control group in chickens exposed to cyclic heat stress. Liu et al. (29) reported that EOA supplementation had no effect on length of tibia and femur. The enhancement of bone characteristics and mineralization could be attributed to the specific composition of the EOAs and their interactions with the broilers' metabolism.

In the evaluation of the results, the dietary inclusion of EOAs could positively affect in various aspects of poultry production such as livability, intestinal health, immunity, and bone mineralization. Further research is needed to optimize the use of EOAs and to better understand the underlying mechanisms responsible for the observed effects. Nevertheless, these findings indicate the potential of EOAs as a viable alternative to antibiotic growth promoters in the poultry industry, supporting intestinal health, immunity, and bone mineralization.

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Ethical Statement

All procedures involving animal care, handling and sampling were approved by the Animal Experiments Local Ethics Committee of the Ankara University, Veterinary Faculty, Ankara, Türkiye (2020-2-14).

Conflict of Interest

The authors declare that there are no conflicts of interest associated with this work.

Author Contributions

The study design and the trial were carried out by İO, HE and SY. All authors performed the analysis. The manuscript was written by İO and SY. All authors approved the final version.

Data Availability Statement

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

Animal Welfare

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

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