Comparative Effects of Herbal Essential Oils, Organic Acids, and Medium-Chain Fatty Acids on Laying Hens: Performance, Egg Quality, and Microbial Activity

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Abstract. *This study compared the effects of herbal essential oils, organic acids, and medium-chain fatty acids, and their sole and decreased mixture on performance, egg quality, blood constituents, and fecal microbial activity in laying hens. A total of 162 Hy-line W80 38-week-old laying hens were allocated into 6 treatment groups with 9 replicates. The treatment groups were as follows: 1) control (C, basal diet, no supplement); 2) supplemented with a herbal essential oil mixture (EOs); 3) 400 mg/kg humic acid (HA); 4) 15 mg/kg medium-chain fatty acids mixture (MCT); 5) EOs + HA + MCT mixture (EHAM); 6) A decreased mixture of EOs + HA + MCT (Mix 1/2).*

The results showed that diet additives did not have a significant effect on the final body weight (BW) of hens (P < 0.05). The feed conversion ratio (FCR) was significantly higher in the control group compared with the groups with supplemented diets (P < 0.01). The egg yield (number and percentage) and the egg mass in supplemented diets were greater than those of the control group (P < 0.01). The Haugh unit, Roche color scale value, eggshell ratio, eggshell surface area, and eggshell unit weight did not show significant differences between the groups. During the whole period, eggshell thickness in all groups with supplemented diets was higher than that of the control group (P < 0.01), and eggshell weight in the Mix 1/2 group was greater than in the control group (P < 0.01). Diet additives did not have a significant effect on egg albumen height and yolk color. Although diet additives did not affect blood parameters, they decreased fecal bacteria counts, such as Escherichia coli, Klebsiella spp., Candida albicans, and Gram-negative bacteria, compared with the control group (P < 0.01). In conclusion, diet additives increased egg yield, egg mass, and eggshell thickness, leading to better feed conversion rates due to their antimicrobial activity, compared with the control group.

Introduction

The increase in world population and changing living standards are the main reasons for improving both the quantity and quality of animal products. Antibiotics were used in animal production as growth promoters, banned in 2006 in Europe and Türkiye, due to the resistance created by antibiotic residues. In the search for natural feed additives, herbal extracts, such as essential oils, organic acids, probiotics, prebiotics, and fatty acids, emerge as promising alternatives to improve not only animal performance and health but also product quality (Buchanan et al., 2008; Świątkiewicz et al., 2013).

Essential oils (EOs) and organic acids have shown antimicrobial and antioxidant properties, contributing to the control of some poultry diseases without the need to use antibiotics, increasing the search for natural feed alternatives to antibiotics (Brenes and Roura, 2010), able to be residue-free (Varel, 2002). Typically, EOs consider combinations of many components, making it difficult to clarify their activities (Russo et al., 1998) Among mixtures, it is possible to find terpenoids, such as geraniol, linalool, thujanol, menthol, borneol, α-terpineol, and citronellal, as well as a variety of reduced-weight molecular aliphatic hydrocarbons, such as phenols thymol, guaiacol, eugenol, carvacrol, cinnamaldehyde, cuminal, and phellandral (Dorman and Deans, 2000).

In addition to the antibacterial, antioxidant, and antifungal properties shown by EOs, they also increase feed intake and digestibility, being used to improve the productive performance in poultry (Chouhan, 2017). Thus, other ingredients originating from plants, consisting of fragrant plant extracts, as well as their purified constituents, have been tested as additives in animal production.

Humic acids (HA) correspond to natural materials with neutral or alkaline pH, capable of transferring electrons and forming chelates with many metal ions. They provide the necessary macro and micronutrients for animals (Arif et al., 2019) and they serve as growth promoters; however, the information about their effects on intestinal health and microbiota composition

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is limited. Kara et al. (2012) and Mudroňová et al. (2021) have reported that HA may positively affect the performance of animals by improving the ecosystem in the gastrointestinal tract which in turn enhances nutrient utilization and poultry health. Furthermore, Disetlhe et al. (2017) have reported that the inclusion of 1.5% HA in canola-based diets positively impacted the absorption and nutrient digestion, also improving bone and immune system advancement in poultry.

Medium-chain triglycerides (MCT) are saturated triglycerides of 4 to 12 carbons, composed mainly of caprylic (C8; 50 ± 80%) and capric fatty acids (C10; 20 ± 50%) with a small contribution of caproic (C6; 1 ± 2%) and lauric (C12; 1 ± 2%) fatty acids (Jadhav and Annapure, 2023). MCTs can be produced from bovine milk, coconut, and palm kernel oil (Jensen, 2002; Nandi et al., 2004). Medium-chain fatty acids are considered the most important type of MCT easily utilizable by non-ruminants, showing a positive impact on gut microbiota, maintaining the intestinal mucosa function, decreasing the pathogen population and inflammation rate, but increasing the useful microbial population in the gut (Jia, 2020).

However, the number of studies reporting the effects of EOs, HA, and MCT in laying hens is scarce. The hypothesis was that herbal essential oils, organic acids, and medium-chain fatty acids have a positive effect on the performance, product quality, and antimicrobial activity in laying hens, especially given in a mixture. This study aimed to determine the effects of herbal essential oils, humic acid, and medium-chain fatty acids and their mixture on the performance, egg quality characteristics, blood parameters, and fecal microbial activity in laying hens.

Materials and methods

Experimental Design and Bird Management

Animal and feeds

Procedures of this animal experiment were approved by the Local Ethics Committee of Erciyes University (Approval No. 20/120). In this study, a total of 162 commercial Hy-line W80, 38-week-old laying hens were used. Before starting the experiment, the live body weights (BW) of the laying hens and followed egg production were recorded and ranked to obtain minimum differences among the groups. The laying hens were placed in cages of 42 × 40 × 46 cm (3 hens/cage), with a total of 54 cages, in 3 floors with 9 replicates (27 hens in each group). After 21 days of adaptation to diets, laying hens were fed for 16 weeks with experimental diets, and the data were collected at 14-day intervals (14 days 8 periods). The lighting schedule was a 16-hour light and 8-hour dark cycle (lighting was obtained from 5:00 am until 9:00 pm). The poultry house had a semi-controlled environmental system.

The diet was formulated according to the nutrient requirements of Hy-line W80 in laying periods. Diet feedstuff and nutritional composition are shown in Table 1. All experimental diets were produced from a basal diet, and all were isocaloric and isonitrogenic; the differences are merely additives. This basal diet showed 89.28% of dry matter, 18.10% of crude protein, 4.70% of crude fat, 4.98% of crude fiber, 12.24% of ash, 2780 Mcal/kg, 3.80% of calcium, 0.66% of available phosphorus, 0.80% of lysine, and 0.40% of methionine. Also, the additives were mixed homogeneously as a premix and added to the experimental feeds (0.75 g/kg). Feed mixtures were prepared every month to be fresh. In determining the doses of feed additives, the values used in practice were taken into account.

Table 1. Nutritional composition of the base formulated diet (LSM + SEM)

Feeds	Ratio, %
Corn	51.62
Sunflower meal (36% CP)	18.00
Soybean meal (46% CP)	9.80
Calcium carbonate	7.95
Dried distiller grain soluble (DDGS)	4.19
Meat-bone meal	3.41
Animal fat	2.24
Molasses	2.00
Salt. NaCl	0.25
Vitamin -mineral premix[1]	0.20
Phytase enzyme 600 U/kg	0.10
DL-Methionine	0.07
L-Lysine	0.07
Sodium bicarbonate	0.05
Mycotoxin binder	0.05
Chemical composition*	
Dry matter, %	89.28
Crude protein, %	18.10
Crude fat, %	4.70
Crude fiber, %	4.98
Ash, %	12.24
Methionine, %[¥]	0.40
Lysine, %[¥]	0.80
Calcium, %[¥]	3.80
Available phosphorus, %[¥]	0.66
Metabolic energy, kcal ME/kg[¥]	2780.0

[1]Vitamin-mineral premix per kilogram of the diet, retinol acetate, 4500 mcg; cholecalciferol, 50 mg; tocopherol acetate, 40.0 mg; menadione, 5.0 mg; thiamine, 3.0 mg; riboflavin, 6.0 mg; pyridoxine, 5.0 mg; cobalamin, 0.03 mg; nicotinic acid, 30.0 mg; biotin, 0.1 mg; calcium d-pantothenate, 12 mg; folic acid, 1.0 mg, choline chloride, 400 mg, manganese, 80.0 mg; iron, 35.0 mg; zinc, 50.0 mg; copper, 5.0 mg; iodine, 2.0 mg; cobalt, 0.4 mg; selenium, 0.15 mg assured. [¥]Compositions were calculated based on NRC (1994) data of feedstuffs.

The base formulated diet for laying hens was included in all the treatments (groups) as follows: 1) control (C, 100% base formulated diet); 2) herbal essential oil (EOs) mixture supplemented diets (including thymol 100 mg/kg, gamma-terpinene 0.77 mg/kg, para-cymene 0.42 mg/kg, carvacrol 1.552 mg/kg, anethole 6.12 mg/kg, limonene 0.81 mg/kg, sabinene 0.44 mg/kg, terpinen-4-0l 0.30 mg/kg): humic acid supplemented diets (0.394 mg/kg, HA); 3) medium-chain fatty acids supplementation (15 mg/kg, MCT); 4) EOs + HA + MCT mixture (EHAM); and 5) a low concentrate (Mix 1/2 of EHAM). The diets and water were offered ad libitum.

Characteristics of performance

The body weight of hens was weighed individually at the beginning and the end of the study. Feed intake was determined every 14 days and feed conversion ratio was calculated (feed consumption: egg mass for each 2, 4, and 8 periods) for each period. Diet intake was figured out by differences between the given feed and the refusals.

Chemical analyses of feed

The chemical composition (dry matter, crude protein, and crude ash) of the feed was analyzed according to established procedures AOAC (2013) of feedstuffs.

Egg production and characteristics

The egg production of hens was recorded daily. Egg yield was calculated through the formula: (number of eggs produced⁄ total number of eggs produced in 14 days) × 100. Egg mass (g/day) was calculated through this formula: egg weight (g) × egg yield (%).

Eggs were collected every 14 days on the last two consecutive days, and 3 eggs were randomly sampled from each cage (27 eggs from each group, a total of 162 eggs) and evaluated for egg weight, shell, and inner egg characteristics. All eggs were numbered to follow during the measurements.

The egg weight, egg yolk Roche color scale values (RSS), albumen height (Hmm), egg yolk height, and Haugh unit (HU) were measured using an Egg Analyzer® (EggAnalyzer, Orka Food Technology LLS, USA). The egg yolk color was defined as brightness (L*), redness (a*), and yellowness (b*), which were measured through a Minolta CR-400 (Minolta Co, Japan) colorimeter. Following the egg evaluation, the shells were washed and dried in an oven at 75°C for 24 hours, and eggshell weights were measured (g) using a precision scale sensitive to 0.1 g. The eggshell ratio was calculated using the formula: (shell weight / egg weight) × 100. The eggshell thickness (µm) was measured with the help of a digital micrometer sensitive to 0.01 mm in the sharp, blunt, and middle parts of the eggshells, and then the shell thickness value was determined through the arithmetic mean of these three measurements. The eggshell weight per unit area (mg/cm^2) was calculated by absolute shell weight (g) / egg surface area (cm^2), according to the formula reported by Carter (1975). The eggshell surface area was calculated using the formula: (3.9782 × egg weight $^{0.7056}$).

Fecal microbial activity

On the 84th day of the experiment, 1 g of fresh fecal samples were taken from each cage to determine fecal *Escherichia coli*, *Klebsiella* spp., *Candida albicans*, *Staphylococcus* spp., total Gram-positive and Gram-negative bacteria populations.

The samples were diluted using 1 mL of saline solution (0.85% NaCl) and were homogenized for 3 minutes. Tenfold dilutions with the sterile solution of physiological salt were prepared from the initial water samples. Then, 5 µL of the examined liquid was taken from each dilution, evenly spread on the surface of the agar media in 3 parallel repetitions and incubated at 37°C for 24 hours. The bacterial microflora concentrations in the samples were determined using 5% sheep blood agar (bioMérieux, France). The eosine methylene blue agar (EMB, bioMérieux, France) was used to determine *Escherichia coli* and total gram-negative bacteria. The chromogenic agar (CHROMagar^{TM}) was used for the identification of *C. albicans*. The microbial counts were determined as colony-forming units (CFU) per gram of samples (Ildız et al., 2018). The microorganism counts were transferred to $\log_{10}$ before statistical analyses.

Blood sampling and analysis of metabolic indicators

At the end of the experiment, 10 cc of blood were taken under the wing from randomly selected laying hens from each group, centrifuged to separate serum, and stored in Eppendorf tubes at –80°C. On the day of analysis, serum was thawed to determine glucose, total protein, triglycerides, cholesterol, high-density lipoprotein (HDL), alanine aspartate aminotransferase (AST), and alanine aminotransferase (ALT) using an autoanalyzer (AMS, VegaSys, Rome Italy) and commercial kits for each parameter.

Statistical analysis

The study was set up and carried out in a completely randomized design. The analysis of variance was performed using the one-way ANOVA procedure of the SPSS statistics program, version 22. Differences between means were analyzed by the Duncan test ($P < 0.05$).

Results and discussion

Performance traits

The initial and final BW, gain, feed intake, and FCR of laying hens with diets supplemented with essential oils, humic acid, and medium-chain triglycerides during 1, 4, 8, and overall periods are shown in Table 2. The LBW did not show any significant effect

Table 2. Effects of essential oils, humic acid, and medium-chain triglycerides on egg performance characteristics of laying hens

Parameter	Treatments						
BW (g)	C	EOs	HA	MCT	EHAM	Mix 1/2	*P*
I-LBW	1594.1 ± 22.20	1579.4 ± 35.34	1566.3 ± 20.67	1585.2 ± 17.82	1630.2 ± 22.87	1600.4 ± 24.02	0.550
F-LBW	1611.6 ± 21.16	1661.1 ± 38.75	1651.7 ± 21.86	1660.3 ± 9.35	1697.8 ± 15.72	1666.1 ± 26.63	0.270
LBW-g	17.5 ± 15.66	81.7 ± 14.64	85.4 ± 15.57	75.2 ± 17.22	67.6 ± 17.75	65.7 ± 21.57	0.090
LBW-%	1.1 ± 0.96	5.2 ± 0.91	5.5 ± 1.01	4.8 ± 1.11	4.2 ± 1.16	4.1 ± 1.39	0.910
Intake(g)							
1	93.41 ± 1.14^{a}	89.54 ± 0.70^{bc}	89.5 5± 0.70^{c}	88.86 ± 0.6^{c}	93.60 ± 0.05^{a}	92.23 ± 1.30 ^{ab}	0.001**
4	102.02 ± 1.07	104.62 ± 1.17	101.55 ± 1.98	100.99 ± 1.08	102.93 ± 1.01	103.41 ± 1.15	0.053
8	108.22 ± 1.3^{b}	112.70 ± 1.2^{a}	113.60 ± 1.4^{a}	107.67 ± 1.6^{b}	114.93 ± 1.1^{a}	113.25 ± 2.0^{a}	0.005**
Total	101.68 ± 0.75^{bcd}	102.40 ± 0.32^{abc}	101.27 ± 0.45^{d}	100.98 ± 0.40^{d}	103.12 ± 0.20^{a}	102.86 ± 0.23^{ab}	0.004**
FCR							
1	1.56 ± 0.36	1.50 ± 0.29	1.48 ± 0.22	1.46 ± 0.23	1.50 ± 0.29	1.48 ± 0.25	0.118
4	1.76 ± 0.047	1.7 7± 0.050	1.66 ± 0.032	1.67 ± 0.032	1.77 ± 0.097	1.69 ± 0.032	0.491
8	2.12 ± 0.11	1.84 ± 0.04	1.82 ± 0.04	1.82 ± 0.05	1.88 ± 0.03	1.90 ± 0.05	0.070
Total	1.81 ± 0.06^{a}	1.69 ± 0.02 ^{b}	1.65 ± 0.02 ^{b}	1.68 ± 0.02 ^{b}	1.70 ± 0.02 ^{b}	1.72 ± 0.02 ^{b}	0.009**

BW (g): body weight; I-LBW: initial live body weight; F-LBW: final live body weight; LBW-g: live body weight gain (g); LBW-%: live body weight gain (%); Intake (g): feed intake; FCR: Feed conversion rate (g feed/g egg mass); C: control (no additive); EOs: essential oils; HA: humic acid; MCT: medium-chain triglycerides; EHAM: a mixture containing 0.75 g/kg of EOs, HA and MCT in the diet; Mix 1/2: a mixture of EHAM at 0.375 g/kg in the diet; Total: total of the 8 periods; *P*: probability; **: $P < 0.01$. $^{a, b, c}$: Differences between the averages are significant in the same column with different letters.

due to the treatments ($P < 0.05$). However, LBW gain almost showed a trend of greater values to the supplemented treatments than the control group ($P > 0.05$). Regarding feed intake, in period 1, it was higher in control and EHAM groups, compared with HA and MCT, but in period 8, the feed intake was greater in all treatments compared with the control group. The total feed intake was higher in EHAM and Mix 1/2 groups, compared with the groups HA and MCT ($P = 0.004$). In period 8, a trend of higher feed conversion ratio (FCR) in the control group compared with the other groups was shown ($P > 0.05$). The total FCR was significantly higher in the control group compared with the supplemented treatments ($P < 0.01$).

Antibiotics have been widely used to promote animal development in livestock production. Nowadays, the use of antibiotics in animals has been restricted only to treatments. Recently, herbal essential oils, organic acids, and some fatty acids have been used instead of antibiotics. However, each of these active substances has different actions. The following should be taken into consideration when using these substances together: firstly, if the activity of one of the substances added provides the expected effects, there may be no need to add another. Secondly, if the additives have a synergistic effect and provide extra benefits when they are added together, and thirdly, if these additives are used together and provide economical contributions by reducing the doses used, it may be beneficial to use more than one active ingredient in the same product, and the products produced in this way can be used commercially and can benefit producers. In this study, the individual and combined effects of using herbal essential oil mixtures, humic acid, and MCT, which have different actions when used as feed additives, were investigated. In the current study, the treatments EOs, HA, and MCT did not affect the final BW of the laying hens. Wang et al. (2019) have reported that 150, 300, and 450 mg/kg of Eos supply had no effect on the LBW of laying hens. Also, in the present study, BW gain was not influenced by the treatments supplemented with EOs, HA, and MCT. It has been reported that essential oil mixture (Olgun, 2016), HA (Hakan et al., 2012), and MCT supply (Wang and Kim, 2011) did not influence the LBW gain of laying hens. Laying hens are not required to receive too much BWG during the feeding period. In addition, live weight losses are not desired. Excessive live weight gain increases basal metabolism and not only consumes more feed but also may cause problems such as prolapse. Decreasing live weight may lead to decreases in egg size and survival rate of the animal. Therefore, the fact that these products do not cause a significant change in live weight can be considered positive. That could be explained as a positive effect of additives on LBW gain; although not significant, there was a trend of higher BW gain in all supplemented treatments compared with the control group.

The total feed intake was higher in EHAM, and Mix 1/2 groups, compared with the HA groups and MCT added groups. Ghanima et al. (2020) have reported that the inclusion of 300 mg/kg of essential oils enhanced the feed intake of laying hens. Also, Marume et al. (2020) have noticed that the addition of 1.0–2.0 g/kg of an essential oil increased the feed intake of laying hens. However, in contrast to others, it has been reported that the addition of 0.1% of an essential oil did not affect the feed intake of laying hens (Bölükbaşi and Erhan, 2007). It has been shown that 2.0, 4.0, and 6.0 mg/kg of HA supply to laying hens' diet (Arafat et al., 2015), and 250, 350, and 450 mL/ton of HA supply in drinking water did not affect the feed intake of laying quails (Kaplan et al., 2018). Regarding the FCR, Liu et al. (2020a) have reported that 300, 600, and 900 mg/kg of EOs supply reduced the FCR of laying hens. Also, it has been reported that 0.5 and 1.0% (Ghanima et al., 2020) or 300 mg/kg of essential oils supply (Radwan et al., 2008) reduced the FCR of laying hens. The current study showed that EO, HA, and MCT additives decreased the FCR compared with the control group. Mudroňová et al. (2021) noted that the addition of HA did not significantly affect feed consumption; however, it positively affected FCR values in laying hens. Similar positive results were observed in FCR values by Arafat et al. (2015) when giving HA by drinking water to laying hens.

Egg production and egg quality traits

The egg characteristics obtained from laying hens with diets supplemented with EOS, HA, and MCT during 1, 4, 8, and overall periods are shown in Table 3. In periods 4 and 8, the egg yield (number and %) was greater in all treatments compared with the control group, following the same trend in egg mass. Although the total egg weight did not show any significant differences between groups ($P > 0.05$), the egg yield (number and %) and the egg mass were greater in all the treatments compared with the control group ($P < 0.01$). The egg weight and the egg mass in period 8 were heavier in all treatments compared with the control group ($P < 0.01$). The

Table 3. Effects of essential oils, humic acid, and medium-chain triglycerides on egg production and egg weights of laying hens

Parameter/ Period	Treatments						
Egg production (number)	C	Eos	HA	MCT	EHAM	Mix 1/2	*P*
1	41.00 ± 0.71	41.56 ± 0.44	41.56 ± 0.34	41.56 ± 0.18	41.89 ± 0.11	41.56 ± 0.29	0.756
4	39.89 ± 0.70[b]	41.44 ± 0.44[a]	41.67 ± 0.17[a]	41.56 ± 0.24[a]	42.00 ± 0.00[a]	41.44 ± 0.38[a]	0.008**
8	37.89 ± 1.57[b]	41.89 ± 0.11[a]	41.89 ± 0.11[a]	40.44 ± 0.44[a]	41.22 ± 0.40[a]	40.78 ± 0.52[a]	0.003**
Total	39.44 ± 0.73[b]	41.67 ± 0.17 [a]	41.56 ± 0.24[a]	41.44 ± 0.24[a]	41.56 ± 0.18[a]	41.00 ± 0.17[a]	0.001**
Egg yield (%)							
1	97.67 ± 1.65	98.89 ± 0.1.11	99.00 ± 0.78	99.11 ± 0.35	99.78 ± 0.22	98.89 ± 0.73	0.750
4	94.78 ± 1.67[b]	98.67 ± 1.11[a]	99.33 ± 0.33[a]	99.00 ± 0.58[a]	100.00 ± 0.00[a]	98.67 ± 0.90[a]	0.005**
8	90.22 ± 3.73[b]	99.78 ± 0.22[a]	99.78 ± 0.22[a]	96.33 ± 1.05[a]	98.11 ± 0.96[a]	97.00 ± 1.28[a]	0.003**
Total	94.22 ± 1.88[b]	99.56 ± 0.34[a]	98.89 ± 0.42[a]	98.22 ± 0.49[a]	98.56 ± 0.38[a]	97.89 ± 0.35[a]	0.001**
Egg weight (g)							
1	61.27 ± 0.79	60.34 ± 0.80	61.49 ± 0.82	61.70 ± 0.82	62.58 ± 0.97	63.12 ± 0.79	0.243
4	61.32 ± 0.58	60.23 ± 0.87	61.84 ± 0.54	61.07 ± 0.81	59.43 ± 0.67	62.12 ± 0.65	0.671
8	57.74 ± 1.00[b]	61.62 ± 0.74[a]	62.51 ± 0.88[a]	61.59 ± 0.75[a]	62.47 ± 0.80[a]	61.43 ± 0.80[a]	0.002**
Total	61.27 ± 0.79	61.05 ± 0.40	61.95 ± 0.62	61.31 ± 0.45	61.77 ± 0.68	61.42 ± 0.61	0.434
Egg mass (g)							
1	59.86 ± 1.52	59.72 ± 1.10	60.82 ± 0.78	61.05 ± 0.85	62.42 ± 1.05	62.48 ± 1.05	0 .318
4	58.08 ± 1.20	59.45 ± 1.21	61.35 ± 0.53	60.41 ± 0.71	59.43 ± 2.67	61.27 ± 067	0.526
8	51.95 ± 1.97[b]	61.46 ± 0.81[a]	62.34 ± 0.91[a]	59.30 ± 0.84[a]	61.31 ± 0.97 [a]	59.62 ± 0.94[a]	0.001**
Total	56.76 ± 109[b]	60.71 ± 0.54 [a]	61.30 ± 0.63[a]	60.22 ± 0.52[a]	60.88 ± 0.75 [a]	60.15 ± 0.68[a]	0.001**

[a, b, c]: Differences between the averages are significant in the same column with different letters. C: control (no additive); EOs: essential oils; HA: humic acid; MCT: medium-chain triglycerides; EHAM: a mixture containing 0.75 g/kg of EOs, HA and MCT in the diet; Mix 1/2: a mixture of EHAM at 0.375 g/kg in the diet; Total: total of the 8 periods; *P*: probability; **: $P < 0.01$.

total egg mass showed heavier values in all treatments compared with the control group ($P < 0.01$).

The diets supplemented with EOs, HA, and MCT increased the egg yield compared with the control group ($P < 0.001$). These EOs results are consistent with the ones reported by Bölükbaşi and Erhan (2007), Ding et al. (2017); Liu et al. (2020a). However, it has also been described that the EOs did not affect the daily egg production of laying hens (Arpášová et al., 2015; Olgun, 2016; Yu et al., 2018). The HA has been shown to improve the egg production of laying hens (Yörük et al., 2004; Arpášová et al., 2016; Mudroňová et al., 2021). In the present study, the egg mass increased when the diets were supplemented with EOs, HA, and MCT compared with the control ($P = 0.001$). Similar results were observed in other studies where the EO (Yu et al., 2018; Ghanima et al., 2020; Marume et al., 2020) and HA (Ozturk et al., 2009; Mudroňová et al., 2021) addition to diets increased egg mass of the laying hens.

The egg yolk characteristics obtained from laying hens with diets supplemented with essential oils, humic acid, and medium-chain triglycerides during 1, 4, 8 and overall periods are shown in Table 4. Although the egg yolk brightness showed significant differences in period 4 ($P < 0.01$). The total egg yolk brightness (L*) did not show any significant differences between the groups in the other and overall period ($P > 0.05$). The egg yolk redness was only significant in period 8 ($P > 0.05$), but the total egg yolk redness was not significant ($P > 0.05$). Although the egg yolk yellowness showed significant differences between the groups in period 1 ($P > 0.01$), , the total egg yolk yellowness was similar in periods 4, 8, and overall ($P > 0.05$). There were no significant differences between the groups in terms of albumen height (Hmm), Roche color scale (RCS), and Haugh unit (HU) in eggs.

The eggshell characteristics obtained from laying hens with diets supplemented with essential oils, humic acid, and medium-chain triglycerides are shown in Table 5. During periods 1, 4, and 8, eggshell ratio and eggshell weight UA did not show any significant differences between the groups ($P > 0.05$). The treatments showed a greater eggshell surface area in period 8 compared with the control ($P > 0.01$). The total eggshell ratio, eggshell surface area (without period 8), and eggshell unit area weight (ESUW) did not show any significant differences between the groups ($P > 0.01$).

The eggshell thickness (during periods 4 and 8) and the total eggshell thickness were higher in all treatments compared with the control group ($P < 0.01$). The eggshell weight of the MCT and Mix 1/2 addition group was significantly greater compared with the control and EOs groups; also, total eggshell weight was greater in Mix 1/2 compared with the control group ($P < 0.01$).

Diet additives did not have any significant effect on egg yolk height, brightness, redness, and yellowness ($P > 0.05$). These results are consistent with the reports showing that MCT and HA did not have any effect on egg yolk color (Arpášová et al., 2016; Bozkurt et al., 2016; Liu et al., 2020; Wang et al., 2019). It was also reported that EOs and MCT could not have any effect on egg yolk color (Wang and Kim, 2011; Klementavičiute et al., 2016). Since the EO, MCT, and HA additives do not contain colorants, egg yolk color was not influenced by these feed additives and their mixture.

The diets supplemented with EOs, HA, and MCT did not have any significant effect on the total RSS, HU, eggshell ratio, eggshell surface area, and eggshell weight UA ($P > 0.05$). Olgun (2016) has reported that the EO mixture addition to the laying hen diet did not have any effect on egg-specific gravity and shell weight. However, it has been reported that the essential oils (Özek et al., 2011; Ding et al., 2017), MCT, and HA (Hakan et al., 2012) addition increased the egg HU according to the control treatment.

The total eggshell thickness was higher in all treatments compared with the control ($P = 0.000$). Olgun (2016) and Torki et al. (2018) have reported that EOs improved the eggshell thickness. In contrast to this result, Florou-Paneri et al. (2005) have demonstrated that the dietary supplementation of rosemary, oregano, and saffron did not affect eggshell thickness. Liu et al. (2020a) reported that medium-chain α-monoglycerides increased the eggshell thickness. However, the HA addition tested no effect on eggshell thickness (Macit et al., 2021). Although it has been reported that the egg weight was increased when the diets were supplemented with essential oils, MCT, and HA addition (Özek et al., 2011; Ghanima et al., 2020; Liu et al., 2020b). In the current study, the total eggshell weight was greater only in the Mix 1/2 group compared with the control group ($P = 0.007$). As reported by Olgun (2016), in the present study, EOs did not show any significant differences in eggshell weight compared with the control (Arpášová et al., 2015; Wang et al., 2019). Also, it has been reported that EOs (Torki et al., 2018) and HA addition (Hakan et al., 2012; Macit et al., 2021) did not have any significant effect on eggshell weight. However, it has also been reported that EOs (Bayram et al., 2007) and HA (Sopoliga et al., 2016) addition to diets could decrease eggshell weight.

Fecal microbial activity

The fecal microbial activity (CFU/mL) in laying hens with diets supplemented with essential oils, humic acid, and medium-chain triglycerides are shown in Table 6. The control group showed the greatest microbial population of *E. coli, Klebsiella* spp., *C. albicans,* and total Gram-negative bacteria than other treatments ($P < 0.01$). Similarly, *Staphylococcus* spp. and the total Gram-positive bacteria were higher in the control and Mix 050 groups, compared with the other groups ($P < 0.01$). On the other hand, HA showed the lowest amounts of *E. coli, Klebsiella* spp., *Staphylococcus* spp.*, and C. albicans*.

In the current study, the diets supplemented with EOs, HA, and MCT addition positively decreased the microbial populations of fecal *E. coli, Klebsiella* spp., *Staphylococcus* spp.*, C. albicans,* total Gram-positive, and total Gram-negative bacteria, compared with the control group of laying hens. It has been reported that the essential oils showed a strong antibacterial activity (Bakkali et al., 2008; Brenes and Roura, 2010; Karásková et al., 2015), which is consistent with the results obtained in the current study. Basile et al. (2006) have reported that the essential oils showed a broad antibacterial spectrum for both, Gram-positive and Gram-negative bacterial strains. Akyurek and Yel (2011) have reported that thymol and carvacrol essential oils showed some antimicrobial properties

Table 4. Effects of essential oils, humic acid, and medium-chain triglycerides on egg internal quality characteristics of laying hens

Parameter/ Period	Treatments						
Albumen height (hmm)	C	EOs	HA	MCT	EHAM	Mix 1/2	*P*
1	3.98 + 0.12	3.83 ± 0.12	3.96 ± 0.17	4.03 ± 0.17	3.69 ± 0.18	4.10 ± 0.23	0.623
4	4.55 ± 0.10	4.39 ± 0.20	4.27 ± 0.20	4.52 ± 0.19	4.24 ± 0.10	4.37 ± 0.21	0.760
8	4.17 ± 0.29	4.29 ± 0.25	4.52 ± 0.15	4.56 ± 0.22	4.39 ± 0.19	4.20 ± 0.29	0.803
Total	4.24 ± 0.10	4.38 ± 0.08	4.15 ± 0.09	4.39 ± 0.10	4.26 ± 0.33	4.30 ± 0.9	0.393
Haugh Unit, HU							
1	57.79 ± 1.21	54.41 ± 2.47	56.56 ± 189	56.76 ± 2.34	53.83 ± 2.59	57.58 ± 1.96	0.699
4	62.96 ± 1.24	60.20 ± 2.68	59.33 ± 2.32	62.41 ± 1.94	58.80 ± 1.62	60.75 ± 2.32	0.672
8	60.50 ± 3.00	58.99 ± 3.19	62.53 ± 1.61	61.66 ± 2.88	58.98 ± 2.62	57.89 ± 3.58	0.858
Total	59.45 ± 0.78	60.25 ± 0.99	57.29 ± 1.19	60.54 ± 1.29	58.56 ± 0.45	59.99 ± 0.87	0.178
Egg yolk RCS							
1	8.30 ± 0.13	7.81 ± 0.26	8.15 ± 0.28	8.15 ± 0.20	8.00 ± 0.22	8.45 ± 0.16	0.391
4	4.89 ± 0.11	5.00 ± 0.29	4.67 ± 0.17	5.11 ± 0.26	4.78 ± 0.15	4.89 ± 0.11	0.657
8	4.89 ± 0.12	4.93 ± 0.17	4.74 ± 0.09	4.71 ± 0.14	4.85 ± 0.13	4.89 ± 0.12	0.805
Total	5.38 ± 0.07	5.35 ± 0.07	5.44 ± 0.09	5.38 ± 0.07	5.36 ± 0.06	5.46 ± 0.11	0.917
Brightness (L*)							
1	65.46 ± 1.21	63.79 ± 0.92	61.98 ± 0.70	63.88 ± 1.50	63.42 ± 1.65	64.29 ± 0.32	0.437
4	64.61 ± 0.33[a]	63.60 ±0.41[ab]	62.62 ± 038[bc]	63.72 ± 0.21[a]	62.25 ± 0.41[c]	59.07 ±0.34[d]	0.001**
8	59.61 ± 0.32	60.50 ± 0.55	60.09 ± 0.53	60.67 ± 1.06	61.48 ± 0.32	61.60 ± 0.56	0.173
Total	63.12 ± 0.24	62.58 ± 0.37	62.06 ± 0.35	62.56 ± 0.22	62.10 ± 0.15	61.46 ± 0.18	0.210
Redness (a*)							
1	5.05 ± 0.17	4.83 ± 0.14	4.71 ± 0.08	4.82 ± 0.12	4.82 ± 0.12	4.80 ± 0.09	0.506
4	4.26 ± 0.07	4.45 ± 0.12	4.59 ± 0.10	4.80 ± 0.39	4.63 ± 0.09	4.40 ± 0.08	0.359
8	4.49 ± 0.15[a]	4.83 ± 0.15[ab]	4.95 ± 0.08[b]	4.72 ± 0.08[ab]	4.96 ± 0.13[b]	4.92 ± 0.08[b]	0.047*
Total	4.53 ± 0.04	4.59 ± 0.09	4.69 ± 0.07	4.62 ± 0.18	4.67 ± 0.08	4.53 ± 0.06	0.788
Yellowness (b*)							
1	46.36 ± 0.98[a]	44.00 ± 0.64[bc]	41.93 ± 0.55[c]	45.18 ± 0.63[ab]	43.94 ± 0.67[bc]	45.49 ±0.89[ab]	0.002
4	42.32 ± 0.80	42.26 ± 0.45	42.86 ± 0.64	42.05 ± 0.62	40.58 ± 0.69	40.31 ± 0.96	0.080
8	40.34 ± 0.54	38.76 ± 0.67	39.51 ± 0.53	38.86 ± 0.86	40.65 ± 0.75	40.55 ± 0.46	0.152
Total	42.26 ± 0.34	41.98 ± 0.23	41.36 ± 0.47	42.16 ± 0.29	41.75 ± 0.36	41.95 ± 0.35	0.417

[a, b, c]: Differences between the averages are significant in the same column with different letters. L*: egg yolk brightness; Redness (a*): egg yolk redness; Yellowness (b*): egg yolk yellowness; C: control (no additive); EOs: essential oils; HA: humic acid; MCT: medium-chain triglycerides; EHAM: a mixture containing 0.75 g/kg of EOs, HA and MCT in the diet; Mix 1/2: a mixture of EHAM at 0.375 g/kg in the diet; Total: total of the 8 periods, *P*: probability; *: $P < 0.05$; **: $P < 0.01$.

after their inclusion in a diet of one-day-old broiler chicks. Essential oils supply increased beneficial microbes, such as *Lactobacillus* and yeast, while decreasing the pathogenic *Escherichia coli* amounts in the gut. Shermer et al. (1998) explained that humates could inhibit the pathogenic microbes and stimulate beneficial microbes in the gut but without any effect on anaerobic microbial amounts. Mudroňová et al. (2020) have reported that HA addition improved the stimulation and engulfing activity of phagocytes and gut health status by reducing the pathogenic microbial, such as *Enterobacteriaceae* amounts. Liu et al. (2020b) have noticed that the medium-chain α-monoglycerides are considered an efficient feed supplement to improve the production performance by modulating intestinal microflora. Medium-chain α-monoglycerides decreased the gut microbial population such as *Schlegelella and Proteobacteria*. Hermans et al. (2011) have reported that different types of MCT supply (lauric acids, caprylic acids, capric acids, and caproic acids) reduced the colonization of *Campylobacter* in the broiler intestine. In other words, essential oils could have a strong antibacterial activity, shown by reducing the pathogenic microbial, such as *E. coli, Klebsiella* spp., *Staphylococcus* spp., *C. albicans,* total Gram-positive, and total Gram-negative bacteria, improving the gut health status and, therefore, the FCR, increasing the egg production and mass and eggshell thickness, but not showing any significant effect on egg yolk characteristics.

Blood parameters

Finally, the plasma indicators (mg/dL) of laying hens supplemented with diets with essential oils,

Table 5. Effects of essential oils, humic acid, and medium-chain triglycerides on eggshell quality characteristics of laying hens

Parameter	Treatments						
	C	EOs	HA	MCT	EHAM	Mix 1/2	*P*
ES thickness (µm)							
1	0.43 ± 0.003	0.43 ± 0.006	0.43 ± 0.002	0.43 ± 0.003	0.44 ± 0.002	0.43 ± 0.003	0.304
4	0.42 ± 0.004^{b}	0.43 ± 0.003^{a}	0.43 ± 0.002^{a}	0.43 ± 0.002^{a}	0.43 ± 0.002^{a}	0.43 ± 0.003^{a}	0.001**
8	0.41 ± 0.005^{b}	0.43 ± 0.003^{a}	0.43 ± 0.003^{a}	0.43 ± 0.003^{a}	0.43 ± 0.003^{a}	0.43 ± 0.002^{a}	0.001**
Total	0.42 ± 0.002^{b}	0.43 ± 0.002^{a}	0.43 ± 0.001^{a}	0.43 ± 0. 001^{a}	0.43 ± 0. 001^{a}	0.43 ± 0. 001^{a}	0.001**
ES weight (g)							
1	6.06 ± 0.11	6.00 ± 0.11	6.19 ± 0.12	6.19 ± 0.06	6.22 ± 0.08	6.28 ± 00.7	0.275
4	6.07 ± 0.08	6.13 ± 0.08	6.17 ± 0.09	6.15 ± 0.05	6.05 ± 0.05	6.12 ± 0.08	0.860
8	5.64 ± 0.09^{c}	6.13 ± 0.06^{bc}	6.16 ± 0.05^{ab}	6.07 ± 0.07^{a}	6.22 ± 0.09^{a}	6.12 ± 0.08^{ab}	0.001**
Total	5.94 ± 0.0 ^{b}	6.03 ± 0.0 ^{ab}	6.09 ± 0.03^{ab}	6.06 ± 0.02^{ab}	6.07 ± 0.02^{ab}	6.11 ± 0.05^{a}	0.007**
ES ratio (%)							
1	9.90 ± 0.28	9.94 ± 0.26	10.09 ± 0.45	10.07 ± 0.50	9.97 ± 0.74	9.95 ± 0.22	0.935
4	10.14 ± 0.19	9.88 ± 0.22	10.07 ± 0.20	9.96 ± 0.15	10.21 ± 0.12	10.20 ± 0.16	0.731
8	10.28 ± 0.19	10.06 ± 0.12	10.15 ± 0.15	10.18 ± 0.10	10.10 ± 0.20	10.09 ± 0.13	0.924
Total	10.28 ± 0.06	10.35 ± 0.04	10.36 ± 0.03	10.36 ± 0.05	10.22 ± 0.06	10.21 ± 0.03	0.052
ESSA (mm)							
1	72.57 ± 0.66^{ab}	71.79 ± 0.67^{b}	72.75 ± 0.69^{ab}	72.92 ± 0.68^{ab}	73.65 ± 0.81^{ab}	74.11 ± 0.65^{a}	0.243
4	72.61 ± 0.49	71.69 ± 0.73	73.05 ± 0.45	72.39 ± 0.68	70.89 ± 2.35	73.27 ± 0.54	0.660
8	69.58 ± 0.85^{b}	72.86 ± 0.61^{a}	73.59 ± 0.73^{a}	72.83 ± 0.62^{a}	73.56 ± 0.66^{a}	72.70 ± 0.67^{a}	0.002**
Total	71.78 ± 0.55	72.38 ± 0.34	73.14 ± 0.52	72.60 ± 0.38	72.99 ± 0.57	72.69 ± 0.52	0.434
ESUW (mg)							
1	8.35 ± 0.09	8.35 ± 0.09	8.51 ± 0.13	8.50 ± 0.11	8.46 ± 0.18	8.48 ± 0.56	0.842
4	8.37 ± 0.14	8.56 ± 0.16	8.45 ± 0.15	8.50 ± 0.11	8.63 ± 0.36	8.35 ± 0.11	0.891
8	8.06 ± 0.09	7.85 ± 0.11	8.00 ± 0.08	8.24 ± 0.06	8.24 ± 0.09	8.06 ± 0.12	0.051
Total	8.28 ± 0.07	8.26 ± 0.02	8.24 ± 0.05	8.31 ± 0.06	8.20 ± 0.06	8.30 ± 0.04	0.709

$^{a, b, c}$: Differences between the averages are significant in the same column with different letters. C: control (no additive); EOs: essential oils; HA: humic acid; MCT: medium-chain triglycerides; EHAM: a mixture containing 0.75 g/kg of EOs, HA and MCT in the diet; Mix 1/2: a mixture of EHAM at 0.375 g/kg in the diet; RSS: Roche color scale values; HU: Haugh unit; ES: eggshell; ESSA: eggshell surface area, ESUW (mg): eggshell weight unit weight; Total: total of the 8 periods; *P*: probability; **: $P < 0.01$.

humic acid, and medium-chain triglycerides are shown in Table 7. The plasma glucose, triglyceride, cholesterol, HDL, and LDL were not affected by the different treatments ($P > 0.05$). Similar to this study, it was shown that the effects of essential oil mixtures (Ghanima et al., 2020), humic acids (Mudroňová et al., 2021), and medium-chain fatty acids (Liu et al., 2022) added to the rations on the blood values of laying hens were not significant. It is desired that feed additives do not cause significant changes in the blood indicators of animals. This is because a stable plasma glucose level indicates that there is no energy metabolism and metabolic diseases in animals. Similarly, triglycerides are important in terms of energy metabolism and the synthesis of new substances in the body and their change is undesirable. Cholesterol is involved in the structure of brain membranes and other cell synthesis, and high blood cholesterol levels are an undesirable blood component in terms of the emergence of cardiovascular diseases.

Conclusion

Compared with the control group, EOs, humic acid, medium-chain fatty acids and their combination and decreased ratios of the combination improved laying hens' performance traits, egg production and eggshell thickness. Also, these additives decreased bacterial loads in feces. So, the addition of EOs, humic acid, medium-chain fatty acids and their combination may act as a performance enhancer and an antibacterial agent. They may benefit the animal health status by reducing the pathogenic microbial activity in the gut. Future studies should also consider other parameters, such as diet digestibility and beneficial bacteria along the productive life of laying hens.

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Table 6. Effects of essential oils, humic acid, and medium-chain triglycerides on fecal microbial activity (CFU/mL) in laying hens

Groups	*Escherichia coli*	*Klebsiella* spp.	*Staphylococcus* spp.	*Candida albicans*	Total Gram + bacteria	Total Gram-bacteria
C	4.8 ± 0.01[a]	4.3 ± 0.04[a]	4.2 ± 0.03[a]	3.9 ± 0.01[a]	4.7 ± 0.02[a]	5.0 ± 0.01[a]
Eos	2.7 ± 0.05[c]	3.3 ± 0.08[c]	2.8 ± 0.13[d]	2.6 ± 0.05[d]	3.5 ± 0.09[c]	3.5 ± 0.08[d]
HA	2.3 ± 0.29[d]	3.0 ± 0.10[d]	2.1 ± 0.27[e]	2.3 ± 0.07[e]	3.1 ± 0.06[d]	3.4 ± 0.04[d]
MCT	3.5 ± 0.10[b]	3.5 ± 0.08[c]	3.4 ± 0.03[c]	2.6 ± 0.10[d]	4.1 ± 0.05[b]	4.5 ± 0.04[c]
EHAM	2.9 ± 0.04[c]	3.3 ± 0.07[c]	3.6 ± 0.06[bc]	3.4 ± 0.04[c]	4.4 ± 0.02[b]	4.4 ± 0.03[c]
Mix 1/2	3.4 ± 0.03[b]	3.7 ± 0.03[b]	3.9 ± 0.01[ab]	3.6 ± 0.02[b]	4.6 ± 0.01[a]	4.6 ± 0.01[b]
P	0.001**	0.001**	0.001**	0.001**	0.001**	0.001**

[a, b, c]: Differences between the averages are significant in the same column with different letters. C: control (no additive); EOs: essential oils; HA: humic acid; MCT: medium-chain triglycerides; EHAM: a mixture containing 0.75 g/kg of EOs, HA and MCT in the diet; Mix 1/2: a mixture of EHAM at 0.375 g/kg in the diet; *P*: probability; **: $P < 0.01$.

Table 7. Effects of essential oils, humic acid, and medium-chain triglycerides on blood serum indicators (mg/dL) of laying hens

Groups	Glucose, mg/dL	Triglyceride, mg/dL	Cholesterol, mg/dL	HDL, mg/dL	LDL, mg/dL
C	205.4 ± 7.03	914.0 ± 217.23	128.2 ± 27.89	16.3 ± 31.61	38.3 ± 52.27
Eos	206.6 ± 7.00	1071.4 ± 187.58	116.0 ± 24.78	24.3 ± 15.38	29.7 ± 29.12
HA	201.6 ± 1.75	1155.4 ± 133.24	117.4 ± 25.04	21.9 ± 19.90	77.7 ± 41.79
MCT	211.2 ± 5.00	1009.0 ± 132.80	105.6 ± 18.84	19.3 ± 15.36	66.1 ± 28.51
EHAM	212.4 ± 3.41	1168.8 ± 124.49	105.0 ± 13.63	12.0 ± 7.97	116.8 ± 19.23
Mix1/2	206.6 ± 3.39	1140.8 ± 102.21	124.8 ± 21.47	38.1 ± 22.15	48.3 ± 35.43
P	0.680	0.833	0.976	0.960	0.573

C: control (no additive); EOs: essential oils; HA: humic acid; MCT: medium-chain triglycerides; EHAM: a mixture containing 0.75 g/kg of EOs, HA and MCT in the diet; Mix 1/2: a mixture of EHAM at 0.375 g/kg in the diet.

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