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Response of laying hens fed diet supplemented with a mixture of olive, laurel, and rosemary leaf powders: Metabolic profile, oxidative status, intestinal histomorphology, and egg quality

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ABSTRACT

This study aimed to evaluate the effects of a mixture of olive, laurel, and rosemary leaf powders, on the oxidative state, biochemical, immune, intestinal morphophysiological parameters, and egg quality of laying hens. One hundred Lohmann Brown hens (28 weeks old) were equally assigned to two groups (n. 50) corresponding to a basal control diet (CON) or the diet supplemented with 6 g/kg feed of leaf powder mixture (LPM) containing olive, laurel, and rosemary leaves (1:1:1), for 60 days. Oxidative status, biochemical indices, immune response, cecal short chain fatty acids (SCFAs), intestinal morphological characteristics, and some egg traits were evaluated at the end of the experiment. The results indicated that LPM improved ($P < 0.05$) the oxidative status (TOS, ROMs), the immune system (IL-6, IL-1β, and TNF-α), the total protein and HDL cholesterol content, whereas it decreased ($P < 0.05$) total cholesterol and LDL cholesterol. Aspartate aminotransferase (AST), alkaline phosphatase (ALP), and alanine aminotransferase were significantly ($P < 0.05$) lower in the LPM than in the CON group. A significant increase ($P < 0.05$) in SCFA content in the caecum, as well as in villi height and crypt depth in both duodenum and ileum of LPM-treated hens, was observed. Egg quality parameters were not influenced ($P > 0.05$) by LPM. These findings indicate that LPM can be considered a candidate as an antioxidant ingredient for functional food in laying hens.

1. Introduction

Various stress factors are linked to poultry production that compromise the health and productivity of laying hens and broilers (Surai et al., 2019; Li et al., 2021). Phytogenic feed supplements, such as intact herbs, spices, and their extracts, have favorable effects on the health and performance of poultry due to their secondary metabolites content, mainly polyphenols (Abdel-Moneim et al., 2020). In poultry, the use of polyphenols has been suggested to counteract oxidative stress (Surai et al., 2019; Mishra and Jha, 2019), in order to prevent the development of metabolic disorders. The beneficial impact of polyphenols includes the improvement of the antioxidant status and the anti-inflammatory effects (Gadde et al., 2017; Abd El-Hack et al., 2022; Seidavi et al., 2022) as well as intestinal health by influencing intestinal microflora, gut morphophisiology, nutrient digestion and absorption, and the performance of laying hens and broilers (Sharma et al., 2020; Mahfuz et al., 2021; Darmawan et al., 2024).

Phytogenic feed additives can be used in solid, dried, and ground form or as extracts (Gadde, 2017). In the present study, a blend of three leaves (*Olea europaea L.*, *Laurel nobilis L.*, and *Rosmarinus officinalis L.*) was used in laying hens because of their reported biological properties, as described below.

Olive leaves have biological characteristics such as antioxidant properties (Bulotta et al., 2011), anti-inflammatory effects (Cavaca

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et al., 2020), and inhibition of low-density lipoprotein (Jemai et al., 2008), attributed to their content in polyphenols. Olive leaves contain >30 phenolic compounds, primary secoroids and oleuropein, which are the most abundant bioactive compounds (Huang et al., 2019; Nediani et al., 2019). It has been observed that dietary supplementation with olive leaf or their extracts positively affects performance in hens (Cayan and Erener, 2015; Papadopoulos et al., 2023) and in broilers (El-Damray et al., 2013; Almuhayawi et al., 2023; Erener et al., 2023).

Laurus nobilis L., is an aromatic and medicinal plant, that belongs to the *Laureacea* family. Abundant phenolic compounds have been found in laurel (Škerget et al., 2005; Lu et al., 2011; Said and Hussein, 2014). Antioxidant activity such as prevention of oxidative stress, anti-inflammatory, antimicrobial, and inhibition of lipid peroxidation properties has been reported (Carocho and Ferreira, 2013; Dias et al., 2014).

Rosemary (*Rosmarinus officinalis* L.), a herb of the family Labiatae, is a spice that has been recognized as an anti-inflammatory, immunomodulatory, and antioxidant agent (De Oliveira et al., 2019). In laying hens, Alagawany and Abd El-Hack (2015) showed that dietary supplementation of rosemary powder improved blood parameters.

Some phytogenic combination treatments are reported to have additive or synergistic effects attributable to the interaction between the active compounds present in the different leaves. In laying hens, dietary supplementation with five types of mixed Chinese medical herbs resulted in increased production performance (Yu et al., 2023). In broilers, a diet supplemented with 14 herbs improved productive efficiency and performance (Guo et al., 2004).

The high levels of antioxidant compounds present in the leaves of olive, laurel, and rosemary plants, along with their antioxidant properties, make them highly promising in combating harmful substances associated with various stressors in poultry production (Dias et al., 2014; Sahin and Bilgin, 2018; Nieto et al., 2018). Therefore, the aim of this study was to determine wether addition of a phytogenic blend constituted by olive, laurel, and rosemary leaves, as a source of antioxidant compounds in the feed, improves the health status of laying hens, by evaluating biochemical blood profile and oxidative status, caecal compound characteristics, intestinal morphology, and some egg quality parameters.

2. Materials and methods

The experimental protocol of the study and implemented animal care procedures were approved by the Institutional Ethics Committee of the Department of Emergencies and Organ Transplantation of the University of Bari (Prot n. 04/2020).

2.1. Plant material collection and analysis

The olive, rosemary, and laurel leaves were harvested during the spring on private farms practing organic farming in the agricultural area of Bari. Subsequently, the collected leaves were properly dried and ground to pass through a 2 mm screen. The individual plant leaves were then evaluated for proximate composition according to AOAC (2002) standards (Table 1). The total content of polyphenols (Fig. 1) was determined for the characterization of individual leaves and their leaf powder mixture (LPM, 1:1:1) using the Folin-Ciocalteau method (Singleton et al., 1999). Extraction of the lipophilic compounds from leaves powder samples was carried out in EtOH (80%; 1:10, *w/v*) for 15 min. The extract was filtered through a 0.45-μm PTFE syringe filter (CH4525-NPL, Fisher Scientific Italia, Rodano, Milano) and stored at −20 °C. All procedures were conducted in the dark to avoid oxidation. For the assay, 2.5 mL Folin–Ciocalteu reagent, 2 mL of 7.5% aqueous sodium carbonate solution, and 0.5 mL of lipophilic extract were mixed well. After 90 min of storage at room temperature (20–25 °C) in the dark, the absorbance of the mixture was read at 765 nm with a UV–Visible spectrophotometer (PerkinElmer Lambda 15; PerkinElmer Italia Spa, Milano). A mixture of solvent and reagents was used as a blank matrix. The TPC was expressed as milligram of gallic acid equivalents (GAE) per gram of dry weight, using a gallic acid standard curve (0–250 mg/L). Data were reported in Table 1.

Table 1
Chemical composition of olive, laurel, and rosemary leaves (% DM; X ± SD).

	Olive	Laurel	Rosemary
Dry matter (%)	60.57 ± 0.95	30.76 ± 0.83	31.91 ± 0.80
Crude protein	9.66 ± 0.61	5.13 ± 0.31	6.40 ± 0.02
Ether extract	2.34 ± 0.51	1.42 ± 0.71	12.85 ± 0.15
Ash	7.15 ± 0.36	3.75 ± 0.34	7.24 ± 0.93
Crude fiber	24.09 ± 1.25	12.67 ± 0.60	26.20 ± 1.80
Neutral detergent fiber	46.12 ± 1.58	23.85 ± 3.93	45.34 ± 1.01
Acid detergent fiber	28.52 ± 0.87	14.69 ± 0.69	33.17 ± 0.69
Acid detergent lignin	19.46 ± 0.81	9.63 ± 1.94	22.23 ± 0.53
Acid insoluble ash	0.20 ± 0.02	0.11 ± 0.09	0.35 ± 0.05

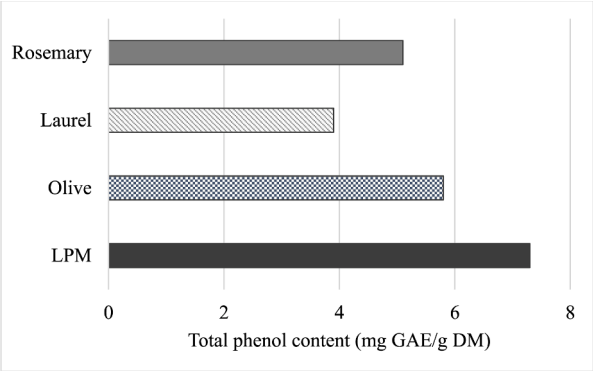
2.2. Animal, diets, and experimental design

The trial was performed in a private farm located in Apulia, South Italy (latitude 41 04′N, 17 05′E, 5 m s.l.m.). The trial was conducted in autumn and lasted 60 days, involving a total of one hundred Lohmann Brown laying hens that were 28 weeks old with an initial body weight of 1820 ± 140 g. All hens were vaccinated according to the vaccination schedule required by commercial systems. The experimental period spanned from 28 to 36 weeks of age.

The hens were allocated to two equal groups based on their weight, corresponding to the dietary treatments. The dietary treatments consisted of a commercial basal diet, designed as the control (CON), and the basal diet supplemented with 6 g/kg feed of the experimental leaf powder mixture (LPM). The dosage of 6 g/kg feed was established based on careful bibliographic research, with a standard ratio among the leaves (1:1:1; 2 g/kg olives leaves +2 g/kg laurel leaves +2 g/kg rosemary leaves). The feed for laying hens was prepared according to the nutritional requirements reported by NRC (1994), and the nutrition levels are shown in Table 2a, 2b.

2.3. Animal housing

Considering that a rearing system with access to an open area offers a high welfare potential for laying hens (Sokołowicz et al., 2020), the two experimental groups of hens were reared using an indoor-outdoor



Total phenol contents were determined as milligrams of gallic acid equivalents (GAE) per gram of dry weight. LPM contained 2 g olive leaves + 2 g laurel leaves + 2 g rosemary leaves.

Fig. 1. Total phenol content of olive, laurel, and rosemary leaves, and leaf powder mixture (LPM). Total phenol contents were determined as milligrams of gallic acid equivalents (GAE) per gram of dry weight. LPM contained 2 g olive leaves +2 g laurel leaves +2 g rosemary leaves.

Table 2a
Composition of the control diet (%).

Ingredients	
Corn	57.60
Soybean meal (46% CP)	22.00
Sunflower meal (36% CP)	6.00
Limestone granular	6.00
Limestone finely ground	3.30
Soyabean oil	2.50
Dicalcium phosphate	1.50
Vitamin and mineral premix[1]	0.50
Sodium chloride	0.20
Sodium bicarbonate	0.15
Methionine (MHA)[2]	0.14
Lysine	0.09
Magnesium oxide	0.02

[1] Supplied per kilogram of diet: vitamin A, 10,000 IU; vitamin D_3, 3000 IU; biotin, 0.08 mg; choline chloride, 350.00 mg; folic acid, 0.80 mg; niacin, 29.50 mg; vitamin B_1, 1.96 mg; vitamin B_{12}, 0.02 mg; vitamin B_2, 4.00 mg; vitamin B_6, 3.96 mg; vitamin E, 25.00 mg; vitamin K_3, 1.79 mg; enzymes: endo-1,4-betaglucanasi; endo-1,4-betaxilanasi; 3-fitasi; iron, 48.00 mg; iodine, 1.02 mg; manganese, 80.60 mg; calcium D-pantothenate, 8.91 mg; selenium, 0.15 mg; zinc, 79.2 mg; copper, 12.50 mg. [2]methionine hydroxy analogue.

Table 2b
Composition of the control diet (%).

Ingredients	
Metabolizable energy (kcal/kg)	2700
Crude protein (%)	17.80
Crude fat (%)	4.40
Calcium (%)	4.10
Phosphorus (%)	0.45
Methionine (%)	0.31
Lisine	0.74
Arginine	0.70
Threonine	0.37
Leucine	0.74
Isoleucine	0.43
Valine	0.46
Histidine	0.25
Phenylalanine	0.48
Triprophan	0.13

rearing system. This system comprised two pens (4 m^2/hen), one for each experimental group, separated by a 2 m high net to prevent their mixing. The surface of the outdoor area consisted of soil without plant cover.

Each enclosure featured a poultry house with a low density of 6 hens/m^2, with windows (1/15 area ratio) placed along one side to allow natural light to enter. The floor inside was made of a mixture of straw and wood chips; there were perches and individually nesting birds (30 × 45 cm, height 60 cm, 1 nest/6 hens), along with 2 circular feeders and bell-shaped drinkers, were provided. Two drinkers and 2 perches also were available in the outdoor area. The hens had free access to the outdoor enclosure through the pop hole (12 in.) located on the wall of the hen house (external option from 08:00 to 18:00). Feed and water were offered ad libitum. A photoperiod of 16 h light and 8 h darkness was maintained using artificial lighting.

2.4. Blood sampling and analyses

At the end of the experimental period (36th week), approximately 2 mL of blood samples were drawn from the brachial vein of 30 hens randomly selected from each experimental group. These samples were collected in plastic vacuum tubes (BD Vacutainer Advance, Becton Dickinson, NJ, USA).

The blood samples were then centrifuged at 3000 rpm (1814 *g*) for 15 min, and the serum aliquots were stored at −20 °C until the analysis of biochemical, immunological, and antioxidtive parameters.

Blood biochemical characteristics were determined using an automated biochemical analyzer (TC-220 TECOM, Jiangxi, China) using commercial kits according to the colorimetric method. The concentration of total protein (TP, monitored at 540 nm), triglycerides (TG, monitored at 505 nm), total cholesterol (TC, monitored at 505 nm), high-density lipoprotein-cholesterol (HDL-C, monitored at 570 nm), low-density lipoprotein (LDL), alkaline phosphatase (ALP, monitored at 405 nm), total bilirubin (monitored at 555 nm) and aspartate aminotransferase (AST) were estimated using diagnostic kits produced by SPINREACT (Sant Esteve de Bas, Girona, Spain). Non esterified fatty acids (NEFA, monitored at 550 nm) were determined in fresh serum immediately after the blood sampling, using a commercial kit produced by Randoz Laboratories Ltd. (Crumlin, County Antrim, UK). Alanine aminotransferase (ALT) was determined (at 340 nm) using a commercial kit produced by PRO-EKO (Petacciato, Campobasso, Italy).

2.5. Determination of oxidative status

The serum oxidant/antioxidant markers were assessed spectrophotometrically. Total oxidative status (TOS) was measured as described by Erel (2004); results were given as micromolar H_2O_2 equivalent/L. Measurements of total antioxidant status (TAS) were represented as described by Erel (2005), using Trolox equivalent/L units. ROM values were determined spectrophotometrically (wavelength 505 nm), using a commercial kit (Diacron, Grosseto, Italy), according to the manufacturer's instruction. Results were expressed in Carr units (1 U/Carr corresponds to 0.024 mmol/L of H_2O_2).

2.6. Determination of cytokines

The level of inflammatory cytokines IL-1β, TNFα, and IL-6 was measured using corresponding assay kits provided by Immunological Sciences (Rome, Italy), following the manufacturer's instructions. A Microplate reader TECAN infinite M1000Pro plate reader (Tecan, Mannedorf, Switzerland) was used for the measurements.

2.7. Intestinal tissue sampling and analyses

At the conclusion of the experiment, a total of 8 animals from each experimental group were slaughtered. The intestinal tract was removed for SCFA analyses and histologic measurements.

2.7.1. Short chain fatty acid (SCFA) analyses

The contents of the caecum of 8 animals were extracted by finger pressure, collected in Eppendorf tubes, and stored at −20 °C until analyzing the short-chain fatty acids (SCFAs).

SCFA concentrations were determined using gas chromatography following a method reported by Lashkari et al. (2014) with slight modification. Briefly, samples were blended with deionized water for 15 min. The homogenate was then filtered through filter paper (Whatman grade 1; Sigma Aldrich, Darmstadt, Germany), and the filtrate (5 mL) had meta-phosphoric acid (25%, wt/vol; 1 mL) added. After shaking (10 min), volatile fatty acids were extracted with toluene and quantified by flame ionization detection gas chromatography using the Agilent 6890 A GC system equipped with an FID detector (Agilent 7890 A GC, Agilent Technology Italia Spa, Roma, Italia) and capillary column (SACtm-5 column 300 cm × 0.25 mm, Supelco, USA). Fatty acids were identified and quantified on the basis of standard elution times (Volatile Free Acid Mix Supelco, Bellefonte, PA, USA).

2.8. Histological morphometry

2.8.1. Sampling and tissue preparation

Segments of approximately 3 cm were collected from the duodenum and ileum of 6 animals and fixed in 4% (*v*/v) phosphate-buffered-saline paraformaldehyde for 24 h at 4 °C. Following fixation, the samples underwent dehydration through a graded series of ethanol and were then embedded in paraffin wax. Serial sections (5 μm thick) were cut, and after de-waxing with xylene and hydratation in a descending ethanol series, they were stained with Hematoxylin-eosin for morphological and morphometric studies.

2.8.2. Morphometry analysis

Hematoxylin-eosin-stained sections of 10 well-oriented villi and crypts of the duodenum and ileum of each animal were photographed using a light microscope (Eclipse Ni-U; Nikon, Japan) with a 4 X lens. These images were utilized to measure the villus height (VH) and crypt depth (CD). Subsequently, the ratio of the villus height to crypt depth (VH:CD) was calculated.

2.9. Recorded egg quality

At the conclusion of the experiment, a total of 30 eggs from each experimental group were randomly collected over 3 days for the analysis of egg quality.

The collected eggs were individually weighed using an electronic balance (0.1 g sensitivity). Upon breaking the eggs, their contents were separated using an egg separator and placed on wet paper to remove the white albumen. Subsequently, the egg white, yolk, and shell were weighed individually using an electronic balance (0.1 g sensitivity). Eggshell thickness was measured from the middle part of the egg using a micrometer (25 M-5, Ames).

The egg yolk coloration was measured visually according to the RYCF (Roche Yolk Colour Fan; Hoffmann-La Roche Ltd., Basel, Switzerland), based on a scale of colors from 1 (light pale) to 15 (dark orange).

The following egg quality indexes were calculated:

$$\text{Yolk ratio} = \text{yolk weight (g)}/\text{egg weight (g)} \times 100;$$

$$\text{Albumen ratio} = \text{albumen weight (g)}/\text{egg weight (g)} \times 100;$$

$$\text{Shell ratio} = \text{shell weight (g)}/\text{egg weight (g)} \times 100.$$

2.10. Statistical analysis

Data collected from biochemical, immunological, and antioxidant assays, as well as the body weight of the animals and egg quality parameters, were analyzed using statistical software (SPSS software for Windows, release 23.0., Inc., Chicago, IL, USA). The difference between the means of the experimental groups was calculated using a one-way analysis of variance (ANOVA). Differences were considered statistically significant at $P < 0.05$, while $0.05 < P < 0.10$ indicated a trend. Data on morphometric measurements were evaluated for statistical significance using Student's *t*-test. *P* values were two-tailed, and a P value <0.05 was considered significant. The values were expressed as mean ± standard deviation (S.D.).

3. Results and discussion

3.1. Biochemical parameters

To our knowledge, this research is the first study on the effects of dietary supplementation with mixtures of olive, rosemary, and laurel leaves in laying hens. Therefore, the discussion of the results will be carried out by comparison with similar studies that have investigated dietary supplementation with individual leaves or their extracts.

Blood parameters represent the main index for evaluating the physiological, nutritional, and general pathological status of animals. Biochemical serum constituents showed significant differences between dietary treatments (Table 3). The results indicated that dietary inclusion of 6 g/kg feed of the LPM resulted in a significant increase in serum total protein ($P < 0.05$), and improvements in the lipid profile for the increase ($P < 0.05$) in HDL cholesterol, and reduction in total cholesterol ($P < 0.05$) and LDL ($P < 0.01$) levels. There was no significant difference ($P > 0.05$) observed between the groups in the serum content of NEFA.

The increased levels of blood proteins in the LPM group may indicate an enhancement in digestibility and metabolism, as previously reported by Attia et al. (2015).

The decrease in cholesterol levels is in agreement with other studies investigating olive leaf supplementation in other avians such as broiler chicks (Erener et al., 2020) and Japanese quails (Sarica and Toptas, 2014). Hypolipidemic effect and antioxidant activities of olive leaves have been attributed to their content in triacetylated hydroxytyrosol, hydroxytyrosol, and oleuropein activities (Cayan and Erener, 2015; Talhaoui et al., 2015). The presence of oleuropein and hydroxytyrosol in olive leaves is known to prevent LDL oxidation and to inhibit 3-hydroxy-3-methyglutaryl coenzyme A, which plays an important role in cholesterol synthesis in the liver (Cayan and Erener, 2015). In addition, Ahmed et al. (2017) indicated that the hypocholesterolemic action of phenolic compounds in olive leaves may be due to the inhibition of dietary cholesterol absorption in the intestine, or by its production by the liver, or stimulating the biliary secretion and fecal excretion of cholesterol. Elazab et al. (2022) reported that a possible explanation for the reduction of cholesterol is the stimulation of the conversion of cholesterol into bile acids that are excreted from the body through enterohepatic circulation. The conversion of cholesterol to bile acids through the stimulation of the hepatic activity of cholesterol-7-hydroxylase is a key pathway for the removal of cholesterol from the body (Chiang and Ferrell, 2020). Our results agree also with Radwan et al. (2008) who showed that adding rosemary to the diet decreased total lipid, total and LDL cholesterol levels in hens. Additionally, Ali and Al-Shuhaib (2021) observed that the addition of crushed laurel leaves (2–3 g/kg of feed) to the diet led to an increase in the concentration of HDL and a decrease in TC, TG, and LDL in broilers.

The current study revealed that the addition of the LPM to the hens' diet had no adverse effect on egg yolk cholesterol content (9.63 vs 9.59 mg/g yolk; data not shown). These results indicate a non-correlative response between blood cholesterol levels and the cholesterol content of egg yolk, aligning with the findings of other authors (Wasburn and Nix, 1974; Rahimi, 2005). A number of studies showed that yolk cholesterol content remains relatively constant, and is not influenced by dietary factors (Bertechini, 2003; Abdel-Moneim et al., 2020; Sharma et al., 2020), because it is required to ensure embryo development (Shafey and Cham, 1994). The liver is the main site of cholesterol

Table 3
Effect of dietary leaf powder mixture (LPM) supplementation on biochemical parameters in laying hens at the end of the experiment.

	Dietary treatment		SEM	*P* value
	CON	LPM		
Animals, n.	30	30		
Total protein, g/L	61.52	62.61	0.23	0.015
Tryglicerides, mg/dL	796.42	669.93	35.70	0.076
Total cholesterol, mg/dL	151.48	125.73	6.19	0.037
HDL cholesterol, mmol/L	20.61	24.36	0.63	0.002
LDL cholesterol, mg/dL	143.69	135.12	1.33	0.001
NEFA, mmol/L	1.39	1.31	0.04	0.331

CON, control basal diet; LPM, diet supplemented with leaf powder mixture, 6 g/kg feed containing 2 g olive leaves +2 g laurel leaves +2 g rosemary leaves. SEM: standard error mean. HDL = high density lipoprotein; LDL = low density lipoprotein, NEFA = not esterified fatty acids.

synthesis, which accumulates cholesterol from the blood during the synthesis of lipoproteins. Plasma LDL is the main carrier transporting endogenous cholesterol. According to Griffin (1992), yolk precursors are synthesized in the liver of laying hens and transported in the plasma to the ovary. Therefore, the cholesterol content of the yolk is primarily dependent on the cholesterol content of triglyceride-rich lipoproteins. Inhibition of cholesterol synthesis can reduce the rate of synthesis and secretion of lipoproteins by the liver, but it has minimal effect on the composition of the lipoproteins that are secreted. Fennema (1993) stated that variations in total yolk lipid content are more influenced by bird genetic strain than dietary factors.

3.2. Liver enzymes

The effects of experimental treatments on liver enzymes are presented in Table 4.

In hens supplemented with the LPM, significant differences ($P < 0.05$) in the serum activity of ALT, AST, and ALP were observed compared to the control group. There were no significant differences between groups in bilirubin levels.

There is little information in the literature on the dietary effects of plant leaves and spices on the liver. Measurement of the serum activities of AST, ALT, and ALP, which are indicative of liver injury, serves as a valuable tool in determining a safe inclusion rate of non-conventional feed or feed additives in birds (Diaz et al., 2003). The high activity of AST, ALP, and ALT in the blood is a bioindicator of the existence of liver injury (Valchev et al., 2014).

The results of this study indicate that LPM may have a hepatoprotective effect in hens, attributable to the antioxidant properties of its components, present in olive and rosemary leaves, as well as to their combined effect (Al-Attar and Shawush, 2015), and in laurel leaves (Mohammed et al., 2021). This finding is consistent with several studies that have reported a significant decrease in AST, ALT, and ALP levels due to the free radical scavenging activity of antioxidant phytochemicals present in plant leaves or extracts, which inhibited physiological and histopathological alterations (Khalil, 2004; Abdel-Wahhab et al., 2011; Al-Attar and Shawush, 2015; Abdel-Azeem et al., 2018).

3.3. Oxidative status

The effect of the dietary LPM supplement on the oxidative status of hens is reported in Table 5.

The serum levels of total oxidative status (TOS) and ROMs were significantly lower ($P < 0.05$) in the hens treated with the LPM compared to the control. Additionally, the LPM group showed a tendency towards higher total antioxidant status (TAS) compared to the control (469.71 vs 393.78 μmol TRX/L; $P < 0.10$).

One of the objectives of this study was to evaluate the effects of the LPM blend of olive, rosemary, and laurel on the oxidative state of the hens. The polyphenol content was higher in olive leaves, followed by rosemary and laurel (Fig. 1), in accordance with the results of other research (Škerget et al., 2005; Vallverdú-Queralt et al., 2014; Falade et al., 2022). Additionally, LPM showed the highest TPC content (Fig. 1). This result highlights the greater effectiveness of the mixture of olive leaves, laurel, and rosemary compared to individual leaves. It has been shown that most phenolic compounds possess antioxidant activity both in vitro and in vivo. Wenk (2002) reported that the performance enhancement associated with the supplementation of poultry diets with plant-origin materials is mainly due to their polyphenolic contents. In this study, the antioxidant activity of the LPM could be attributed to its major polyphenol constituents, and probably mainly to oleuropein and its derivative hydroxytyrosol for olive leaves (Benavente-García et al., 2000; Sahin and Bilgin, 2018), to 1.8 cineole for laurel leaves (Chahal et al., 2017; Singletary, 2021), and to the antioxidant properties of carnosol and carnosic acid for rosemary (Aruoma et al., 1992; Loussouarn et al., 2017). In particular, most phenolic compounds in the olive leaves showed antioxidant activity in vitro (Silva et al., 2006) and in vivo (Jemai et al., 2008). Oleuropein supplementation increased TAS and reduced TOS in quails subjected to heat stress (Sarica et al., 2017).

The lower level of ROMs observed in the serum of LPM-treated hens (Table 5) expresses the capability of LPM to reduce free radical production and oxidative stress, which is associated with favorable TOS and TAS levels. Positive effects on oxidative status have been observed in other species of animals using supplementation with rosemary or laurel leaf (lambs, Khayyal et al., 2021; rabbits, Elazab et al., 2022), or their aqueous extracts (rats, Falade et al., 2022). On the other hand, mixed proportions of herbs and plants would influence the antioxidant properties (Liu et al., 2016) since the total antioxidant capacity is attributed to their additive and/or synergistic effect (Wang et al., 2019), which may align with the results obtained in this study.

3.4. Immunomodulatory effects

In our study, LPM supplementation decreased ($P < 0.05$) IL-6, IL-1β and TNF-α concentrations compared to the CON (Table 6), suggesting its suppressive effects on systemic inflammation in hens.

Inflammation represents a common final pathway related to

Table 4
Effect of dietary leaf powder mixture (LPM) supplementation on serum levels of total bilirubin and ALT, AST, ALP in laying hens at the end of the experiment.

	Dietary treatment		SEM	P value
	CON	LPM		
Animals, n.	30	30		
ALP, IU/L	275.71	261.98	2.92	0.017
AST, IU/L	186.27	176.10	1.22	<0.001
ALT, IU/L	17.16	16.18	0.11	<0.001
Bilirubin, mmol/L	0.99	1.03	0.30	0.299

CON, control basal diet; LPM, diet supplemented with leaf powder mixture, 6 g/kg feed containing 2 g olive leaves +2 g laurel leaves +2 g rosemary leaves. SEM: standard error mean.
ALP = alkaline phosphatase; AST = aspartate aminotransferase; ALT = alanine aminotransferase enzyme.

Table 5
Effect of dietary leaf powder mixture (LPM) supplementation on oxidative status of laying hens at the end of the experiment.

	Dietary treatment		SEM	P value
	CON	LPM		
Animals, n.	30	30		
TOS, μmol H_2O_2 Eq/L	23.06	17.97	0.67	0.002
TAS, Trolox Eq/L	393.78	469.71	24.74	0.11
ROMs, UCarr	39.29	28.07	1.74	0.001

CON, control basal diet; LPM, diet supplemented with leaf powder mixture, 6 g/kg feed containing 2 g olive leaves +2 g laurel leaves +2 g rosemary leaves. SEM: standard error mean. TOS = total oxidative status; TAS = total antioxidant status; ROMs = reactive oxigen metabolites.

Table 6
Effect of dietary leaf powder mixture (LPM) supplementation on serum levels of TNF-α, IL-1β and IL-6 in laying hens at the end of the experiment.

	Dietary treatment		SEM	P value
	CON	LPM		
Animals, n.	30	30		
TNF-α, pg/mL	25.85	21.07	1.10	0.029
IL-1β, pg/mL	69.71	57.42	2.66	0.02
IL-6, pg/mL	21.61	15.95	1.16	0.013

CON, control basal diet; LPM, diet supplemented with leaf powder mixture, 6 g/kg feed containing 2 g olive leaves +2 g laurel leaves +2 g rosemary leaves. SEM: standard error mean.
TNF-α = tumor necrosis factor; IL-1β = interleukine-1β; IL-6 = interleukine-6.

oxidative processes. The molecular mechanisms of the interaction between oxidative stress and inflammation, and their interaction with cell death are unclear (Wu et al., 2020). The antioxidant properties and immunological effect of phytochemical dietary additives deserve attention as strong oxidation affects the health of laying hens.

Miliaraki et al. (2022), in an oxidant/antioxidant pathway study, reported an expression of the cytokines TNF-α and IL-6 strongly correlated with an increase in the TOS/TAC ratio. This finding is in agreement with our results where the leaf powder blend supplementation in the laying hens' diet was effective in enhancing their antioxidant ability and immune system.

Our results are in line with previous findings regarding the separate use of individual plants and their effects on the immune system (Vezza et al., 2017). Beneficial effects on the immune system are reported for olive leaf extract (Kaneko et al., 2019; De Cicco et al., 2020), laurel leaves (Lee et al., 2019), and rosemary leaf powder (Farouk et al., 2022).

3.5. Histology of the small intestine

The intestinal mucosa consisted of villi, which are finger-like projections, and basal crypts (Fig. 2A,B). Villi were covered by a simple columnar epithelium in which goblet cells were scattered (Fig. 2C). Histological observations revealed a clear decrease in villi height from the duodenum to the ileum (Fig. 2A, B) in both the CON and LPM fowls, with no histological lesions observed.

Histology measurements of the intestine were performed to monitor the effects of the LPM on villus height or crypt depth. The results of the morphometric analysis of the small intestine are displayed in Table 7.

Supplementation with leaf powder mixture induced a significant ($P < 0.05$) increase in the VH in the duodenum (1046.57 ± 19.55 μm vs 1121.73 ± 20.80 μm) and ileum (482.44 ± 10.84 μm vs 593.15 ± 15.71 μm). Moreover, LPM supplementation significantly increased the CD of the duodenum (303.44 ± 11.41 μm vs 245.78 ± 5.99 μm) and the ileum (201.05 ± 7.05 μm vs 148.24 ± 3.08 μm). The treatment induced a decrease of VH: CD in the duodenum (3.69 ± 0.06 vs 4.25 ± 0.03, $P < 0.05$) and in the ileum (2.95 ± 0.01 vs 3.25 ± 0.03, $P = 0.145$).

The histomorphometric analysis revealed that LPM induces a significant increase in the VH of the duodenum and the ileum of laying hens. This increase in intestinal VH is related to the enhancement of the absorptive surface area, attributed to a higher expression of brush border enzymes capable of facilitating greater digestion and absorption of available nutrients (Rezaei et al., 2018). Furthermore, LPM supplementation produced a significant increase in the CD of the duodenum and the ileum. This result supports the increase in VH, as intestinal crypts consist of proliferating cell types that differentiate into villus epithelial cells (see Galosi et al., 2021 for references).

Table 7
Effect of the dietary leaf power mixture (LPM) supplementation on the intestinal morphology of laying hens ($n = 3$) (x ± SD).

Item	CON	LPM	*P*-value
Duodenum			
Villus height (μm)	1046.57 ± 199.55	1121.73 ± 211.15	0.004
Crypt depth (μm)	245.78 ± 59.35	303.44 ± 11.84	<0.001
Villus height: crypt depth	4.25 ± 1.39	3.69 ± 1.24	0.046
	Ileum		
Villus height (μm)	482.44 ± 102.28	593.15 ± 148.24	<0.001
Crypt depth (μm)	148.24 ± 30.58	201.05 ± 77.25	<0.001
Villus height: crypt depth	3.25 ± 1.09	2.95 ± 0.61	0.145

CON, control basal diet; LPM, diet supplemented with leaf powder mixture, 6 g/kg feed containing 2 g olive leaves +2 g laurel leaves +2 g rosemary leaves.

It has been shown that an increase in the depth of crypts, which serve as a cellular source for intestinal epithelium, is an indicator of the cell renewal rate. Villus height and crypt depth represent an indirect indication of the maturity and functional capacity of enterocytes; longer villi and crypts indicate a greater number of enterocytes (Hampson, 1986). Crypt lengthening is known to be influenced by a range of factors including local, immune, and neuro–humoral factors, as well as diet composition (Desantis et al., 2021; Galosi et al., 2021; Pearson and Brownlee, 2010). It has been reported that CD is directly representative of the intestinal environment and may be used as an indicator of intestinal health (Meimandipour et al., 2011).

Compared to the control group, the VH:CD ratio decreased in LMP-supplemented laying hens in both the duodenum and ileum. There are no studies on the histological effects of adding rosemary and laurel to the diet on the avian intestine, whereas a few studies have investigated the effect of adding olive on the small intestine. As in this study, an increase in CD and a lower VH:CD ratio has been found in the duodenum and jejunum of broilers supplemented with olive pomace (Herrero-Encinas et al., 2021). This result was interpreted as a presumed increase in epithelial turnover. However, our findings do not completely match the results obtained by Pirman et al. (2021) in broilers fed with olive leaf extract. In that investigation, no difference was detected in the VH:CD ratio of the duodenum, whereas a reduction of the VH:CD ratio was observed in the ileum. It has been suggested that the variation in the results in different trials may depend on the composition of the

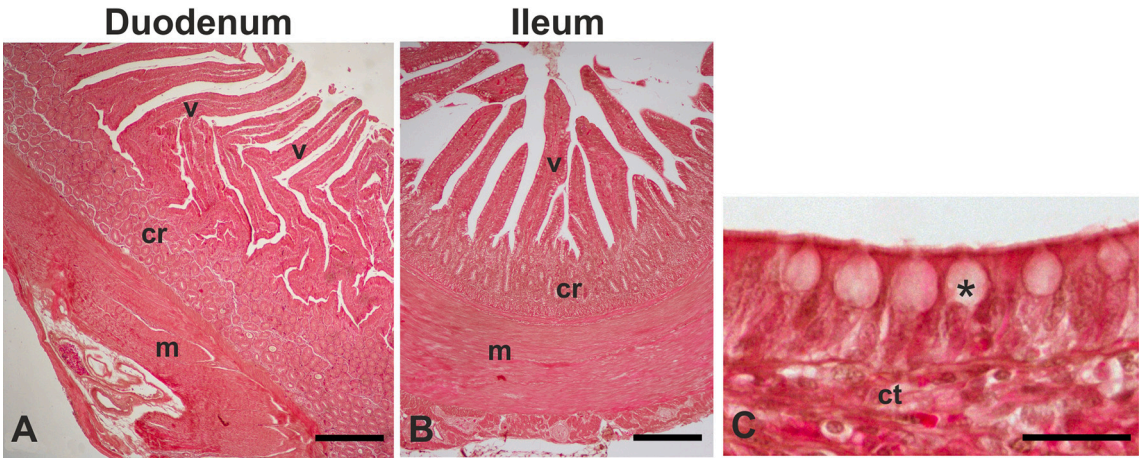


Fig. 2. Representative histological view of the duodenum (A) and ileum (B) of the laying hens. These low-magnification pictures show the height of villi is larger in the duodenum than in the ileum; on the contray, the duodenum presents a lower muscular thickness than the ileum. Note the crypts located at the base of the villi. C, Image at high magnification of villus epithelium showing the presence of the goblet cells. cr, crypts; ct, connective tissue; m, muscularis; v, villum; asterisk, goblet cell. Hematoxylin-Eosin staining. Scale bars: A = 500 μm; B, = 250 μm; C = 20 μm.

phytogenic feed additives, the level of application, or methods for extracting bioactive compounds from herbs (Cross et al., 2007).

3.6. Concentration of short chain fatty acids (SCFAs) in caecum contents

The caecum is the main site for microbial fermentation, where undigested carbohydrates are transformed into short-chain fatty acids (Jamroz et al., 2002). Their concentrations may vary according to the dominant microbiota (Gong et al., 2019), which in turn affects the gut function.

The results of concentrations of SCFAs are shown in Table 8.

Most of the SCFAs analyzed in the caecum chymus varied according to the diet of the laying hens. The acetate concentration in the LPM group, accounting for the largest proportion of total SCFAs, was significantly higher ($P < 0.05$) than that in the CON group. Likewise, a higher ($P < 0.05$) concentration of propionic acid, butyric acid, and isobutyric acid was found in the LPM group compared to the CON group. No significant differences in the levels of isocaproic and heptanoic acids were observed between the experimental groups.

In this study, acetate production was the highest, followed by propionate, with butyrate showing the lowest cumulative production, consistent with findings from other researchers (Gong et al., 2019; Oost et al., 2021). Isobutyric acid was significantly higher ($P < 0.05$) in the LPM group than in the CON group.

The production of propionate and acetate seems to be associated with the increased presence of *Bacteroides* spp. (Salonen et al., 2014), while butyrate is produced mainly by the *Firmicutes phylum* (Baothman et al., 2016). Lastly, isobutyric acid is mainly synthesized by genera *Bacteroides* and *Clostridium* (Aguirre et al., 2016).

The observed increase in SCFAs in the LPM group may have contributed to improve intestinal functionality and metabolism. Short chain fatty acids are an important source of energy for enterocytes: up to 50% of their daily requirement (Awad et al., 2016). The three main SCFAs play a role in modulating intestinal epithelial cell function promoting intracellular permeability through the regulation of tight junction and zonulin expression or distribution (Feng et al., 2018). Acetic acid suppresses intestinal apoptosis, promotes mucin production (Liu et al., 2017), inflammasome activation (Macia et al., 2015) and intestinal barrier integrity (Nowarski et al., 2015). Butyrate has been shown to promote gut microbiota balance (Borda-Molina et al., 2021), enhance intestinal barrier function (Mathewson et al., 2016), and increase mucin activity via MUC2 gene expression (Gaudier et al., 2004). The protective effects of propionate are also evident in the intestinal barrier (Tong et al., 2016). The short-chain fatty acids derived from the intestine are assimilated in the metabolism of host carbohydrates and lipids (Besten et al., 2013). Propionate can become a substrate for gluconeogenesis in the liver (Brüssow and Parkinson, 2014), in which acetate can contribute to cholesterol synthesis (Boets et al., 2017).

In this study, the effect of LPM on gut microbiota was not investigated. However, it is worthy of consideration that the polyphenol content in leaves can modulate the microbial composition. It has been reported that olive leaf extract significantly stimulates the abundance of beneficial *Lactobacillus* and *Bifidobacterium* spp. while reducing the abundance of *Escherichia coli* in the caecum (Xie et al., 2022).

Table 8
Effect of dietary leaf powder mixture (LPM) supplementation on cecal concentration of short chain fatty acids (mg/g DM) in laying hens at the end of the experiment.

	Dietary treatment		SEM	P value
	CON	LPM		
Animals, n.	8	8		
Acetic acid	20.71	28.11	1.34	0.022
Propionic acid	4.79	10.85	0.62	0.046
Isobutyric acid	0.83	2.40	0.15	<0.001
Butyric acid	0.54	1.40	0.08	0.048
Isocaproic acid	1.29	1.66	0.23	0.304
Eptanoic acid	2.26	2.35	0.31	0.867

CON, control basal diet; LPM, diet supplemented with leaf powder mixture, 6 g/kg feed containing 2 g olive leaves +2 g laurel leaves +2 g rosemary leaves. SEM: standard error mean.

3.7. Egg quality

The effect of the olive, rosemary, and laurel leaf powder mixture on selected egg quality traits of laying hens during the last days of the experimentation are reported in Table 9, and shows that this dietary supplementation did not change the investigated parameters.

Similar results to this study have been reported in previous studies on egg quality traits related to feed supplemented with only olive leaves, at 3% of the diet (Cayan and Erener, 2015) or 5–10 g/kg diet (Botsoglou et al., 2013) in laying hens. As for laurel leaves, their supplementation (2 or 4 g/kg feed) in quails had no effect on external and internal egg quality traits (Karaalp and Elmastas, 2011). Moreover, rosemary leaf powder supplementation (5 g/kg diet) did not induce changes in egg quality characteristics (Botsoglou et al., 2005). In contrast, in other studies, the supplementation of olive leaves resulted in an improvement in yolk colour (Cayan and Erener, 2015).

4. Conclusions

This is the first study on the dietary use of a mixture of olive, rosemary, and laurel leaves in laying hens. LPM showed higher TPC than single leaves, suggesting a potentially greater antioxidant effectiveness of the combined mixture. The results highlighted the effectiveness of the LPM in enhancing the antioxidant ability and immune system, the serum lipid profile, and the functionality of the liver, most likely due to its high polyphenolic content. The few investigated egg quality parameters were not influenced by the LPM supplementation. These findings provide new insights on the beneficial effects of dietary administration of a leaf powder combination of olive, laurel, and rosemary.

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Table 9
Effect of dietary leaf powder mixture (LPM) supplementation on egg characteristics ($n = 30$) in laying hens at the end of the experiment.

	Dietary treatment		SEM	P-value
	CON	LPM		
Egg weight, g	60.64	61.03	0.53	0.722
Shell weight, g	7.73	7.79	0.09	0.783
Yolk weight, g	15.82	15.68	0.22	0.747
Albumen weight, g	35.60	35.56	0.41	0.960
Yolk ratio, %	26.15	25.67	0.30	0.440
Albumen ratio, %	58.67	58.26	0.38	0.591
Shell ratio, %	12.76	12.78	0.14	0.933
Yolk albumen ratio, %	44.75	44.21	0.66	0.690
Egg shell tickness, mm	0.43	0.40	0.025	0.602
Yolk colour fan (1–15 scale)[1]	7.69	7.86	0.16	0.598

CON, control basal diet; LPM, diet supplemented with leaf powder mixture, 6 g/kg feed containing 2 g olive leaves +2 g laurel leaves +2 g rosemary leaves. SEM: standard error mean. [1]1: pale yellow; 15: deep orange.

Declarations of competing interest

none.

CRediT authorship contribution statement

Angela Gabriella D'Alessandro: Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Salvatore Desantis:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Giuseppe Fracchiolla:** Writing – review & editing, Methodology, Investigation. **Riccardo Porrelli:** Methodology, Investigation. **Roberta Savina Dibenedetto:** Writing – review & editing. **Alessio Di Luca:** Writing – review & editing, Investigation. **Giovanni Martemucci:** Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

The authors of this manuscript have no financial or personal relationships that could potentially influence the content and findings presented in the manuscript.

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