Research Article

The Effects of Administering a Combination of Lavender, Pine, and Eucalyptus Oils on the *In Vivo* Health Status and Productivity of Laying Hens

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Abstract | Laying hens often experience digestive disturbances during their egg-laying period, which can noticeably affect their production levels. To explore potential improvements, this study examined how a natural blend of essential oils derived from pine, lavender, and eucalyptus (referred to as PLOE) influences gut condition, blood parameters, immune responses against Avian Influenza (AI) and Newcastle Disease (ND), as well as overall egg performance. A total of 2,400 hens were randomly allocated into four treatment groups and provided either plain drinking water (control; without PLOE) or PLOE at concentrations of 10, 20, or 30 mL per 10 L of water (v/v) daily for 30 days. After the treatment period, blood profiles, bacterial shedding, and egg productivity were evaluated. Supplementation with 30 mL of PLOE per 10 L of water resulted in higher packed cell volume, elevated but physiologically acceptable liver enzyme activity, improved egg weight and egg production, and reduced bacterial shedding, particularly *E. coli* and coliforms ($P < 0.05$). Despite these benefits, antibody titers against AI and ND remained unchanged ($P > 0.05$), suggesting that the additive did not directly influence humoral immunity. Overall, PLOE supported gut health and productivity in laying hens without altering their vaccine-induced antibody responses. Further research is warranted to determine its long-term application, optimal inclusion rate, and potential integration with vaccination or probiotic strategies to promote sustained flock health and performance.

Keywords | *Canalis alimentarius*, Essential oils, Egg production, Hematology, Laying hens

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INTRODUCTION

One of the biggest challenges faced by today's livestock and poultry industries is how to increase production to satisfy the world's growing need for animal protein without sacrificing sustainability or efficiency. Improving animal growth and feed efficiency remains a key approach to achieve this (Grigore *et al*., 2025). Yet, this progress is increasing undermined by the spread of antimicrobial resistance (AMR), which has largely emerged from the long-term use of antibiotics as growth enhancers. AMR is now recognized as a serious global health issue that, if uncontrolled, could lead to millions of deaths each year (Islam *et al*., 2024; Matheou *et al*., 2025). Overusing and misusing antibiotics have accelerated the rise of resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA), threatening both human and animal health (Arulappen *et al*., 2025; Matuszewska *et al*., 2022). Although new antibiotics continue to be developed, their discovery cannot keep up with the rapid evolution of resistance, highlighting the urgency of alternative solutions (Desai *et al*., 2025; Konwar *et al*., 2022). In poultry, this resistance problem can even interfere with vaccine performance, reducing antibody responses to diseases like Newcastle disease (ND) and avian influenza (AI). For this reason, many researchers are turning to natural products with antimicrobial potential. Among them, *Eucalyptus globulus* essential oil has shown remarkable effectiveness against MRSA (El-Shall *et al*., 2020; Elangovan and Mudgil 2023; Mohebodini *et al*., 2025). Findings from twenty studies have consistently supported its strong antibacterial activity, which becomes even more potent when blended with other essential oils or antibiotics (Romo-Castillo *et al*., 2023; Simbu *et al*., 2024).

Eucalyptus oil has long been valued for its potent antimicrobial characteristics, positioning it as a valuable bioactive compound that may benefit both human and animal health. Within the livestock sector particularly in poultry production its application as a natural feed supplement for broilers and laying hens has drawn increasing scientific attention (Fu *et al*., 2025; Movahedi *et al*., 2024). Broilers are generally reared for rapid body weight gain and superior feed conversion, whereas laying hens undergo a considerably longer developmental period before achieving peak egg-laying performance (Heines *et al*., 2022; Pirgozliev *et al*., 2024). Despite these production contrasts, egg farming tends to offer greater stability over time, as hens can sustain egg output well beyond 49 weeks of age (Arulnathan *et al*., 2024; van Eck *et al*., 2024). In contrast, broiler enterprises often generate faster economic returns when management and environmental conditions are favorable (Sa'ad *et al*., 2025; Ramukhithi *et al*., 2023). The inclusion of Eucalyptus oil in poultry feed formulations may contribute to improved productivity and immune robustness while simultaneously reducing the industry's reliance on conventional antibiotic growth promoters (Akue *et al*., 2024; Aminullah *et al*., 2025; Salinas-Chavira and Barrios-García, 2024). The performance efficiency of laying hens is determined by multiple interrelated factors chiefly feed intake, growth dynamics, and the feed conversion ratio (FCR) during the early growth and rearing phases. Optimizing these performance parameters serves as an indicator of effective nutrition and husbandry management (Bezahegn *et al*., 2025; Elsherbeni *et al*., 2024), which are vital for sustaining consistent egg output (Bist *et al*., 2024; Ishak *et al*., 2024). Moreover, the formulation strategy, cost-efficiency, and nutritional quality of feed remain central considerations affecting economic viability in contemporary poultry systems (Pesti and Choct, 2023; Tavares *et al*., 2022; Vlaicu *et al*., 2024). Efficient feed formulation plays a vital role in achieving desirable growth and egg quality in poultry. Feed typically represents 70–80% of total production costs, emphasizing the importance of optimizing nutrient utilization (Gao *et al*., 2025; Katu *et al*., 2025). The inclusion of functional feed additives has been proposed as a key strategy to enhance feed efficiency through modulation of gut microbiota (Khasanah *et al*., 2024; Pangga *et al*., 2025).

Growth promoters, widely used in poultry production, have been shown to improve performance, reduce mortality, and maintain intestinal integrity (El-Fateh *et al*., 2024; Lu *et al*., 2020). However, the extensive use of antibiotic growth promoters (AGPs) has raised concerns regarding antimicrobial resistance and residue accumulation, leading to their prohibition in several countries, including Indonesia since 2018 (Hedman *et al*., 2020; Ishaq *et al*., 2025; Wen *et al*., 2022). Consequently, research has shifted toward natural alternatives such as probiotics, prebiotics, organic acids, essential oils, and enzymes (Chowdhury *et al*., 2025; Movahedi *et al*., 2024; Qui, 2023). Among these, PLOE a blend of pine, lavender, and eucalyptus oils has gained attention for its potential antimicrobial and performance-enhancing effects. Lavender oil, rich in linalool, possesses antibacterial activity (Bălașoiu *et al*., 2024; Pandur *et al*., 2021); eucalyptus oil provides cineole with broad-spectrum effects (Elangovan and Mudgil, 2023; Shiekh *et al*., 2025), and pine oil contributes isobornyl acetate with antioxidative and anti-inflammatory properties (Ancuceanu *et al*., 2024; Asbabou *et al*., 2024). Nevertheless, limited data are available on their combined influence on layer productivity and immune response. This study aimed to evaluate the effects of PLOE supplementation on hematological parameters, health status, egg production, and antibody responses to Newcastle disease and avian influenza in laying hens.

MATERIALS AND METHODS

Animal ethics

Prior to the commencement of the study, ethical approval for all animal-related procedures was secured from the Animal Ethics Commission, Faculty of Veterinary Medicine, Udayana University, Indonesia, as stated in approval document B/186/UN14.2.9/PT.01.04/2024.

Research design

A total of 2,400 laying hens were used in this experiment, arranged under a Completely Randomized Design (CRD). The laying hens were 20 weeks old at the start of the experiment and were evenly and randomly allocated into four groups, designed as P0-P3, with 600 hens per group (six replications), each replicate consisted of 100 hens housed in adjacent cages. Those in P0 (control, received 10L of water without PLOE), P1 (10 mL PLOE per 10 L water), P2 (20 mL PLOE per 10 L water), and P3 (30 mL PLOE per 10 L water). All chickens in each group, except the control group, were continuously supplied with drinking water containing PLOE at concentrations of 10, 20, and 30 mL per 10 L of water. The experimental hens were kept in battery-type cages and received a commercial feed—HI-PRO-VITE 521 for the starter phase and either HI-PRO-VITE 524AX or CP 524 TA during the production phase (Phokphan-Indonesia). Drinking water was provided freely throughout the study period. Standard vaccination programs were carried out against Newcastle Disease (ND; Hitchner BI, Lasota, ND-EDS), Coryza, Fowl Pox, Infectious Bronchitis (IB), and Avian Influenza (AI).

Blood sample collection

One day after the 30-day PLOE administration period, for reasons related to biosafety and research funding limitations, a total of 80 hens (20 birds per treatment group (P0-P3) were randomly sampled for hematological and serological analyses. Therefore, hematological and serological findings were interpreted as indicative trends rather than definitive population-level estimates. Blood samples for hematological evaluation were collected in Vacuette K_3EDTA tubes (Greiner Bio-One, Austria), whereas blood samples for serological analysis were collected in plain BD Vacutainer tubes (BD, USA). Serum samples were separated and stored at −20 °C for no longer than three weeks prior to analysis. Blood samples containing anticoagulant were analyzed on the same day they were collected.

Hematological profile

A comprehensive hematological analysis was performed, including measurements of total leukocyte, erythrocyte count, hemoglobin concentration and packed cell volume (PCV) or hematocrit value, following the protocol for the use of the LICARE 3-Part Vet Auto Hematology Analyzer (Licare Biomedical Limited, China). The differential leukocyte count was determined using Giemsa-stained blood smears, observed under a microscope at 100× magnification, and analyzed by the straight-edge counting method (Andreea *et al.*, 2022). The evaluated leukocyte types comprised heterophils, lymphocytes, eosinophils, and monocytes, as well as the calculation of the heterophil-to-lymphocyte ratio (Andreea *et al.*, 2022; Haile and Chanie, 2014). The standard units used for the normal hematological values were derived broiler (Edeh *et al.*, 2023), these ranges are commonly applied as provisional references in layer studies due to limited layer-specific data.

Detection of antibody responses

Antibody titers against Newcastle disease virus and avian influenza virus were examined using hemagglutination inhibition (HI) assay based on a published method (Merdana *et al.*, 2024) with slight modifications. A 1% erythrocyte suspension was prepared from specific pathogen-free hens. Approximately 2 mL of blood was collected from the brachial vein of laying hens into tubes containing Heparin. Subsequently, 5 mL of phosphate-buffered saline (PBS, pH 7.2) was added to each sample, before being centrifuged at 2500 rpm for 10 minutes. The supernatant was discarded, and the erythrocyte pellet was washed and centrifuged three times with PBS. The resulting erythrocyte pellet was evaluated for concentration using a microhematocrit to determine the packed cell volume (PCV) for preparing a 1% erythrocyte suspension in PBS, served as the sample for the HI assay. The test was initiated by adding 0.025 mL of PBS to each well of the microplate, before 0.025 mL of serum was being added to the first well and serially diluted across the plate. Subsequently, 0.02 mL of 4 HAU antigen was introduced into each well, and the plate was incubated at room temperature for 30 minutes. Following this, approximately 0.025 mL of 1% erythrocyte suspension was added to each well, and the plate was left at room temperature for another 40 minutes. The HI titer was read by tilting the microplate at a 45° and observing whether erythrocyte agglutination (tear-shaped formation) occurred. The HI antibody titer was defined as the highest serum dilution that completely inhibited 1% erythrocyte agglutination.

Detection of serum biochemical parameters

Serum biochemical parameters including Glutamic Pyruvic Transaminase (GPT)/Alanine Aminotransferase (ALT) and Serum Glutamic Oxaloacetic Transaminase (SGOT)/ Aspartate Aminotransferase (AST) were measured using an automated clinical chemistry analyzer (Hitachi 902, Roche Diagnostics, Germany) according to the manufacturer's protocol to assess the hens' physiological status.

Detection of bacteria in fecal samples

Fecal samples were collected from birds in each treatment group. A total of 10 fecal samples per group (P0, P1, P2, and P3) were analyzed in this study. Sampling was performed aseptically by directly collecting fresh feces from the cloaca using sterile swabs. The swabs were then placed into sterile tubes containing transport medium, stored in a cool box with ice gel, and immediately transported to the laboratory for isolation and identification according to standardized laboratory protocols (Indonesian National Standard, 2018). Initial screening of enteric bacteria was carried out by inoculating fecal samples onto MacConkey Agar, followed by incubation at 37°C for 18–24 hours. Bacterial growth was evaluated based on colony morphology and lactose fermentation ability, and the screening results were recorded for further analysis. For the isolation of *Escherichia coli*, fecal samples were first inoculated into selective enrichment medium EC Broth using a sterile loop and incubated at 44°C for 18–24 hours. The cultures were then sub cultured onto Eosin Methylene Blue Agar (EMBA) and incubated at 37°C for 18–24 hours. The presence of *E. coli* was determined based on characteristic colony morphology, including a distinctive metallic green sheen. Suspected *E. coli* colonies were further confirmed using IMViC tests (indole, methyl red, Voges–Proskauer, and citrate). For the isolation of *Salmonella* spp., fecal samples were inoculated into Selenite F Broth as a selective enrichment medium and incubated at 37°C for 18–24 hours. After incubation, the cultures were sub cultured onto Salmonella–Shigella Agar (SSA) and incubated again at 37°C for 24 hours. Suspected *Salmonella* colonies were subsequently confirmed through biochemical and serological tests.

The measurement of egg production

Egg production was evaluated by recording daily egg counts and individual egg weights throughout the experimental period. A total of 200 laying hens were used in this study, randomly allocated into four experimental groups.

Data analysis

Data analysis was performed using one-way Analysis of Variance (ANOVA) to evaluate the effects of treatments on hematological parameters (total erythrocyte and leukocyte counts, hemoglobin concentration, and packed cell volume), differential leucocyte counts, serum SGPT and SGOT activities, egg production, and egg weight. When significant differences were found ($P < 0.05$), mean comparisons were conducted using Duncan's multiple range test as described by Steel and Torrie (1980). Antibody titers against Newcastle disease (ND) and avian influenza (AI), together with bacterial detection results, were analyzed descriptively.

RESULTS AND DISCUSSION

Hematological profile

Generally, the administration of PLOE to laying hens produced no adverse effects on hematological parameters. As presented in Table 1, most measured values remained within normal physiological ranges. The reference intervals applied in this study were derived from broilers but are commonly used as general benchmarks in poultry hematology due to the limited availability of layer-specific references (Edeh *et al.*, 2023). Total leukocyte counts were significantly lower in the P1 and P2 groups compared with the control (P0) and P3 groups ($P < 0.01$), although all values remained within normal limits. Differential leukocyte analysis showed increased lymphocyte percentages and reduced monocyte levels ($P < 0.05$), whereas heterophils (neutrophils) and eosinophils were not significantly affected ($P > 0.05$) (Figure 1). The reduction in leukocyte counts at 10–20 mL/10 L, while still physiologically normal, likely reflects immunomodulatory or anti-inflammatory effects rather than immunosuppression. This interpretation is consistent with previous reports demonstrating reduced inflammatory markers alongside preserved adaptive immune responses in essential oil–supplemented birds (Movahedi *et al.*, 2024; Swaggerty *et al.*, 2025; Zhang *et al.*, 2022). Importantly, antibody titers against avian influenza (AI) and Newcastle disease (ND) were not negatively affected, although minor quantitative variations cannot be excluded due to the qualitative nature of the HI assay. Regarding erythrocytic parameters, erythrocyte counts were significantly higher in the P1 and P2 groups

Table 1: Blood profile of laying hens after adding different volumes of PLOE to 10 L of drinking water (x± SD, n = 20, 30 days duration of the experiment).

Parameters	Normal values (Edeh et al., 2023)	Group			
		Control (P0)	Experimental (P1)	Experimental (P2)	Experimental (P3)
Total leukocytes (×10³/ µL)	3.7–11.9	8.37±0.84^{C}	7.87±0.79^{B}	7.27±0.72^{A}	8.44±0.84^{C}
Total erythrocytes (×10⁶/µL)	2.5–3.5	2.18±0.17^{A}	3.16±0.25^{B}	3.36±0.27^{B}	2.24±0.18^{A}
Hemoglobin (Hb, g/dL)	7.3–10	7.78±0.62^{A}	8.62±0.69^{A}	11.96±0.96^{A}	7.63±0.61^{A}
Packed cell volume (PCV,%)	24–43	26.06±2.61^{B}	25.84±2.58^{B}	20.44±2.04^{A}	31.83±3.18^{C}

Note: means within a row followed by the same letters do not differ significantly ($P > 0.05$), while means with different letters indicate significant differences ($P < 0.01$).

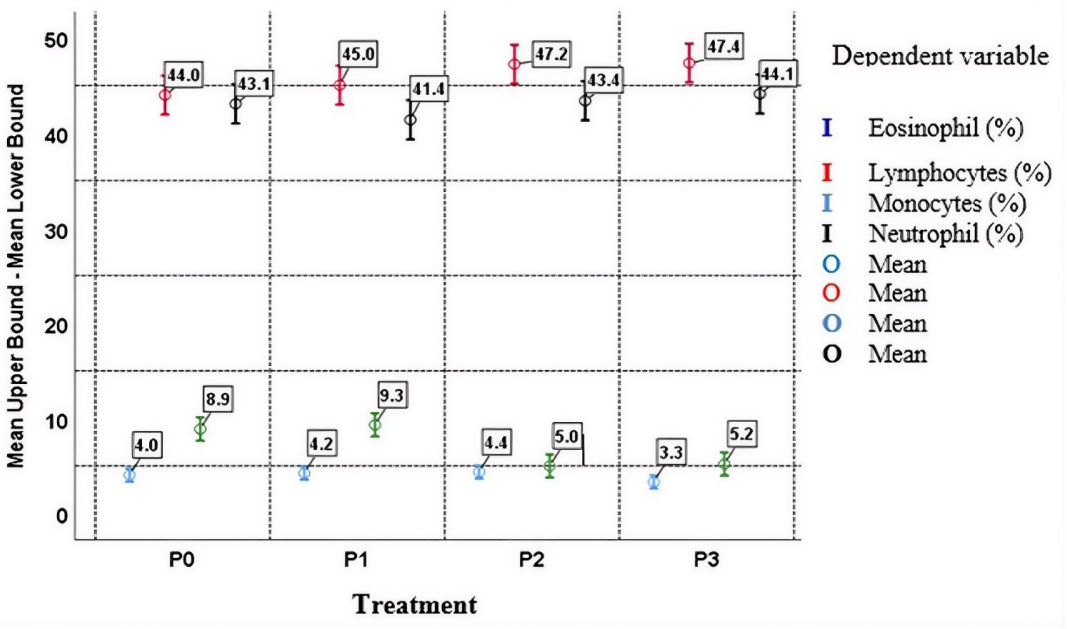

Figure 1: The mean percentages of differential leukocytes (eosinophils, lymphocytes, monocytes, and heterophils) across four treatment groups (P0–P3). Lymphocytes consistently show the highest values (44.0–47.4%) with a slight increase, while heterophils remain stable (41–44%). Monocytes vary moderately at P1 and P3, and eosinophils stay low (3.3–5.0%) without a clear trend. Overall, treatments have minimal effect on leukocyte distribution, with lymphocytes dominating in all groups.

Table 2: Serological HI test results for AI and ND vaccination (n = 5, 30 days duration of the experiment).

Group	Description	ND Antibody titer (log_2)	Interpretation	AI Antibody titer (log_2)	Interpretation
Control (P0)	received 10L of water without PLOE	≤4	Low positive	log_2: ≤4	Low positive
Experimental (P1)	10 mL PLOE per 10 L water	≤4	Low positive	log_2: ≤4	Low positive
Experimental (P2)	20 mL PLOE per 10 L water	≤4	Low positive	log_2: ≤4	Low positive
Experimental (P3)	30 mL PLOE per 10 L water	≤4	Low positive	log_2: ≤4	Low positive

Notes: HI (hemagglutination inhibition); Ab (antibody); AI (avian influenza); ND (Newcastle disease). No significant differences were observed between the control and experimental groups (P>0.05), suggesting that PLOE supplementation at 10–30 mL per 10 L of drinking water does not interfere with humoral immune responses.

compared with the control ($P < 0.05$). Hemoglobin concentrations did not differ significantly among treatments ($P > 0.05$), although values in P2 slightly exceeded commonly cited reference ranges without clinical signs of erythrocytosis. Packed cell volume (PCV) remained within normal physiological limits in most groups. The P2 group exhibited a slight decrease below the lower reference limit, but this was not accompanied by clinical signs of anemia. Conversely, PCV in the P3 group increased significantly compared with P1 and P0 ($P < 0.01$), yet still remained within normal limits. These non-linear, dose-dependent responses highlight the importance of dose optimization, as both beneficial and transient hematological changes have been reported depending on essential oil concentration (Khalaf *et al.*, 2025; Nhara and Marume, 2025). The modest increases observed in erythrocyte indices may be associated with improved nutrient absorption, enhanced antioxidant protection of red blood cells, and reduced oxidative stress mechanisms widely supported in recent reviews on phytogenic feed additives (Merdana *et al.*, 2024; Paredes-López *et al.*, 2024; Wandscheer *et al.*, 2024). Overall, the observed patterns slightly elevated erythrocytes and hemoglobin in some treated groups, reduced leukocytes at intermediate doses, and dose-responsive PCV variation are consistent with previous findings indicating that essential oils administered at practical doses do not induce hematological stress and are compatible with normal poultry blood physiology (Darmawan *et al.*, 2024; Merdana *et al.*, 2024; Mohebodini *et al.*, 2025). In summary, properly formulated PLOE supplementation did not negatively affect total erythrocytes, hemoglobin concentration, or PCV and helped maintain normal hematological function in laying hens.

Detection of antibody responses

Serum biochemical parameters are summarized in Table 2. Most parameters did not differ significantly among groups. However, AST levels were significantly higher in

P2 and P3 compared to P0 ($P < 0.05$). Administration of PLOE did not affect antibody responses to AI and ND (Table 2). A total of 20 hens were randomly selected, and the mean antibody titers did not differ significantly from those of the control group ($P > 0.05$). At 20 weeks of age, the HI test indicated comparable antibody titers against AI and ND across all groups (data not shown). After 30 days of PLOE supplementation, antibody titers remained similar among groups ($P > 0.05$). The HI test confirmed low positive antibody titers against ND and AI, indicating absence of interference with vaccine-induced antibody maintenance. This test is less sensitive than ELISA and was therefore used only for screening-level interpretation.

Detection of serum biochemical parameters

Liver enzymes (ALT and AST) showed dose-dependent increases at certain treatment levels in this study; however, all values remained within accepted physiological ranges (Table 3). This pattern is more consistent with adaptive metabolic responses rather than overt hepatotoxicity at the tested concentrations (Khalafalaa *et al*., 2025; Watanabe *et al*., 2023; Yilmaz and Gul, 2024). Specifically, PLOE administration at 10–20 mL per 10 L of drinking water (groups P1–P3) significantly increased ALT levels compared to the control group ($P<0.05$), with a more pronounced elevation observed at the 30 mL per 10 L dose (P3 group) ($P<0.01$). In contrast, AST levels remained unchanged at the 10 mL dose, whereas supplementation at 20–30 mL resulted in a significant increase ($P<0.05$). Despite these statistically significant elevations, the observed increases in ALT and AST were not indicative of hepatocellular injury, as all measured values remained within established physiological limits for chickens. The 20 mL dose produced a slight elevation compared to the control but still fell within normal reference ranges (AST: 84–342 U/L; ALT: 0–24 U/L) (Zálešáková et al., 2025). For comparison, supplementation with 300 ppm chitosan in laying hens resulted in ALT levels of 26.4 U/L, which were also considered safe. Taken together, these findings suggest that PLOE supplementation up to 20 mL per 10 L of drinking water appears safe with respect to liver function, although the observed biochemical shifts warrant continued monitoring in longer-term trials or at higher dosage levels.

Table 3: Serum liver enzyme levels (x ± SD, n = 20 experimental duration 30 days).

No	Treatment	ALT (U/L)	AST (U/L)
1	P0	14.15 ± 1.13^{A}	174.05 ± 17.41^{A}
2	P1	15.55 ± 1.24^{B}	184.35 ± 18.44^{A}
3	P2	15.05 ± 1.20^{B}	217.15 ± 21.72^{B}
4	P3	16.45 ± 1.32^{C}	222.30 ± 22.23^{B}

Note: superscript letters (A, B, C) indicate significant differences among treatments ($P<0.05$).

Detection of bacteria in fecal samples

Fecal analysis demonstrated that supplementation with PLOE at doses of 10–30 mL per 10 L of drinking water was associated with an absence of detectable intestinal bacteria under the applied qualitative bacteriological screening protocol, using special selective media for difference bacteria compare with reference bacterial standard, *E. coli* ATCC 25922 and *Salmonella enteritidis* ATCC 13976. Fresh fecal samples were collected aseptically from individual laying hens in each experimental group at the end of the 30-day treatment period. Samples were obtained concurrently from different individual hens within each group, rather than repeatedly from the same birds across groups, and were subjected to standard qualitative bacteriological examination.

In contrast to the treated groups, bacterial presence was observed in the untreated control group (P0). As summarized in Table 4, qualitative examination of fecal samples revealed noticeable differences among treatment groups. In the control group, several samples tested positive for *Escherichia coli* and coliform bacteria, while other samples were negative, indicating variability in bacterial presence among individual hens. Specifically, *E. coli* was detected in samples 2, 4, 7, and 10, whereas coliform bacteria were observed in samples 3 and 9.

Table 4: Results of bacteriological examination of poultry fecal samples (30 days duration of the experiment).

Sample ID	Treatments			
	P0	P1	P2	P3
1	Negative	Negative	Negative	Negative
2	*E. coli* (+)	Negative	Negative	Negative
3	Coliform (+) *E.coli* (+)	Negative	Negative	Negative
4	*E. coli* (+)	Negative	Negative	Negative
5	Negative	Negative	Negative	Negative
6	Negative	Negative	Negative	Negative
7	*E. coli* (+)	Negative	Negative	Negative
8	Negative	Negative	Negative	Negative
9	Coliform (+)	Negative	Negative	Negative
10	*E. coli* (+)	Negative	Negative	Negative

Notes: Sample ID refers to individual fecal samples collected from different animals and serves only as an identifier. A total of 10 individual fecal samples were examined per treatment group. Negative: No Salmonella, *E. coli*, and coliform bacteria were observed.

Conversely, all fecal samples obtained from the PLOE-treated groups (P1, P2, and P3) consistently tested negative for *Salmonella*, *E. coli*, and coliform bacteria under the applied qualitative screening conditions. Overall, these findings indicate that administration of PLOE in drinking water at doses of 10–30 mL per 10 L was associated with

Table 5: Laying percentage and egg weight of hens given different doses of PLOE in drinking water daily (x ± SD, n = 200, experimental duration 30 days).

Parameters	Treatment			
	P0	P1	P2	P3
Production percentage (%)	77.2±3.09^{A}	77.0±3.08^{A}	78.7±3.15^{B}	78.9±3.16^{B}
Egg weight (grams)	44.83±2.24^{A}	47.87±2.39^{B}	57.57±2.88^{C}	63.73±3.19^{D}

Note: Different uppercase letters in the same row indicate statistically significant differences between treatments (P < 0.05).

the absence of detectable *Salmonella*, *E. coli*, and coliform bacteria in laying hen feces when assessed using qualitative bacteriological screening methods.

The assessment of egg production

Supplementation of laying hens with PLOE at a concentration of 10 mL per 10 L of drinking water resulted in an egg production percentage that did not differ significantly from the control group (P > 0.05). Nevertheless, increasing the supplementation levels to 20 mL and 30 mL per 10 L of drinking water led to a significant improvement in egg production compared with the control, as corroborated by the trends presented in Table 5. Conversely, the mean egg weight of hens receiving PLOE at 10 mL per 10 L of drinking water was already significantly greater than that of the control group (P < 0.05), and a further increase in egg weight was observed at the higher supplementation levels of 20 mL and 30 mL per 10 L of drinking water, with highly significant differences (P < 0.01).

Liver enzymes (ALT and AST) showed dosage-dependent rises at certain treatment levels in this study, but values stayed inside accepted physiological ranges a pattern more consistent with adaptive responses than frank hepatotoxicity at the concentrations tested (Khalafalaa *et al*., 2025; Watanabe *et al*., 2023; Yilmaz and Gul, 2024). Even so, these biochemical shifts suggest the need for ongoing observation in extended trials or at increased dosage levels. The microbiological findings showing reduced presence of *E. coli* and coliform bacteria in the feces of treated birds are consistent with numerous recent reports describing the antimicrobial and microbiota-regulating effects of essential oils in poultry. Such actions are thought to enhance intestinal integrity, reduce systemic inflammation, and ultimately support improved production outcomes, including egg output and body weight, as reflected in the current results (Movahedi *et al*., 2024; Pham *et al*., 2023; Salinas-Chavira and Barrios-García 2024). The combination of preserved antibody titers, unchanged leukocyte differentials (except for lymphocyte/monocyte shifts), and reduced fecal pathogen detection suggests PLOE acts through local antimicrobial and anti-inflammatory mechanisms without impairing humoral immunity (Khukhodziinai *et al*., 2024; Movahedi *et al*., 2024). Taken together, the present results are concordant with a growing recent evidence base that carefully formulated essential-oil blends delivered at efficacious but non-toxic doses are compatible with normal hematology and can support intestinal health and production parameters in laying hens. However, heterogeneity in EO composition, bird strain, administration route (feed vs water vs encapsulated), dose and trial length across studies means that direct comparisons should be made cautiously; standardized reporting of dose (mg/kg feed or mL/L water), active compound concentrations, and exposure duration will improve comparability in the literature (Yilmaz and Gul, 2024).

CONCLUSION AND RECOMMENDATIONS

Thirty days combined supplementation of lavender, pine, and eucalyptus oils did not induce hematological or biochemical toxicity in laying hens. All measured blood and liver parameters remained within normal physiological ranges, and minor fluctuations were consistent with adaptive responses rather than harmful effects. The reduction in fecal *E. coli* and coliform counts further highlights the antimicrobial and gut health–promoting potential of the essential oil blend. Altogether, these findings suggest that properly formulated essential oils, administered at practical dietary levels, are safe and compatible with normal hematological, hepatic, and immune functions in poultry.

Future research should extend the supplementation period to cover complete production cycles and different stages of laying performance. Higher sample sizes and dose response evaluations would help define optimal inclusion rates and safety margins. Additional endpoints such as egg quality, microbiota profiling, immune markers, and long-term liver histopathology are also recommended to better understand efficacy and ensure sustained safety under commercial conditions.

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NOVELTY STATEMENT

To our knowledge, limited field-scale investigations have explored single/specific PLOE formulation that combines pine, lavender, and eucalyptus oils in laying hens maintained under real farm conditions. Most available publications examine each oil separately or rely on small-scale laboratory trials. In contrast, the present study followed a more comprehensive approach by observing blood characteristics, liver status, vaccine responses to avian influenza and Newcastle disease, together with actual egg production performance within the same trial. Another point that distinguishes this work is the evaluation of different inclusion rates, which made it possible to identify that only the highest supplementation level produced noticeable gains in egg weight and a decline in fecal bacterial counts. The lack of changes in antibody titers against both viral vaccines suggests that the improvement in productivity is more likely linked to gut function and metabolic adjustment rather than an enhanced immune response. Overall, the outcomes of this study offer a fresh perspective on how essential-oil blends may be incorporated alongside vaccination programs in commercial laying flocks.

AUTHOR'S CONTRIBUTION

IBKA and IWMT: Coordinated the project from start to finish, interpreted the main outcomes, and drafted the primary version of the manuscript. AASGK and RMDM: Handled procedures related to animal care, and helped supervise the laboratory phase. IMM and NLAKMPS: Worked on collecting data, preparing biological samples, and arranging the statistical dataset. IPTCP and IAAP: Contributed to animal sampling, routine monitoring on the farm, and helped with laboratory procedures and record keeping. HS and INTA: Carried out the virological testing and participated in reviewing vaccine-related responses. IMS: Critical comments on the methodology and refinement of the final manuscript. All authors have read the manuscript and agreed with its final form.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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