

Essential Oils as Feed Additives for Reducing Fungal Load and Improving Poultry Health

Iqra Ikram

Department of Biochemistry and Biotechnology, University, The Women University Multan, Punjab, Pakistan

Fahad Umer

Department of Animal Nutrition, University of Veterinary and Animal Sciences, Lahore, Punjab, Pakistan

Irtaza Hussain (Corresponding Author)

Department of Pathobiology, Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan, Punjab, Pakistan Email: dirtazasahu@bzu.edu.pk

Mirza Fahad Baig

Department of Zoology, University of Okara, Punjab, Pakistan

Syed Najaf Hasan Naqvi

College of Ecology and Environment, Chengdu University of Technology, Chengdu, China

Zaheer Ahmad

Department of Livestock Production and Management, The University of Veterinary and Animal Sciences (UVAS) Swat, Khyber Pakhtunkhwa, Pakistan.

Tahira Bibi

Department of Botany, Sardar Bahadur Khan Women's University, Quetta

Mian Muhammad Salman

Department of Pathobiology, College of Veterinary Sciences & Animal Husbandry, Abdul Wali Khan University Mardan, Khyber Pakhtunkhwa, Pakistan

Maqsood Ahmad

Veterinary Disease Investigation Laboratory, Nankana Sahib, ADDRS L&DD Punjab, Pakistan

ABSTRACT

Fungal contamination of the feed is one of the most significant problems which can impact on poultry health, productivity and feed safety and this is related to the risk of mycotoxin exposure. The present study aimed at assessing the effect of dietary essential oils on fungal load, mycotoxin concentrations, growth performance, antioxidant status, immune responses, gut microbiota and gut morphology in broiler chickens. The feeding experiment lasted 42 days and 240 broilers were distributed equally into four dietary treatments (six replicate pens per treatment): Control, oregano essential oil, thyme essential oil, or a blend of both essential oils. Dietary essential oils showed a reduction in total fungal load and *Aspergillus spp.* contamination, the most significant of which was seen in the birds fed with EO blend. The concentration of AFB1, OTA, DON and ZEN was also found to be lower in the groups supplemented with essential oil compared to the control. The supplementation

with essential oils increased final body weight, body-weight gain, average daily gain and feed conversion ratio without having any significant effect on feed intake. The antioxidant response was strengthened with an increase in SOD, CAT, GSH-Px, T-AOC and a decrease in MDA levels. The immunoglobulin levels were raised, and the levels of IL-6 and TNF- α were lowered. Essential oils also enhanced villus height, VH:CD ratio, beneficial bacteria and expression of genes related to intestinal barrier function, such as MUC2, ZO-1, and OCLN. EO blend tended to give the most significant responses. In summary, the results suggest the potential for essential oils to offer several benefits, including their ability to enhance the safety of feed, antioxidant activity, immune regulation, intestinal health, and broiler productivity. Further controlled studies are recommended to confirm these effects and to determine optimum inclusion rates for application in poultry production.

Keywords: Essential Oils; Broiler Chickens; Fungal Contamination; Mycotoxins; Gut Health

Introduction

Poultry production plays a significant role in the food security of the world due to the chicken meat being a cheap and nutritious source of animal protein. Feeds are becoming more contaminated in intensive poultry production systems, and dysbiosis of the gut, oxidative stress, immune dysfunction and exposure to mycotoxins are increasingly becoming important challenges (Olariu et al., 2025). Fungal contamination of feed is important among these challenges as cereal grains or ingredients of feed can facilitate growth of *Aspergillus*, *Penicillium* and *Fusarium* species. They can also produce mycotoxins that can have a negative impact on poultry productivity and health, such as aflatoxin B1 (AFB1), ochratoxin A (OTA), deoxynivalenol (DON), and zearalenone (ZEN) (Okasha et al., 2024). Moreover, AFB1 is of particular importance as it compromises the growth, feed efficiency, immune response, antioxidant status, and gastrointestinal health of broilers (Pożarska et al., 2024). The need of alternatives to the use of antibiotic growth promoters has been growing and this has led to an increased interest in the use of phytogetic feed ingredients such as essential oils. Essential oils are complex mixtures of plant-derived compounds that have antimicrobial, antifungal, antioxidant, anti-inflammatory and immunomodulatory properties (Grazul et al., 2023). Oregano essential oil is richer in carvacrol and thymol while thyme oil is richer in thymol and related phenols. The main compound in cinnamon oil is Cinnamaldehyde. These compounds can affect microbial membranes, membrane permeability, cellular metabolism and can inhibit fungal growth and the production of mycotoxins (Cai et al., 2022). In addition, recent evidence point to the potential of using phytogetic compounds in poultry nutrition as multifunctional alternatives to traditional antimicrobial growth promoters (Aminullah et al., 2025). Furthermore, essential oils can have an effect on poultry health other than antimicrobial. The dietary essential oils increased the beneficial population of lactobacilli and decreased the *E. coli* population in broilers, and that it also increased body-weight gain and feed conversion ratio. Oregano essential oil led to better growth performance, antioxidant status, intestinal microbiota, digestive enzyme activity, and intestinal health in broilers (Zhang et al., 2021). Following the intake of oregano EO,

the antioxidant capacity, immunity, abundance of the *Lactobacillus* and expression of Claudin-1 and MUC2 in the intestine improved. Similarly, thyme essential oil supported the intestinal barrier integrity, antioxidant capacity, IgA concentration and immune activity of broilers (Hossain et al., 2021). This antifungal activity of essential oils is especially important in the safety of poultry feed. Thymol, carvacrol, cinnamaldehyde, eugenol and citral inhibited the growth of *Aspergillus flavus* and *cinnamaldehyde* and citral inhibited AFB1 production in the poultry feed. The essential oils chosen could simultaneously control fungal contamination and the associated mycotoxins. The individual oil and combination effects can vary depending on the chemical composition, concentration, formulation and delivery method (Almeida et al., 2025). Although several studies have focused on individual essential oils, there is relatively little research that has simultaneously looked at the fungal load, mycotoxin exposure, growth performance, antioxidant status, immunity, intestinal microbiota, and intestinal barrier characteristics in a single experimental system. Hence, the present study was undertaken to assess the effects of oregano essential oil, thyme essential oil, and a mixture of essential-oil blend on fungal contamination and poultry health. It was hypothesized that there would be a reduction in fungal and mycotoxin levels, growth performance, antioxidant defense, immune regulation, intestinal morphology, microbial balance, and intestinal barrier function in fish when supplemented with essential oils, especially the combination of essential oils.

Materials and Methods

Study Design

A 42-day randomized controlled feeding experiment was conducted with broiler chickens to determine the influence of dietary essential oils as phytochemical feed additives on fungal load, mycotoxin contamination, growth performance, antioxidant status, immune response, intestinal microbiology and intestinal morphology. A total of 240 one day old broiler chicks were randomly distributed into four dietary treatments each with six replicate pens and 10 birds per pen. The experimental groups were: basal-diet control (T1), oregano essential oil (T2), thyme essential oil (T3) and combined essential-oil blend (T4). For the statistical analysis of growth performance and treatment levels, the pen was taken as the experimental unit.

Experimental Birds and Management

A total of 240 one day old commercial broiler chicks were collected from one hatchery and randomly placed in 24 experimental pens. Treatment consisted of 6 replicate pens (10 birds per pen). Standard environment and management conditions were maintained for birds during the 42-day experimental period. The standard commercial broiler husbandry and management practices were followed for temperature, ventilation, lighting, stocking density, sanitation and general husbandry. Food and clean drinking water were freely available during the experiment. Birds were observed for health status, abnormal behavior, morbidity and mortality daily and all mortality was documented during the experiment.

Dietary Treatments

A corn-soybean meal based basal diet was prepared that provided a nutritionally adequate diet for the starter, grower and finisher phases of broiler chicken production. The basal diet was fed to the control group, while the experimental diets were fed with oregano essential oil, thyme essential oil or a mixture of both. The essential oil from oregano and thyme were incorporated at 250 mg/kg of diet and the combined essential oil blend was incorporated at 250 mg/kg of diet. The mixture was a combination of oregano, thyme and cinnamon essential oils. The basal diets were synthesized to be nutritionally equivalent between the treatments, and the essential oils were mixed with a carrier suitable for the feed before being well mixed with the basal feed to achieve uniformity.

Essential-Oil Characterization

The essential oils of oregano, thyme and cinnamon were extracted from sources that were acknowledged and kept in tightly closed and light protected containers at the recommended storage conditions. Essential oils' chemical composition was determined by gas chromatography–mass spectrometry (GC-MS). The major bioactive components such as carvacrol, thymol, cinnamaldehyde, eugenol and other volatile compounds were identified and quantified as applicable. The characterization aimed to give information about the main bioactive compounds, which are likely to be responsible for the anti-microbial, anti-fungal, anti-oxidant and anti-immunomodulatory effects of the essential-oil preparations.

Experimental Period and Feeding Phases

The feeding experiment was carried out for a period of 42 days and was divided into three production phases: starter (1-14 days); grower (15-28 days); and finisher (29-42 days). The feeding and management conditions were kept identical for all experimental groups, except for the presence or absence of the essential-oils treatments.

Growth-Performance Measurements

Body weight was determined by using a calibrated digital weighing balance at days 1, 14, 28 and 42. The feed offered and feed left in each replicate pen was noted during each feeding phase to calculate feed intake. Body-weight gain was calculated as final body weight minus initial body weight, while average daily gain was determined as body weight gain/number of experimental days. Feed conversion ratio was determined as feed intake/body-weight gain. The daily mortality was recorded and reported as a percentage of birds allocated to each treatment at the beginning of the experiment.

Feed Sampling and Fungal-Load Assessment

Samples were taken from the different food treatments at fixed time intervals over the course of the experiment to obtain representative feeds. Samples were taken from various sectors of feed batches in an aseptically way and then were pooled, homogenized and stored in proper conditions before analysis of microbiological status. The assessment of fungal contamination was achieved with the determination of the total fungal load and the occurrence of the major fungal groups such as *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., yeasts and molds. Fungi levels

were reported in log₁₀ colony forming units per gram of feed (log₁₀ CFU/g).

Fungal Identification

The fungal colonies were first identified according to the characteristics of the colonies, the color of the spores, growth, and the morphology and pigmentation of the microcultures obtained during the fungal counting. Where necessary, molecular identification of representative isolates was then performed. A total of 20 fungal isolates were selected and DNA was extracted from each isolate, followed by the amplification of internal transcribed spacer (ITS) with suitable primers. To find the taxonomic identification, purified PCR products were sequenced and sequences matched with curated reference databases.

Mycotoxin Analysis

The eight most relevant mycotoxins in poultry feed were analyzed such as the Aflatoxins B₁ (AFB₁) and total Aflatoxins, ochratoxin A (OTA), deoxynivalenol (DON) and zearalenone (ZEN). Depending on the availability of the laboratory, the concentrations of mycotoxins were determined by enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC) or liquid chromatography–tandem mass spectrometry (LC-MS/MS). The levels of individual mycotoxins expressed as µg/kg in feed. Since AFB₁ is strongly linked to aspergillosis and has been recognized as a feed-associated mycotoxin in poultry production, special attention was given to this compound.

Blood Collection and Serum Preparation

Blood samples were taken from representative birds at day 21 and 42 by wing vein. Blood was put into proper serum-separation tubes and left to clot at room temperature. The serum was then separated by centrifugation at standard conditions. The serum was then transferred into sterile labeled microtubes and were stored in the freezer at –80°C for biochemical, antioxidant and immunological analyses.

Antioxidant and Oxidative-Stress Biomarkers

The activities or levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), total antioxidant capacity (T-AOC), reduced glutathione (GSH) and malondialdehyde (MDA) were measured to assess the antioxidant and oxidative stress status of serum. Commercially validated assay kits or established analytical procedures were followed in accordance with manufacturer's instructions. The higher activities of SOD, CAT, GSH-Px and the lower concentrations of MDA were interpreted as a better antioxidant defense and decreased oxidative stress.

Immune and Inflammatory Biomarkers

The level of the serum immune and inflammatory responses were assessed through measurement of immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA), interleukin-6 (IL-6), interleukin-10 (IL-10) and tumour necrosis factor-α (TNF-α). It was quantified using chicken-specific ELISA kits or

other validated analytical methods. The aim of the study was to examine the effect of dietary supplementation with essential oils on humoral immunity and inflammatory responses in broiler chickens.

Intestinal Sample Collection

Representative birds were taken from each replicate pen at day 42 of the experiment for examination of the intestine. Birds were humanely euthanized as per approved animal care procedure; and intestinal tissue was taken at the same time. The jejunal and ileal sections were harvested for histological and molecular analyses, while the cecal contents were aseptically collected for microbial analysis. Tissues for histological examination were immediately placed in 10% neutral buffered formalin, and tissues for molecular analyses were rapidly frozen and stored at -80°C until analysis.

Intestinal Morphology

The intestinal tissues were fixed and then processed by standard histological methods, embedded in paraffin, cut into sections, and stained with hematoxylin and eosin. The villus height, crypt depth, villus height/crypt depth and goblet-cell density were measured using digital image-analysis software. Intestinal structures were measured in the orientations that would decrease the amount of variation due to sectioning artifacts. Villus height and crypt depth were measured, and the ratio of these two was used as an indicator of the intestinal absorptive and structural condition.

Cecal Microbiological Analysis

The contents of the ceca were retrieved aseptically and examined for complimentary and/or pathogenic bacterial species. The main bacterial groups tested were *Lactobacillus* spp., *Bifidobacterium* spp., *Escherichia coli*, and *Salmonella* spp. Log₁₀ (CFU/g cecal content) was used to report the microbial concentrations. In molecular facilities, quantitative PCR was used to give more specific quantification of selected populations of bacteria. The aim of the microbial study was to find out if there was any link between the use of essential-oils and improving the balance of micro-flora in the gastrointestinal tract.

Intestinal Barrier Gene Expression

Intestinal tissue was taken for RNA extraction with a validated RNA extraction procedure, and the quality of the RNA and its concentration was determined prior to complementary DNA synthesis. Relative expression of selected genes related to intestinal barrier function (MUC2, ZO-1, OCLN, CLDN1) and inflammation (IL-6 and IL-10) was next assessed by quantitative real-time PCR. A stable housekeeping gene was used as an internal reference and the relative gene expression was determined by the comparative 2^{-ΔΔCt} method. Gene-expression analysis provided mechanistic information on the effect of supplementation with essential-oils on the integrity of the intestinal barrier and inflammatory regulation.

Primary and Secondary Outcomes

Total fungal load, *Aspergillus* spp. load and AFB1 concentration in feed were the

major outcomes of the study. Secondary responses were body weight, body-weight gain, feed intake, feed conversion ratio, antioxidant markers, immune markers, intestinal morphological parameters, cecal microbial counts, and intestinal barrier gene expression. This outcome hierarchy was designed to maintain the focus on the main research question (fungal-load reduction) to assess the overall effects of essential oils on the health of poultry.

Statistical Analysis

Data was analysed using statistical software R or IBM SPSS Statistics. Data for continuous variables were presented as mean $\pm$ standard deviation (SD). The replicate pen was considered the experimental unit for growth-performance measurements. A linear mixed-effects model was applied to variables collected over time, with the fixed effects of treatment, time, and treatment $\times$ time being included, and the random effect of replicate pen. After checking the assumptions of normality and homogeneity of variance, one-way analysis of variance (ANOVA) was conducted for terminal measurements to determine differences between the dietary treatments. If significant treatment effects were found, then pairwise comparisons were made using Tukey's multiple-comparison test. Where appropriate, fungal and bacterial counts (CFU/g) were log₁₀ transformed prior to statistical analysis. The associations between fungal loads, mycotoxins concentrations, antioxidant biomarkers, intestinal characteristics and growth-performance parameters were analyzed by Spearman's rank correlation analysis. All statistical tests were two-tailed and a P value of <0.05 was regarded as being statistically significant.

Results

Determination of the Hatchability of Broiler Eggs

The growth performance of broiler chickens was improved with dietary supplementation with essential oils (Table 1). There were no significant differences between experimental groups in initial body weight ($P = 0.842$), suggesting comparable initial weights. Essential oils group and essential-oil blend group birds had more final body weight than control birds at the 42nd day of the experiment. The final body weights of the control group, oregano group, thyme group, and EO blend group were 2,341.7, 2,478.3, 2,512.5, and 2,596.7 g/bird respectively, and were significantly higher in the EO blend group ($P = 0.018$). The same pattern with significantly greater body weight gain was seen in the EO-supplemented groups compared to the control group ($P = 0.015$). The same effects of the EO blends were observed in average daily gain with the EO blend group recording the highest gain (61.6 g/bird/day), followed by the thyme group (59.6 g/bird/day), oregano group (58.8 g/bird/day) and the control group (55.7 g/bird/day). There was also an increase in feed intake in the supplemented groups but the difference was statistically insignificant ($P = 0.126$). However, FCR was significantly better for essential-oil supplementation ($P = 0.021$), with the lowest FCR in the EO blend (1.56) followed by thyme EO (1.61) and oregano EO (1.64) than in the control group (1.72). There was no significant difference among treatments in mortality ($P = 0.771$) and it stayed low.

Table 1. Growth performance of broiler chickens receiving different dietary essential-

oil treatments during the 42-day experimental period

Parameter	T1 Control	T2 Oregano EO	T3 Thyme EO	T4 EO Blend	P-value
Initial BW (g/bird)	43.2 ± 1.4	43.5 ± 1.6	43.3 ± 1.5	43.6 ± 1.3	0.842
Final BW (g/bird)	2341.7 ± 74.6 ^a	2478.3 ± 68.2 ^{ab}	2512.5 ± 71.4 ^b	2596.7 ± 63.8 ^c	0.018
BW gain (g/bird)	2298.5 ± 74.1 ^a	2434.8 ± 67.5 ^{ab}	2469.2 ± 70.6 ^b	2553.1 ± 63.2 ^c	0.015
ADG (g/bird/day)	55.7 ± 1.8 ^a	58.8 ± 1.6 ^{ab}	59.6 ± 1.7 ^b	61.6 ± 1.5 ^c	0.014
Feed intake (g/bird)	3952.4 ± 91.5	3998.6 ± 87.3	3976.8 ± 94.2	3981.5 ± 89.7	0.126
FCR	1.72 ± 0.04 ^a	1.64 ± 0.03 ^{ab}	1.61 ± 0.03 ^b	1.56 ± 0.03 ^c	0.021
Mortality (%)	3.33 ± 2.11	3.33 ± 2.11	1.67 ± 1.05	1.67 ± 1.05	0.771

Values are presented as mean ± SD (n = 6 replicate pens per treatment). Different superscript letters within a row indicate significant differences among treatments according to Tukey's post-hoc test (P < 0.05). EO, essential oil; BW, body weight; ADG, average daily gain; FCR, feed conversion ratio.

Fungal Load and Mycotoxin Concentrations

The supplementation of essential-oils in the diet significantly decreased the level of fungal contamination in the experimental diets compared to the control group (Table 2). The control group had the highest total fungal load, which was significantly reduced by the use of essential-oil supplementation (with the oregano group being 4.82 log₁₀ CFU/g, the thyme group 3.96 log₁₀ CFU/g and the EO blend group 3.31 log₁₀ CFU/g, P < 0.001). The EO blend in turn was the most effective in reducing the total fungal load. The same pattern was seen with the concentration of *Aspergillus* spp. which dropped from 3.91 log₁₀CFU/g in the control group to 2.64 log₁₀CFU/g in the EO blend group (P < 0.001). Essential-oil supplementation also significantly decreased the populations of *Penicillium* spp. and *Fusarium* spp. EO blend treatment had the lowest levels of both groups of fungi. This was also the case for yeast counts (P = 0.031). In addition, dietary treatment had a significant effect on mycotoxin concentrations. The concentration of AFB₁ dropped by 81% and 67% in oregano and thyme groups, respectively, compared to the control group (18.6 µg/kg by 13.9 and 12.5 µg/kg, respectively). In a similar way, the concentrations of OTA, DON and ZEN were reduced in the essential-oil treatments compared to the control group, and the highest reductions were observed in the EO blend group. Overall, the results suggest that the dietary essential oils, especially the essential oils preparation, decreased fungal contamination and levels of the most abundant feed associated mycotoxins (Figure 1).

Table 2. Fungal load and mycotoxin concentrations in experimental diets following essential-oil supplementation

Parameter	T1 Control	T2 Oregano EO	T3 Thyme EO	T4 EO Blend	P-value
Total fungal load (log ₁₀ CFU/g)	4.82 ± 0.18 ^c	3.96 ± 0.15 ^b	3.72 ± 0.14 ^b	3.31 ± 0.12 ^a	<0.001
<i>Aspergillus</i> spp. (log ₁₀ CFU/g)	3.91 ± 0.17 ^c	3.08 ± 0.13 ^b	2.94 ± 0.12 ^b	2.64 ± 0.11 ^a	<0.001
<i>Penicillium</i> spp. (log ₁₀ CFU/g)	3.52 ± 0.14 ^c	2.94 ± 0.12 ^b	2.78 ± 0.10 ^b	2.41 ± 0.09 ^a	<0.001
<i>Fusarium</i> spp. (log ₁₀ CFU/g)	3.17 ± 0.13 ^c	2.71 ± 0.11 ^b	2.58 ± 0.10 ^b	2.26 ± 0.08 ^a	<0.001
Yeasts (log ₁₀ CFU/g)	3.24 ± 0.16 ^c	2.89 ± 0.13 ^b	2.76 ± 0.12 ^{ab}	2.53 ± 0.11 ^a	0.031
AFB ₁ (µg/kg)	18.6 ± 1.4 ^c	13.9 ± 1.1 ^b	12.5 ± 1.0 ^b	9.8 ± 0.8 ^a	<0.001
OTA (µg/kg)	7.42 ± 0.61 ^c	5.86 ± 0.48 ^b	5.41 ± 0.44 ^b	4.38 ± 0.37 ^a	0.002
DON (µg/kg)	421.5 ± 28.7 ^c	368.2 ± 24.6 ^b	351.7 ± 22.9 ^b	309.4 ± 20.8 ^a	0.004
ZEN (µg/kg)	68.4 ± 5.3 ^c	57.2 ± 4.7 ^b	54.6 ± 4.1 ^b	47.3 ± 3.8 ^a	0.006

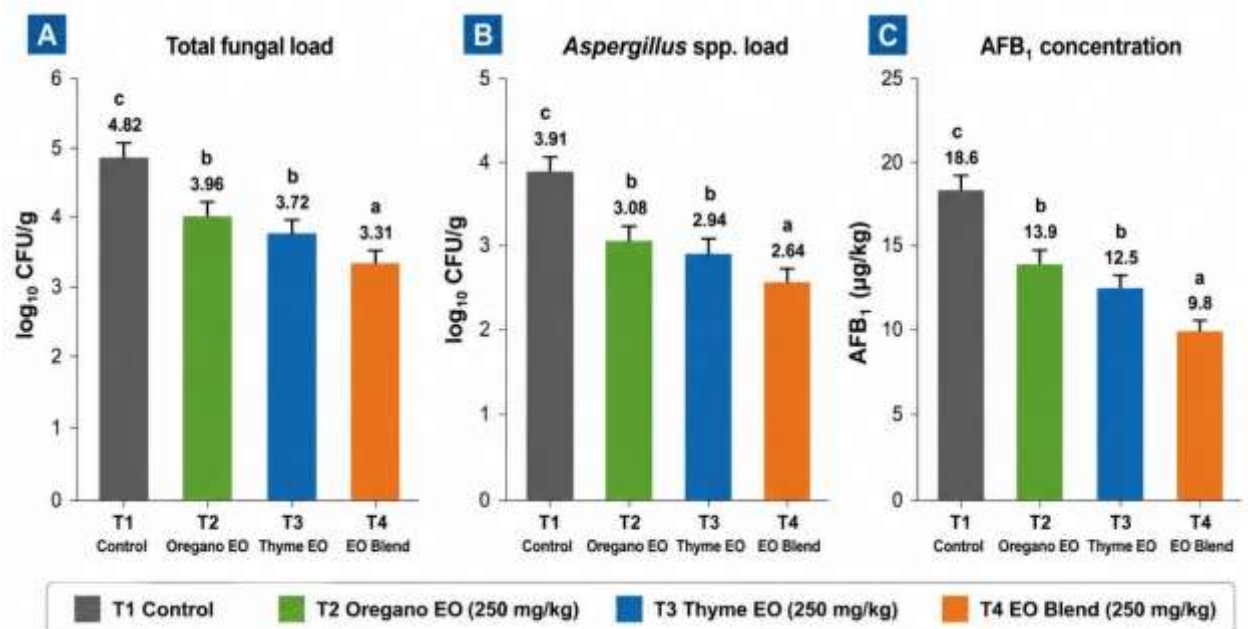


Figure 1. Influence of essential-oil supplementation in experimental diets on the total load of fungi, load of *Aspergillus* spp. and AFB₁ concentration. EO blend was the most effective treatment for decreasing the fungal contamination and AFB₁ concentration in comparison to control group.

Antioxidant and Immune Responses

The antioxidant and immune status of broiler chickens (Table 3) were significantly

affected by dietary supplementation with essential oils. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) enzyme activities were significantly higher in the essential-oil-supplemented groups when compared to the control group ($P < 0.05$). Birds given the EO blend had the highest activities throughout. There was a significant rise in SOD activity in the oregano group (151.8 U/mL), thyme group (157.4 U/mL) and EO blend group (169.2 U/mL) ($P < 0.001$). Likewise, MDA concentration was significantly decreased after supplementation with essential-oil ($P < 0.001$) while T-AOC and GSH were significantly increased ($P > 0.001$), suggesting that essential-oil supplementation may reduce oxidative stress. Essential-oil supplementation also resulted in positive effects on immune-related parameters. All the EO-treated groups showed significant increase in the levels of IgG, IgM, and IgA as compared to the control group, with the highest levels being recorded in the EO blend treatment. The concentration of IgG was higher in the EO blend group compared to the control group (10.31 mg/mL vs. 8.42 mg/mL, respectively; $P = 0.006$). Of these inflammatory biomarkers, IL-6 and TNF- α concentrations were significantly lower in EO-supplemented groups and IL-10 concentration significantly higher. EO blend group showed the most significant changes, indicating a beneficial modulation of antioxidant and immune response after combined essential-oil supplementation.

Table 3. Effects of dietary essential-oil supplementation on antioxidant and immune biomarkers of broiler chickens

Parameter	T1 Control	T2 Oregano EO	T3 Thyme EO	T4 EO Blend	P- value
SOD (U/mL)	128.6 $\pm$ 6.8 ^a	151.8 $\pm$ 7.2 ^b	157.4 $\pm$ 6.5 ^b	169.2 $\pm$ 7.1 ^c	<0.001
CAT (U/mL)	42.7 $\pm$ 2.8 ^a	49.6 $\pm$ 2.5 ^b	51.8 $\pm$ 2.7 ^b	56.4 $\pm$ 2.6 ^c	0.001
GSH-Px (U/mL)	61.4 $\pm$ 3.9 ^a	69.8 $\pm$ 3.5 ^b	72.6 $\pm$ 3.7 ^b	78.9 $\pm$ 3.8 ^c	<0.001
T-AOC (U/mL)	10.8 $\pm$ 0.7 ^a	12.6 $\pm$ 0.6 ^b	13.1 $\pm$ 0.7 ^b	14.4 $\pm$ 0.6 ^c	0.002
GSH (μ mol/L)	4.18 $\pm$ 0.31 ^a	4.86 $\pm$ 0.28 ^b	5.02 $\pm$ 0.32 ^b	5.47 $\pm$ 0.29 ^c	0.001
MDA (nmol/mL)	4.72 $\pm$ 0.34 ^c	3.91 $\pm$ 0.29 ^b	3.68 $\pm$ 0.27 ^b	3.21 $\pm$ 0.25 ^a	<0.001
IgG (mg/mL)	8.42 $\pm$ 0.52 ^a	9.36 $\pm$ 0.48 ^{ab}	9.71 $\pm$ 0.55 ^b	10.31 $\pm$ 0.49 ^c	0.006
IgM (mg/mL)	1.84 $\pm$ 0.16 ^a	2.07 $\pm$ 0.14 ^{ab}	2.16 $\pm$ 0.15 ^b	2.34 $\pm$ 0.13 ^c	0.012
IgA (mg/mL)	0.92 $\pm$ 0.08 ^a	1.06 $\pm$ 0.07 ^{ab}	1.11 $\pm$ 0.08 ^b	1.22 $\pm$ 0.07 ^c	0.009
IL-6 (pg/mL)	42.8 $\pm$ 3.4 ^c	36.7 $\pm$ 2.9 ^b	34.9 $\pm$ 2.8 ^b	30.6 $\pm$ 2.5 ^a	0.003
IL-10 (pg/mL)	18.4 $\pm$ 1.7 ^a	21.7 $\pm$ 1.5 ^b	22.8 $\pm$ 1.6 ^b	25.1 $\pm$ 1.8 ^c	0.002
TNF- α (pg/mL)	31.6 $\pm$ 2.6 ^c	27.8 $\pm$ 2.2 ^b	26.4 $\pm$ 2.1 ^b	23.1 $\pm$ 1.9 ^a	0.004

Intestinal Morphology, Microbial Populations, and Intestinal-Health Indicators

The essential-oil supplementation had a significant effect on the intestinal morphology and microbial characteristics of broiler chickens (Table 4). The height of the villus was significantly higher in the EO supplemented groups than in the control group ($P < 0.001$), with the EO blend group showing the highest villus height. The height of the villus was found to be 1,214.6 μm in the EO blend group when compared to the control group (1,028.4 μm). Villus height to crypt depth ratio was also moderately decreased after supplementation, and increased in the oregano, thyme and EO blend group ($P = 0.002$). The goblet-cell density also showed a similar trend, which suggested that the intestinal structure was stronger. Essential-oil supplementation also beneficially altered the micro flora of the cecal contents. *Lactobacillus* spp. and *Bifidobacterium* spp. levels were significantly higher in the birds fed EO while *E. coli* levels were significantly lower than the control group. The EO blend was the most effective treatment in terms of increasing the numbers of beneficial bacteria and reducing the concentration of *E. coli*. A similar numerical reduction was noted with *Salmonella* spp. but was not statistically significant ($P = 0.068$). The expression of genes associated with intestinal barrier was also increased following the addition of essential oils. The expression level of MUC2, ZO-1 and OCLN was significantly increased in the EO-supplemented groups, especially EO blend group. Conversely, the expression of IL-6 was decreased and expression of IL-10 was increased after supplementation. The results showed that the intestinal morphology, the microbial profile and the intestinal barrier-related responses were improved with the combined treatment of essential oil.

Table 4. Effects of dietary essential-oil supplementation on intestinal morphology, microbial populations, and intestinal-health indicators of broiler chickens

Parameter	T1 Control	T2 Oregano EO	T3 Thyme EO	T4 EO Blend	P- value
Villus height (μm)	1028.4 $\pm$ 48.6 ^a	1126.8 $\pm$ 45.2 ^b	1168.3 $\pm$ 47.5 ^b	1214.6 $\pm$ 43.8 ^c	<0.001
Crypt depth (μm)	178.6 $\pm$ 9.4 ^c	166.2 $\pm$ 8.7 ^b	162.8 $\pm$ 8.3 ^b	154.7 $\pm$ 7.9 ^a	0.003
VH:CD ratio	5.76 $\pm$ 0.31 ^a	6.78 $\pm$ 0.34 ^b	7.18 $\pm$ 0.36 ^b	7.86 $\pm$ 0.38 ^c	0.002
Goblet cells (cells/field)	18.6 $\pm$ 1.8 ^a	21.4 $\pm$ 1.7 ^b	22.6 $\pm$ 1.9 ^b	24.8 $\pm$ 1.6 ^c	0.004
<i>Lactobacillus</i> spp. (log10 CFU/g)	7.21 $\pm$ 0.24 ^a	7.64 $\pm$ 0.21 ^b	7.78 $\pm$ 0.23 ^b	8.06 $\pm$ 0.20 ^c	0.001
<i>Bifidobacterium</i> spp. (log10 CFU/g)	6.18 $\pm$ 0.21 ^a	6.52 $\pm$ 0.19 ^b	6.67 $\pm$ 0.20 ^b	6.91 $\pm$ 0.18 ^c	0.005
<i>E. coli</i> (log10 CFU/g)	6.42 $\pm$ 0.27 ^c	5.78 $\pm$ 0.24 ^b	5.61 $\pm$ 0.22 ^b	5.18 $\pm$ 0.20 ^a	<0.001
<i>Salmonella</i> spp. (log10 CFU/g)	3.42 $\pm$ 0.19	3.18 $\pm$ 0.17	3.09 $\pm$ 0.18	2.91 $\pm$ 0.16	0.068
MUC2 relative	1.00 $\pm$	1.28 $\pm$ 0.14 ^b	1.36 $\pm$	1.57 $\pm$	0.001

expression	0.12 ^a		0.13 ^b	0.15 ^c	
ZO-1 relative expression	1.00 ± 0.10 ^a	1.24 ± 0.11 ^b	1.31 ± 0.12 ^b	1.48 ± 0.13 ^c	0.002
OCN relative expression	1.00 ± 0.11 ^a	1.21 ± 0.10 ^b	1.29 ± 0.11 ^b	1.45 ± 0.12 ^c	0.003
IL-6 relative expression	1.00 ± 0.13 ^c	0.82 ± 0.10 ^b	0.77 ± 0.09 ^b	0.64 ± 0.08 ^a	0.004
IL-10 relative expression	1.00 ± 0.11 ^a	1.23 ± 0.12 ^b	1.31 ± 0.13 ^b	1.49 ± 0.14 ^c	0.002

Integrated Relationships Among Fungal Load, Mycotoxin Exposure, Antioxidant Status, Intestinal Health, and Growth Performance

The consistent relationships between variables of fungal contamination, mycotoxin exposure, antioxidant status, intestinal characteristics and growth performance were verified through correlation analysis (Figure 2). The total fungal load strongly positively correlated with AFB1 concentration ($\rho = 0.89$, $P < 0.001$) and MDA concentration ($\rho = 0.76$, $P < 0.001$), suggesting that the more the fungi are contaminated, the higher the exposure to the mycotoxins and the higher the oxidative stress. In contrast, fungal load was negatively correlated with final body weight ($\rho = -0.71$, $P < 0.001$), body-weight gain ($\rho = -0.70$, $P < 0.001$), villus height ($\rho = -0.73$, $P < 0.001$), and the VH:CD ratio ($\rho = -0.69$, $P = 0.001$). Similar negative relationship was observed between AFB1 concentration and growth and intestinal health markers. Higher AFB1 concentrations were associated with reduced final body weight ($\rho = -0.68$, $P = 0.001$), poorer feed conversion efficiency ($\rho = 0.64$, $P = 0.002$), lower villus height ($\rho = -0.67$, $P = 0.001$), and reduced Lactobacillus abundance ($\rho = -0.61$, $P = 0.004$). On the other hand, antioxidant biomarkers were positively correlated with growth performance. The relationship between SOD, GSH-Px, T-AOC and final body weight was positive while the relationship between MDA concentration and both SOD and GSH-Px was negative. There were also strong associations between the intestinal-health parameters and productive performance. Final body weight ($\rho = 0.79$, $P < 0.001$) was found to be positively correlated with villus height while FCR ($\rho = -0.66$, $P = 0.001$) was found to be negatively correlated with villus height. Similarly, the abundance of Lactobacillus was positively correlated with body-weight gain ($\rho = 0.63$, $P = 0.003$), while abundance of *E. coli* was negatively correlated with body-weight gain ($\rho = -0.59$, $P = 0.005$). In general, the overall correlation pattern revealed a close relationship between the fungal exposure and antioxidant defense, intestinal barrier integrity, microbial balance, and growth performance, whereas the mycotoxin exposure was associated with reduced growth performance, intestinal barrier integrity, and microbial balance, and neutralized antioxidant defense capabilities.

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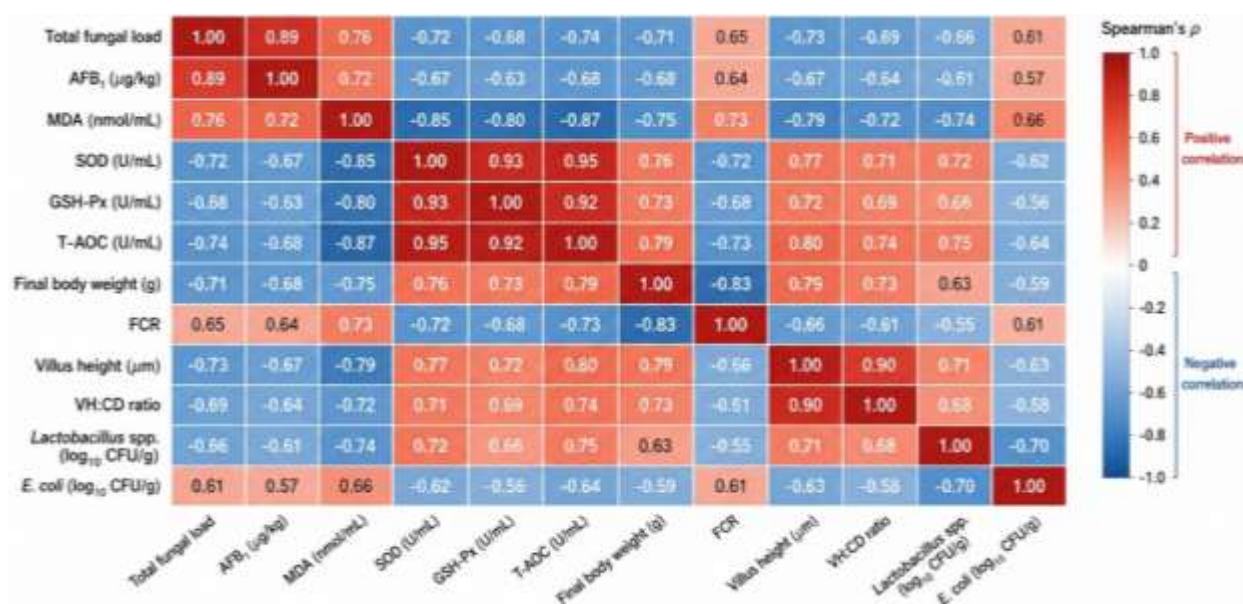
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Figure 2. The relationships among fungal contamination, mycotoxin exposure, antioxidant status, intestinal health, and growth performance were analyzed.

Discussion

Based on the present results, dietary essential oils were seen to exert beneficial growth performance, lesser fungal contamination and AFB₁, higher antioxidant, immune, microbial and intestinal health parameters in broiler chickens. Overall, the EO blend proved to be the most responsive, indicating that the synergistic effect of a blend of oregano, thyme and cinnamon oils might be more effective than the individual components. The improvements in final body weight, weight gain, ADG and FCR were not accompanied by a corresponding increase in feed intake, suggesting that the improvement in performance was due to improved nutrient utilisation and intestinal function rather than increased feed intake. This is consistent with the results of Irawan et al. (2020) who conducted a meta-analysis of 41 articles and 55 experiments to show that dietary essential oils can enhance BWG and FCR, and increase beneficial bacteria such as *Lactobacillus* and decrease *E. coli* bacteria. In broilers, oregano essential oil supplementation was also shown to enhance growth performance, antioxidant status and intestinal health by Zhang et al. (2021). One of the significant observations was the significant decrease in the overall fungal burden and *Aspergillus* spp. after supplementation with EO. This is especially important because AFB₁ is an important mycotoxin produced by *A. flavus* and a significant feed-associated mycotoxin that can negatively affect the growth, feed efficiency and health of poultry. Mateo et al. (2025) showed that thymol, carvacrol, cinnamaldehyde, eugenol and citral possess inhibitory activity against *A. flavus* with higher inhibitory effect of cinnamaldehyde and citral on the growth and AFB₁ production in poultry feed. Satterlee et al (2023) also showed that a combination of cinnamon, oregano and lemongrass essential oils and organic acids was inhibitory and synergistic against contamination of *A. flavus*. The decreased percentage with the EO blend might therefore be an indication of the synergic effect of the components in its antifungal activity. Importantly, the reduction of fungal

counts does not necessarily mean the elimination of mycotoxin risk as mycotoxin can remain after fungal viability has been reduced, so direct mycotoxin analysis should be performed when evaluating interventions for feed-safety. The antioxidant profile is better too, which further reinforces the beneficial effect of EO supplementation. The high levels of SOD, CAT, GSH-Px, T-AOC, and GSH and the low level of MDA indicate that the lipid peroxidation and endogenous antioxidant defense levels are increased. Similar antioxidant status enhancements have been seen when oregano EO was supplemented (Zhang et al., 2021). Moreover, thyme EO exhibited a reduction of MDA, an increase in intestinal IgA antibody, an improvement of intestinal barrier integrity and an immune stimulatory effect in broilers, as reported by Placha et al. (2014). Biswas and Kim (2025) also found that oregano EO boosted antioxidant capacity of the intestine and up-regulated the expression of Claudin-1 and MUC2, suggesting its protective effect on the intestine through its antioxidant effect as well as its protective effect against epithelial damage. This negative correlation between fungal load and antioxidant biomarkers, and the positive correlation with MDA and fungal/mycotoxin measures, indicates that the decrease in fungal exposure could possibly have led to an improvement in antioxidant balance. The immune responses seen in the present study were also quite favorable. A rise in IgG, IgM, IgA and IL-10, along with a decrease in IL-6 and TNF- α , is indicative of immune regulation, not immune stimulation. This modulation could be advantageous as too much inflammatory activity could lead to overproduction of oxidative stress and competition for metabolic resources for growth. According to Placha et al. (2014), thyme EO supplementation resulted in an increase of IgA. The intestinal morphological and microbial changes are another reason for the performance improvement. A more favorable intestinal environment is suggested by increased villus height, VH:CD ratio, increased abundance of *Lactobacillus* and *Bifidobacterium*, and decreased abundance of *E. coli*. In addition, Irawan et al. (2021) also found that the essential oils in the diet increased the *Lactobacillus* and decreased *E. coli*, as well as changed intestinal morphology. In addition, enhanced nutrient digestibility and digestive enzyme activity in broilers fed EO has been linked to increased feed efficiency (Adil et al., 2024), offering another possible mechanism of action for improved feed efficiency. In the present study, the expression of MUC2, ZO-1 and OCLN were all increased, which further indicated an enhanced intestinal barrier function. The biological significance of these changes is that the preservation of epithelial integrity will limit microbial translocation, ensure nutrient absorption and limit inflammatory stimulation. These responses were interpreted as a whole by conducting a correlation analysis. AFB₁ and MDA were strongly positively correlated with total fungal load, while final body weight, villus height and VH:CD ratio and *Lactobacillus* had a negative correlation. Likewise, the negative correlations of AFB₁ with growth and gut-health markers were found. On the other hand, growth performance was positively related to SOD, GSH-Px, T-AOC, villus height and beneficial bacteria. The correlations do not imply causation, but do suggest a potential feed safety–gut health–productivity pathway that suggests that less fungal/mycotoxin exposure leads to less oxidative and inflammatory pressure, leading to better gut integrity, which in turn leads to better nutrient utilization. The enhanced answer of the EO mix is noteworthy particularly. Irawan et al. (2021) found that these compounds,

including thymol, carvacrol and cinnamaldehyde, could have a powerful effect on productive performance and feed efficiency when added to EO blends. A synergistic effect among the selected EO compounds was also shown in the study by Satterlee et al. (2023) who confirmed the synergistic effect of cinnamon and oregano oil against *A. flavus*. However, the efficacy of EO is highly dependent on the botanical source, chemical composition, concentration, formulation and delivery method. Therefore, any apparent benefits of the blend should be demonstrated by dose response experiments and chemical standardization and should not be assumed to hold true for all. In summary, the results suggest that essential oils are promising phytogetic feed supplements offering a one-for-all solution against fungal contamination, oxidative stress, immune regulation, microbial balance in the gut, barrier function, and productive efficiency. Future experimental studies should include GC-MS characterization, wider fungal and microbiome profile, validated mycotoxin assay, dose-response assessment and economic analysis. These strategies could be used to establish if essential-oil mixtures can be used as an additive to promote poultry health, as well as being used as a viable means of controlling the risk of fungal and mycotoxin growth in poultry feeds.

Conclusion

The outcomes of dietary essential-oil supplementation showed a positive impact on broiler performance, safety of feed, antioxidant status, immune response and intestinal health. Essential oils of oregano and thyme decreased fungal contamination and mycotoxin levels and increased growth performance, antioxidant defense, microbial balance, intestinal morphology and barrier related responses. The overall effect was the highest with the combined essential oil mixture, which may indicate synergistic activity of the bioactive components in the combined essential oil mixture. The results are encouraging essential oils as potential multifunctional phytogetic feed additives in poultry production and feed safety management. To further validate these effects and set practical inclusion levels, additional controlled experiments with standardized preparations of essential oils, dose-response design, microbiome analysis, and validated mycotoxin analysis are necessary.

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