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# EVALUATION OF DIETARY ESSENTIAL OILS AS NATURAL ANTIFUNGAL AGENTS AGAINST ASPERGILLUS INFECTION IN BROILER CHICKENS

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*Avian Aspergillosis, Broiler Nutrition, Essential Oils, Origanum vulgare, Phytogenics, Thymus vulgaris.*

**Abstract**

***Background:*** *Avian aspergillosis, primarily caused by Aspergillus fumigatus, poses a significant threat to global broiler production, leading to respiratory distress, immunosuppression, elevated mortality, and reduced feed efficiency. With growing restrictions on synthetic antimicrobials and antifungal resistance concerns, dietary phytogenic compounds offer a viable, natural therapeutic alternative.*

***Objective:*** *This study evaluated the protective efficacy of dietary supplementation with thyme oil and oregano oil, individually and in combination, against experimentally induced Aspergillus infection in broiler chickens.*

***Methods:*** *A total of 100 broiler chickens were randomly allocated to four treatment groups: Control (0 mg/kg), Thyme Oil (350*

*mg/kg), Oregano Oil (350 mg/kg), and Thyme+Oregano Combination (350 mg/kg total). Birds were monitored over 42 days. Parameters evaluated included growth performance, pulmonary Aspergillus fungal burden, culture positivity rate, and pulmonary lesion severity scores. Data were analyzed using One-Way ANOVA, Tukey's HSD post-hoc test, and Kruskal-Wallis tests.*

***Results:*** *Oregano Oil and Thyme Oil significantly improved final body weight (2008.77 ± 74.02 g and 1985.00 ± 64.71 g vs. 1919.80 ± 74.05 g in Control; $p<0.01$). FCR significantly improved in Oregano Oil (2.04 ± 0.08) and Thyme Oil (2.06 ± 0.07) groups ($p<0.01$). Fungal load was markedly reduced by Oregano Oil (1.26 ± 0.64 log10 CFU/g) and Thyme Oil (1.45 ± 1.17 log10 CFU/g) compared to Control (3.46 ± 2.52 log10 CFU/g; $p<0.001$). Culture positivity dropped from 48% in Controls to 8% in Oregano Oil and 12% in Thyme Oil groups. Lesion scores were lowest in Oregano Oil (0.96 ± 0.79) and Thyme Oil (1.52 ± 0.87) compared to Control (2.20 ± 0.58; $p<0.05$). The combination treatment showed less efficacy, indicating potential competitive antagonism.*

***Conclusion:*** *Dietary supplementation with individual oregano or thyme essential oil at 350 mg/kg significantly mitigates Aspergillus infection in broiler chickens. Monotherapy with oregano oil demonstrated the highest efficacy.*

## INTRODUCTION

Avian aspergillosis is one of the greatest respiratory disease problems for the world's broiler poultry industry, and is commonly associated with the opportunistic fungal pathogen Aspergillus fumigatus. Because aspergillus spores are everywhere in the environment and litter and feed in a high-density commercial poultry operation, susceptible birds are continually exposed. Clinically the disease presents as either an acute or chronic respiratory distress with dyspnea, gasping, cyanosis and typical pulmonary granulomatous lesions [1]. In addition to the welfare consequences, avian aspergillosis causes significant economic losses, due to increased mortalities, lower weight gain, poor feed conversion ratio, carcass condemnation at slaughter and uniformity losses in the flock. In addition, the infection has an immunosuppressant effect, allowing secondary bacterial infections to occur, which further contributes to production losses. In spite of the high prevalence and economic impact of this disease, there are few effective, cost-effective and commercially available therapeutics for it [2]. Synthetic antimicrobial agents and antifungals have long been used in poultry production to control bacterial and fungal pathogens. However, rising consumer preferences for antibiotic-free poultry products, rising concerns for antimicrobial resistance, and tightening regulations have significantly reduced the use of traditional prophylactic and therapeutic antibiotics. Since the European Union (EU) banned the use of antibiotic growth promoters (AGP) in 1998 and other countries followed suit, there has been an increased need for alternatives to antibiotic fed for effective growth enhancement [3]. In addition, no systemic antifungal agents are approved for use and are cost-effective for commercial broiler production. Although effective, azole antifungal drugs are associated with problems such as the appearance of resistance in Aspergillus species, high cost and regulatory constraints for food producing animals. This therapeutic gap creates a need for the exploration of alternative strategies to be able to achieve both fungal pathogens control and flock health and productivity [4].

As a result, especially essential oils extracted from aromatic plants, phytogenic feed additives (PFA) have become promising natural alternatives in the field of poultry nutrition. Of these, thyme oil (Thymus vulgaris) and oregano oil (Origanum vulgare) have gained significant attention as a result of their strong antimicrobial, antioxidant and immunomodulatory activities [5]. Thymol (2-isopropyl-5-methylphenol) and carvacrol (5-isopropyl-2-methylphenol) are the two main phenolic components present in thyme and

oregano essential oils, respectively, and are considered to be the main contributors to the bioactive activity of these oils. These volatile monoterpenoid phenols have multiple modes of action as fungicides, which include disrupting the integrity of fungal cell membranes, inhibiting the synthesis of ergosterol, preventing fungal spore germination and inducing oxidative stress to fungal cells [6]. Their lipophilic property allows them to pass through the fungal cell membrane, increasing permeability, efflux of cell ions and ATP, and disrupting the integrity of the cell membrane. Moreover, there is evidence that these compounds are immunomodulatory and could improve the host's defense mechanisms against fungal pathogens [7]. Although thymol and carvacrol have been shown to have in vitro antifungal effects, limited information exists in the commercial literature for the effects of in vivo dietary supplementation with these EOs on fungal challenge in the lungs of poultry. Most of the studies conducted so far have used essential oils for the control of bacterial pathogen or as a general growth promoter and little has been done on evaluating the effectiveness of essential oils against respiratory fungal infection in broilers [8]. Also, the individual efficacy of oils and combination formulations, including possible antagonistic interactions between active oils has not been sufficiently examined. The optimum dose, period of supplementation, and synergism/antagonism of blending thyme and oregano oils in relation to aspergillosis challenge has yet to be identified. Therefore, the protective effect of feeding broiler chickens alone and in combination thyme oil and oregano oil against an experimental challenge with Aspergillus fumigatus was tested in this study [9]. Specific aims included evaluating growth performance, pulmonary fungal clearance, and tissue protection, and it was hypothesized that dietary essential oil supplementation results in significantly lower fungal burden and higher growth efficiency in addition to protecting the pulmonary tissues from pathological damage as compared to non-supplemented controls.

## Materials and Methods

### 2.1 Study Design and Ethical Approval

Four dietary treatment groups were established and the experiment was set up as a completely randomized design (CRD). All animal procedures were approved by the Institutional Animal Care and Use Committee in accordance with the guidelines and regulations for the ethical use of animals in research.

### 2.2 Experimental Animals, Housing, and Management

100 one-day-old commercial broiler chickens were bought from a commercial hatchery and divided into four dietary treatment groups of 25 chickens each. Birds were kept in controlled-environment floor pens containing new wood shavings as litter. The standard brooding management was followed in which the brooding temperature was slowly decreased from 33°C (Day 1) to 22°C (Day 42). The lighting schedule was 23 hours per day, which was the same for the trial period. Feed and fresh water were available to birds at all times. Standard commercial vaccination regimes were followed for common viral diseases for all birds.

### 2.3 Dietary Treatments and Essential Oil Preparation

The four dietary treatment groups were as follows:

- **Control Group:** Basal diet with no essential oil additive (0 mg/kg)
- **Thyme Oil Group:** Basal diet + 350 mg/kg Thymus vulgaris essential oil
- **Oregano Oil Group:** Basal diet + 350 mg/kg Origanum vulgare essential oil
- **Thyme + Oregano Combination Group:** Basal diet + 175 mg/kg Thymus vulgaris + 175 mg/kg Origanum vulgare (350 mg/kg total inclusion)

Basal Diets for broiler starter (day 1 to 14), broiler grower (day 15 to 28) and broiler finisher (day 29 to 42) were prepared based on the NRC recommendations. The essential oils were standardized by the content of their active phenolic compounds: thymol in thyme oil ≥ 55 % and carvacrol in oregano oil ≥ 60 % and they were added to the feed via spray mixing with a small fraction of the basal diet before being thoroughly mixed with the complete ration. Ad libitum provision of feed and fresh water allowed diets to be offered in a mash form.

**2.4 Aspergillus Fungal Challenge Model**

All birds were inoculated intratracheally with a standardized suspension of the spores of Aspergillus fumigatus on day 7 of the trial to induce acute pulmonary infection. Fungal inoculum was prepared by growing A. fumigatus in Sabouraud Dextrose Agar at 37°C for 5-7 days, harvesting the spores in sterile phosphate-buffered saline containing 0.05% Tween 80, and counting them using a hemocytometer in order to obtain a concentration of 1 x 10^6 spores/mL. The spore suspension was delivered direct into the trachea of each bird by the use of a sterile cannula and syringe using 0.2 mL per bird. The model chosen for challenge was based on this rationale, as it would provide a consistent and reproducible infection in all treatment groups.

**2.5 Performance Monitoring**

Individual body weights were recorded at days 1, 14, 28, and 42 using a calibrated digital scale. Feed intake per replicate pen was recorded daily throughout the trial period. Performance metrics were calculated as follows:

- **Weight Gain (g)** = Final Body Weight (Day 42) - Initial Body Weight (Day 1)
- **Feed Conversion Ratio (FCR)** = Cumulative Feed Intake (g) / Weight Gain (g)

**2.6 Mycological Evaluation**

All the birds at day 42 of the trial were humanely sacrificed by cervical dislocation and right lung was collected for mycological evaluation under strict aseptic condition. Homogenization of the lung tissues in sterile phosphate buffered saline and the subsequent inoculation onto the sabouraud dextrose agar with chloramphenicol (to suppress bacterial multiplication) was used to assess culture positivity. Plates were incubated at 37°C for 48 hours and then samples with identified and typical Aspergillus growth (greenish-black colonies with typical morphological features) were scored as positive (1) or negative (0). For fungal load, serial 10-fold dilutions of tissue homogenates were made and poured onto Sabouraud Dextrose Agar. The number of fungal colony forming units (CFU) was determined after incubation and expressed as log10CFU/g lung tissue.

**2.7 Pathological Lesion Scoring**

Left lung lobes were visually evaluated for gross fungal lesions, including nodules, consolidation, and necrosis, using a standardized 5-point scoring scale:

- **Score 0:** Normal tissue architecture; no visible lesions
- **Score 1:** Mild infection; 1 to 2 small localized focal lesions (<2 mm)
- **Score 2:** Moderate infection; 3 to 5 multifocal lesions (2 to 5 mm)
- **Score 3:** Severe infection; extensive multifocal to coalescing lesions (>5 mm)
- **Score 4:** Extremely severe infection; diffuse consolidation and widespread necrotic granulomas across the entire lung lobe

**2.8 Statistical Analysis**

IBM SPSS Statistics (Version 26.0, Armonk, NY, USA) was used for data analysis. The Shapiro-Wilk test was used to assess normality and the Levene's test to test for the homogeneity of variance. One-Way Analysis of Variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) post hoc was performed on continuous performance and microbiological data for pairwise comparisons. Both ANOVA and the non-parametric Kruskal-Wallis H test were used to compare the ordinal lesion scores. Categorical data (culture positivity rates) were analyzed using Chi-Square test of independence. The differences obtained were considered statistically significant at $P < 0.05$. Results are presented as Mean ± Standard Deviation (SD).

**Results**

**3.1 Growth Performance and Feed Utilization Efficiency**

Dietary supplementations of essential oils significantly improved growth performance parameters of broiler chickens under Aspergillus fumigatus stress. At day 14, birds in the Thyme Oil and Oregano Oil

groups exhibited significantly higher body weights (385.12 ± 12.04 g and 388.94 ± 11.85 g, respectively) compared to the Control group (373.18 ± 13.91 g; p = 0.0001). The intermediate value was obtained for the combination group (378.40 ± 14.10 g) which failed to show any significant difference between the Control and combination group.

The positive effects of the single essential oils treatments were more evident at day 28. The Thyme Oil group (1165.80 ± 38.50 g) and Oregano Oil group (1178.45 ± 36.12 g) both significantly exceeded the Control group (1124.32 ± 41.20 g; p < 0.001). The efficacy of the combined formulation was not effective compared to the Control group at this growth stage as no significant differences were found between the Combination group (1135.10 ± 40.80 g) and the Control group.

At final weight on day 42, the Oregano Oil group achieved the highest body weight (2008.77 ± 74.02 g), followed closely by the Thyme Oil group (1985.00 ± 64.71 g), both significantly exceeding the Control group (1919.80 ± 74.05 g; p = 0.0001 and p = 0.0051, respectively). However, there was no significant difference between the Combination group (1935.42 ± 60.03 g) and the Control group.

Total weight gain from day 1 to day 42 followed a similar pattern, with Oregano Oil (1965.13 ± 74.53 g) and Thyme Oil (1942.57 ± 64.58 g) groups demonstrating significantly higher weight gains compared to Control (1876.83 ± 74.03 g; p < 0.001). There was no significant difference between the Combination group (1892.19 ± 59.51 g) and the Control group.

However, there were no significant differences in feed intake during the different treatment groups (p = 0.854) suggesting that the greatest differences in body weight were due to feed efficiency rather than greater feed intake. Feed conversion ratio (FCR) significantly improved in the Oregano Oil (2.04 ± 0.08) and Thyme Oil (2.06 ± 0.07) groups compared to the Control group (2.14 ± 0.08; p = 0.0006 and p = 0.0058, respectively). The Combination group (2.11 ± 0.09) had an intermediate FCR, which was not significantly different from the Control group. Complete growth performance data are presented in Table 1.

**Table 1: Broiler Growth Performance and Feed Efficiency (Days 1 to 42)**

| Parameter | Control (n=25) | Thyme Oil 350mg/kg (n=25) | Oregano Oil 350mg/kg (n=25) | Thyme+Oregano 350mg/kg (n=25) | ANOVA F-Value | p-Value |
|---|---|---|---|---|---|---|
| Initial Weight (g) | 43.34 ± 2.05 | 43.43 ± 1.83 | 43.64 ± 1.88 | 43.23 ± 2.10 | 0.224 | 0.879 (NS) |
| Day 14 Weight (g) | 373.18 ± 13.91b | 385.12 ± 12.04a | 388.94 ± 11.85a | 378.40 ± 14.10ab | 7.821 | 0.0001* |
| Day 28 Weight (g) | 1124.32 ± 41.20b | 1165.80 ± 38.50a | 1178.45 ± 36.12a | 1135.10 ± 40.80b | 11.230 | < 0.0001* |
| Final Weight Day 42 (g) | 1919.80 ± 74.05b | 1986.00 ± 64.71a | 2008.77 ± 74.02a | 1935.42 ± 60.03b | 9.331 | < 0.0001* |
| Total Weight Gain (g) | 1876.83 ± 74.03b | 1942.57 ± 64.58a | 1965.13 ± 74.53a | 1892.19 ± 59.51b | 9.210 | < 0.0001* |
| Cumulative Feed Intake (g) | 4002.44 ± 51.16 | 3993.33 ± 64.24 | 4009.14 ± 75.84 | 3993.29 ± 101.63 | 0.260 | 0.854 (NS) |
| Feed Conversion Ratio (FCR) | 2.14 ± 0.08a | 2.06 ± 0.07b | 2.04 ± 0.08b | 2.11 ± 0.09a | 7.303 | 0.0002* |

Note: Values with different superscript letters within a row differ significantly (p < 0.05). NS = Not Significant.

### 3.2 Mycological Evaluation: Fungal Load and Culture Positivity

Dietary supplementation with essential oil proved to be highly effective in controlling Aspergillus fungus, significantly decreasing the fungal load in the lungs and the positivity rate in the culture. The percentage of birds with culture positivity (viability of aspergillus organisms in lung tissue) was higher in the Control

group (12/25, 48.0%) than in the other groups. The lowest culture positivity was the Oregano Oil group at 8.0% (2/25 birds) followed by the Thyme Oil group with a culture positivity of 12.0% (3/25 birds). Culture positivity was much higher for the Combination group (40.0% (10/25 birds) than for any of the single oil treatments (Chi-Square = 14.82, p = 0.0020).

Colonization of lungs was considered to be high in the Control group with the mean value of log10 CFU/g (3.46 ± 2.52). Single application with both essential oils significantly reduced fungal load with Oregano Oil group having the lowest meanLog10CFU/g (1.26 ± 0.64, P<0.001) and Thyme Oil group 1.45 ± 1.17 log10CFU/g (P<0.001). The fungal loads in the Combination group (2.58 ± 1.97 log10 CFU/g) were not significantly different from the Control group but they were significantly higher than the two single oil treatments, indicating antagonistic effect between the combined oils. These results were confirmed by median log10 CFU/g values: Controls 3.91, Oregano Oil 1.02, and Thyme Oil 1.02. Significant differences between the treatment groups were confirmed by a Kruskal-Wallis test (H = 11.75, p = 0.0083). Full mycological data are given in Table 2.

**Table 2: Pulmonary Aspergillus Microbial Burden and Culture Positivity**

| Parameter | Control (n=25) | Thyme Oil 350mg/kg (n=25) | Oregano Oil 350mg/kg (n=25) | Thyme+Oregano 350mg/kg (n=25) | Test Statistic | p-Value |
|---|---|---|---|---|---|---|
| Culture Positive Birds (n, %) | 12 (48.0%) | 3 (12.0%) | 2 (8.0%) | 10 (40.0%) | Chi-Square = 14.82 | 0.0020* |
| Log10 CFU/g (Mean ± SD) | 3.46 ± 2.52a | 1.45 ± 1.17b | 1.26 ± 0.64b | 2.58 ± 1.97a | F = 8.809 | 0.00003* |
| Log10 CFU/g (Median) | 3.91 | 1.02 | 1.02 | 1.02 | H = 11.75 | 0.0083* |
| Log10 CFU Range (Min - Max) | 1.02 - 6.56 | 1.02 - 5.89 | 1.02 - 3.84 | 1.02 - 6.20 | - | - |

Note: Values with different superscript letters within a row differ significantly (p < 0.05).

### 3.3 Pathological Lesion Severity

There were substantial variation in the severity of lesions between the various treatments according to gross pulmonary pathology evaluation. The Control group had the highest mean lesion score (2.2 ± 0.6), with moderate to severe pulmonary lesions that were multifocal and consisted of multifocal nodules and consolidation. The Oregano Oil group had the lowest mean lesion score (0.96 ± 0.79) and these were significantly lower compared with all other groups (p < 0.0001). Lesion severity was also significantly lower in the Thyme Oil group (1.52 ± 0.87) than in the Control group of chickens (p = 0.0057). Combination group (1.64 ± 0.81) had intermediate scores which did not significantly differ from the Control group.

A distribution analysis of the lesion scores was also made to provide further information on the efficacy of treatment. None (0%) of the birds in the Control group had a normal lesion score (0), compared to 8 birds (32%) in the Oregano Oil group with no pulmonary lesions and 4 (16%) in the Thyme Oil group. The highest percentage of birds (birds in the Control group) had severe lesions (Score 3; 7/25 birds) and the Oregano Oil group had no birds with Score 3 lesions. Lesion scores were distributed more closely to the Control group in the Combination group with 56% of birds scoring 2 and 8% at Score 0.

The parametric ANOVA results were confirmed by non-parametric Kruskal-Wallis analysis which showed significant differences between median lesion scores for the different treatments (H = 25.03; p < 0.0001). Full pathological information is shown in Table 3.

**Table 3: Pulmonary Gross Lesion Scores Across Treatment Groups**

| Parameter | Control (n=25) | Thyme Oil 350mg/kg (n=25) | Oregano Oil 350mg/kg (n=25) | Thyme+Oregano 350mg/kg (n=25) | Test Statistic | p-Value |
|---|---|---|---|---|---|---|
| Mean Lesion Score (± SD) | 2.20 ± 0.58a | 1.52 ± 0.87b | 0.96 ± 0.79c | 1.64 ± 0.81ab | F = 10.899 | < 0.0001* |
| Median Lesion Score | 2.00 | 2.00 | 1.00 | 2.00 | H = 25.03 | < 0.0001* |
| Score 0 Frequency (n, %) | 0 (0%) | 4 (16%) | 8 (32%) | 2 (8%) | - | - |
| Score 1 Frequency (n, %) | 2 (8%) | 6 (24%) | 10 (40%) | 7 (28%) | - | - |
| Score 2 Frequency (n, %) | 16 (64%) | 13 (52%) | 7 (28%) | 14 (56%) | - | - |
| Score 3 Frequency (n, %) | 7 (28%) | 2 (8%) | 0 (0%) | 2 (8%) | - | - |

Note: Values with different superscript letters within a row differ significantly ($p < 0.05$).

**Graphical Representation**

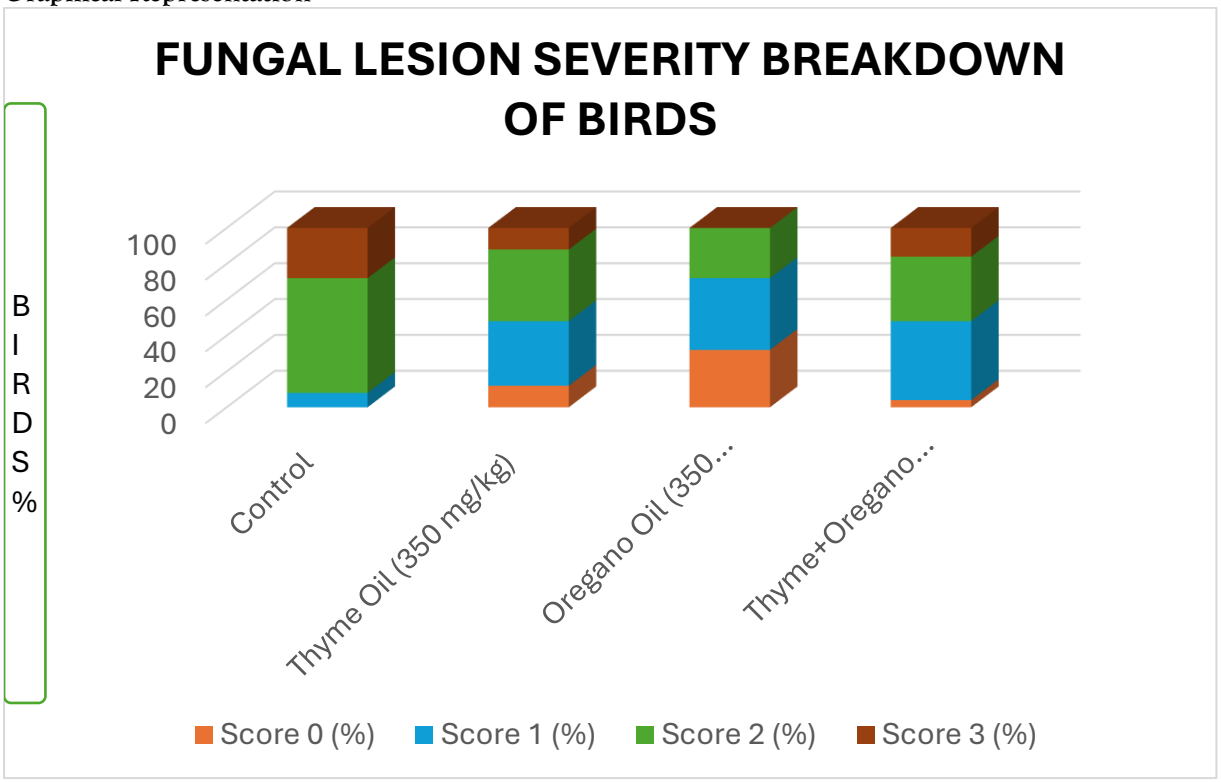



**Figure 1.0: Fungal Lesion Severity Breakdown of Birds Across Treatment Groups**

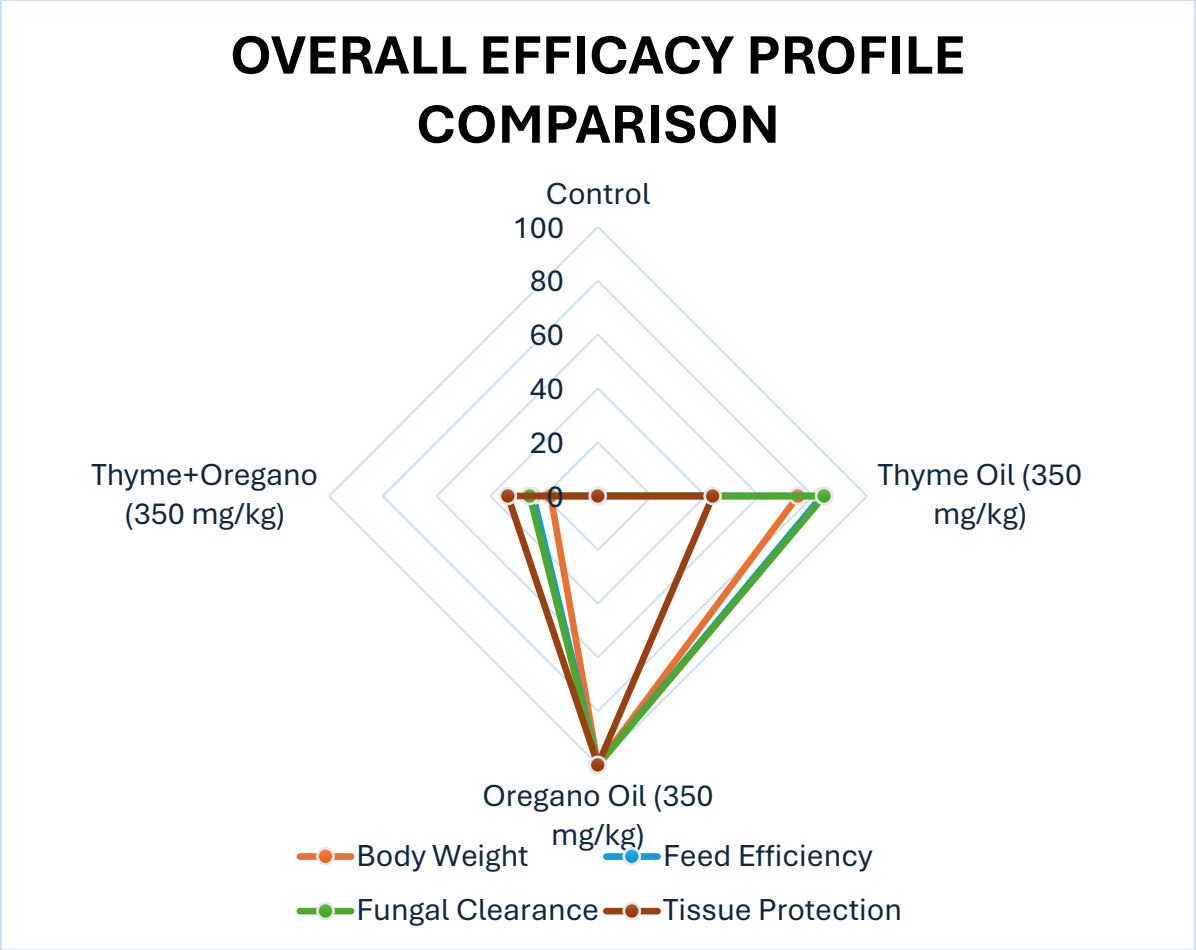



**Figure 2.0: Overall Efficacy Profile Comparison Across Treatment Groups**

**Discussion**

The use of thyme oil and oregano oil at a dosage of 350 mg/kg feed could significantly decrease the fungal burden of aspergillus fungus in the lungs and culture positivity in experimental chickens. The significant decrease in fungal load among the Control group (3.46 log10 CFU/g) and Oregano Oil group (1.26 log10 CFU/g) and Thyme Oil group (1.45 log10 CFU/g) is evidence of the strong in vivo antifungal effect of these essential oils [10]. These effects are similar to the mechanisms of action of the main constituents of the EO: thymol and carvacrol. These monoterpenoid phenols are lipophilic and can efficiently permeate the lipidic hydrophobic part of fungal cell membranes, thereby compromise the integrity of the membrane and induce its permeability. This creates a leakage of several important cellular components, such as ATP, $Ca^{2+}$ and $K^{+}$, leading to a depolarization of the membranes, disruption of the proton motive force and membrane degradation, ending in membrane rupture and cell death [11]. Also, thymol and carvacrol block the synthesis of ergosterol, the sterol present in fungal cells, by inhibiting the enzymes that are involved in the biosynthesis of fungal cell wall lipids in which the synthesis is mediated by enzymes of cytochrome P450 enzymes, further impairing the integrity of the cells. The overall decrease in culture positivity (from 48%, Control to 8%, Oregano Oil; and 12%, Thyme Oil) demonstrates that the compounds not only inhibit fungal growth, but are able to kill the fungal organisms in pulmonary tissues [12]. The growth performance was significantly improved in dietary essential oil supplementation groups (Oregano Oil and Thyme Oil), whose final body weights were 88.97 g and 65.20 g higher than the Control group, respectively. The growth improvements observed are due to multiple mechanisms. First, less fungal burden and accompanying pulmonary pathology meant less energy expended for immune activation and tissue repair, and more net energy was available for skeletal muscle deposition [13], [14], [15]. Aspergillosis causes chronic inflammation, leading to catabolic processes such as degradation of muscle protein and increased maintenance energy requirement. Essential oils conserved protein synthesis and growth

efficiency by decreasing the inflammatory burden. Second, essential oils are known to stimulate the secretion of such enzymes as lipase, amylase and trypsin at the intestinal tract and pancreas and increase the digestibility of nutrients and absorption of these nutrients into the body. This is indicated by a better FCR value for Oregano Oil (2.04) and Thyme Oil (2.06) in comparison with Control (2.14) [16]. The lack of treatment differences in cumulative feed intake supports the idea that the better weight gain was due to better feed efficiency and not to more feed. Third, essential oils' modulation of gut microbiota decreases the subclinical bacterial competition for nutrients and prevents the elaboration of bacterial toxins that negatively affect intestinal barrier function and nutrient absorption. The effects of fungal burden reduction, enzyme stimulation and microbiome modulation are all combined in the single EO treatment groups to account for the significant benefits in growth performance [17].

The most interesting result of this study was that the combination treatment of Thyme + Oregano was less effective than the treatments with the individual oils alone. The Combination group significantly had more fungal load (2.58 log10 CFU/g), more culture positive (40%) and higher lesion scores (1.64) than the single oil treatments and equal to the Control group. These unexpected antagonism indicates that the two major active compounds, Thymol and Carvacrol, have a competitive interaction in co-administration at the doses used in this study [18]. Thymol and carvacrol (phenolic monoterpenoids, respectively with isopropyl and methyl substituents on the benzene ring) may bind competitively to hydrophobic target sites on the fungal cell membranes based on their similar structures. Each compound alone can be applied to a plant in sufficient amounts to reach satisfactory levels of saturation at the target sites and cause loss of membrane integrity. Co-administered at the same doses (175 mg/kg each) though, the concentration of each compound is cut half (350 mg/kg). This dilution may affect bioactivity, for instance if an important concentration should be reached for the antifungal activity [19]. In addition, the combined effect of both compounds could involve a competitive inhibition at the level of penetration of the fungal cell membrane or of the effect of targets within the cell. For practical application, they emphasize an important point: the use of combination of essential oils does not necessarily result in additive and/or synergistic effect; it may lead to a decrease in the effect in some cases. The complicated dynamics of phytogenic active substances, which can relate to several target points in cells, require careful optimization in ratios and concentrations for the particular application [20]. The results of this study have ramifications for commercial broiler production especially in the light of the growing move towards restricted use of synthetic antimicrobials. In conclusion, the potential of oregano and thyme essential oils as an effective treatment for avian aspergillosis is demonstrated, suggesting a natural alternative to treat this economically important respiratory disease. The inclusion rate of 350 mg/kg in this study is considered to be a practical and cost friendly dose level which can easily be incorporated into commercial broiler feed [21], [22], [23].

The study is well-designed with a controlled challenge, standardized essential oil formulations, full performance measures and several complementary outcome measures (growth, microbiology, pathology). Both parametric and non-parametric statistical analysis have been carried out, thus enabling to validate the findings. The following caveats, however, should be made. The intratracheal inoculation model may not accurately simulate natural exposure situations because birds naturally inhale Aspergillus spores when they breathe in contaminated litter and feed. Only a single inclusion rate (350 mg/kg) was tested in the study and dose-response trials would be beneficial to define inclusion rate effective levels and threshold(s). This study lasted 42 days, which is the time period used for broiler production and does not consider possible effects on reproductive performance or long-term health. Lastly, the study showed a fungal effect, the mechanisms of which were not directly examined. Barrier function of the fungal membrane, levels of ergosterol and fungal gene expression should be examined in the future using molecular analyses to gain deeper insights into the mechanisms.

**Conclusion**

Oregano and thyme essential oils can be used as dietary supplements at 350 mg/kg feed to significantly reduce the impact of Aspergillus infection in broiler chickens by curbing fungal growth, reducing tissue damage, and maintaining growth performance. Oregano oil was the most effective overall as it reduced the fungal burden to 1.26 log10 CFU/g, reduced pulmonary lesion score to 0.96 and had the best final

body weight of 2008.77 g. Mixing the two oils, namely thiole and oregano at equal ratio, showed a therapeutic antagonism with high percentage of reduction in efficacy than any single oil treatment therefore it is not recommended for practical application. The results indicate that oregano essential oil could be considered as a natural antifungal agent in commercial broiler feed to help minimize the use of synthetic antimicrobials without compromising flock health and productivity.

The following are recommendations for practice and further studies based on these findings. Oregano essential oil supplementation in broiler feed at the level of 350 mg/kg could be considered as a cost-effective natural approach to mitigate the effects of pulmonary fungal challenges by commercial broiler producers. Systematic management of litter quality and environmental sanitation should be implemented in conjunction with the nutritional interventions to reduce exposure to Aspergillus spores. Research to determine optimal and possibly lower levels of inclusion needs to be examined. Longitudinal studies on the impact of essential oil supplementation on carcass quality, meat composition and consumer acceptance, would help in the regulation approval and commercial use of the supplementation. Search for the possible synergism between oregano oil and other phytogenic chemicals such as saponins, flavonoids, and organic acids can help to determine part of the possible true synergisms without antagonistic effects. Lastly, research on essential oil effect on immune function and potential decrease in severity of disease in naturally occurring outbreaks would provide further evidence of the effect and support evidence-based use in commercial poultry production.

**Authors' Contribution**

AS conceptualized and designed the study, developed the fungal challenge model, performed the investigation, curated the data, and drafted the manuscript. Author 2 conducted the laboratory and mycological analysis, while EU supervised the project, administered the dietary formulation, and critically revised the manuscript. PB, MA, AA, RMS, ST, MS, Q, and MA contributed to data analysis, animal management, lesion scoring, feed preparation, oil characterization, literature review, microbiological assessment, and statistical analysis. All authors read and approved the final version and agree to be accountable for all aspects of the work.

**Conflict of Interest**

The authors declare no competing financial or non-financial interests.

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Generative AI tools were used solely for language editing and grammar refinement; all scientific content, data analysis, and interpretation were conceived and written entirely by the authors, who take full responsibility for the final manuscript.

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