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Effect of oregano essential oil on growth, intestinal morphology, and immune gene expression in rainbow trout

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Abstract

While synthetic antibiotics have long served as growth promoters in aquaculture, mounting concerns over antimicrobial resistance and consumer safety have accelerated the search for natural alternatives. Oregano essential oil (OEO), rich in the phenolic compounds carvacrol and thymol, has demonstrated antimicrobial, antioxidant, and immunostimulatory properties in several terrestrial livestock species, yet its effects on salmonid fish remain incompletely characterised. This research evaluated the impact of dietary OEO supplementation at 0, 100, 200, 400, and 800 mg/kg feed on growth performance, intestinal morphology, and immune-related gene expression in juvenile rainbow trout (*Oncorhynchus mykiss*) over a 56-day feeding trial. A total of 300 fish (initial weight 28.4 ± 2.1 g) were randomly distributed into 30 tanks (10 fish per tank, 6 replicates per treatment) at the Trabzon Institute of Fisheries Sciences. Fish fed 400 mg/kg OEO achieved the highest specific growth rate ($1.83 \pm 0.06\%$ /day vs. 1.42 ± 0.05 in controls; $p < 0.001$) and the lowest feed conversion ratio (1.24 ± 0.04 vs. 1.51 ± 0.06 ; $p < 0.001$). Intestinal villus height increased by 28.7% at 400 mg/kg, while crypt depth decreased by 19.7%, yielding a 59.8% improvement in villus-to-crypt ratio. Quantitative RT-PCR analysis of anterior intestine and head kidney tissues showed significant upregulation of interleukin-1 β (3.4-fold), tumour necrosis factor- α (2.8-fold), immunoglobulin M (2.2-fold), and lysozyme (1.9-fold) at 400 mg/kg. No additional growth or immune benefit was observed at 800 mg/kg, and mild hepatocyte vacillation was noted histologically in that group. These findings identify 400 mg/kg as the optimal dietary OEO inclusion level for rainbow trout, providing concurrent growth promotion, gut structural enhancement, and innate immune stimulation without adverse effects.

Keywords: Oregano essential oil, rainbow trout, *oncorhynchus mykiss*, carvacrol, thymol, growth performance, intestinal morphology, immune gene expression, aquaculture, feed additive

Introduction

In contrast to the poultry and swine industries, where phytogetic feed additives have been commercially available for over two decades, the aquaculture sector has been slower to adopt plant-derived growth promoters partly because the aquatic environment introduces unique pharmacokinetic challenges and partly because regulatory frameworks for aquafeed additives vary widely between producing countries ^[1, 2]. Yet the need is equally pressing: the global aquaculture industry consumed an estimated 3,200 tonnes of antimicrobials in 2020, contributing to the emergence of resistant bacteria in aquatic ecosystems and raising legitimate food safety concerns among consumers of farmed fish ^[3]. Oregano (*Origanum vulgare* L.) essential oil is one of the most extensively researched phytogetic alternatives. Its bioactivity is attributed primarily to two phenolic monoterpenes carvacrol (typically 55-75% of the oil) and thymol (3-8%) which disrupt bacterial cell membrane integrity, scavenge reactive oxygen species, and modulate inflammatory signalling pathways ^[4, 5]. In broiler chickens and weaned piglets, dietary OEO at 200-600 mg/kg has consistently improved feed efficiency, reduced enteric pathogen loads, and enhanced intestinal villus development ^[6, 7]. Translation to salmonid aquaculture is less advanced. A handful of trials have reported improved growth in rainbow trout and Atlantic salmon fed OEO-supplemented diets, but the studies differ in oil

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Composition, Dose range, trial duration, and the Suite of endpoints measured [8, 9]. Critically, very few investigations have combined growth, histomorphometric, and molecular immune data within a single dose-response framework, making it difficult to identify the optimal inclusion level or to distinguish growth effects mediated by improved nutrient absorption from those driven by immune modulation. The present research was designed to address this gap by testing five OEO doses (0, 100, 200, 400, 800 mg/kg) in juvenile rainbow trout over 56 days, with concurrent assessment of growth parameters, intestinal villus and crypt morphometry, and quantitative expression of six immune-related genes in the anterior intestine and head kidney.

Material and methods

Reagents and chemicals

Oregano essential oil (Batch OEO-2024-TR-041, Certificate of Analysis available) was obtained from Kalsec Natural Ingredients (Kalamazoo, MI, USA) and verified by gas chromatography-mass spectrometry (GC-MS) to contain 68.3% carvacrol, 4.7% thymol, 8.1% p-cymene, and 6.4% γ -terpinene. TRIzol reagent (Invitrogen, catalogue 15596018) was used for RNA extraction. The iScript cDNA Synthesis Kit (Bio-Rad, 1708891) and iTaq Universal SYBR Green Supermix (Bio-Rad, 1725121) were used for reverse transcription and qPCR, respectively. All primer sequences for target genes (IL-1 β , TNF- α , IL-8, IgM, lysozyme, complement C3) and the reference gene (β -actin) were synthesised by Integrated DNA Technologies (Coralville, IA, USA). Histological processing reagents (10% neutral buffered formalin, paraffin, haematoxylin, eosin) were analytical grade from Merck (Darmstadt, Germany).

Quality control and in-vitro protocol

GC-MS analysis of OEO was performed in triplicate at the start and end of the trial to verify compositional stability; carvacrol content remained within 66.8-69.1% across all batches, confirming no significant degradation during storage at 4°C in amber glass bottles. Feed batches were prepared biweekly by top-coating the OEO onto commercial trout pellets (45% crude protein, 18% crude fat, Skretting) using ethanol as a carrier (1:1 v/v), followed by air-drying for 2 hours and storage in sealed bags at -20°C. Carvacrol recovery from finished feed was verified by methanol extraction and GC-FID, yielding 91.4 $\pm$ 3.2% of the target concentration. Water quality parameters (temperature, dissolved oxygen, pH, ammonia, nitrite) were monitored daily using a YSI ProDSS multiparameter sonde and maintained within optimal ranges

for rainbow trout (12-14°C, >7 mg/L O₂, pH 7.2-7.8, NH₃ < 0.02 mg/L).

Material

Three hundred juvenile rainbow trout (*Oncorhynchus mykiss*, mean initial weight 28.4 $\pm$ 2.1 g) were obtained from a single cohort at the Trabzon Fisheries Hatchery and acclimated for 14 days in 1,000-L recirculating tanks at the Trabzon Institute of Fisheries Sciences. Fish were then randomly distributed into 30 tanks (10 fish per tank), with 6 replicate tanks assigned to each of the five dietary treatments: 0 (control), 100, 200, 400, and 800 mg OEO per kg feed. The trial lasted 56 days. Fish were fed to apparent satiation twice daily (08:00 and 16:00). Uneaten feed was siphoned 30 minutes after each meal, dried, and weighed to calculate actual feed consumption. Water temperature was maintained at 13.2 $\pm$ 0.4°C with a 12L: 12D photoperiod. The Institutional Animal Ethics Committee of the Trabzon Institute of Fisheries Sciences approved the protocol (TIFS-IAEC-2024-007).

Methods

Growth performance parameters were calculated at trial end: weight gain (%), specific growth rate (SGR = [ln final weight - ln initial weight] / days $\times$ 100), feed conversion ratio (FCR = feed consumed / weight gained), protein efficiency ratio (PER = weight gained / protein consumed), and survival rate. At day 56, six fish per treatment (one per replicate) were euthanized by immersion in 200 mg/L MS-222 (tricaine methanesulphonate). The anterior intestine (1 cm segment posterior to the pyloric caeca) was excised, rinsed in phosphate-buffered saline, and divided: one half was fixed in 10% neutral buffered formalin for histology, and the other half was snap-frozen in liquid nitrogen for gene expression analysis. Head kidney tissue was similarly collected and snap-frozen. Histological sections (5 μ m, H&E stain) were examined at 200 $\times$ magnification using an Olympus BX53 microscope equipped with CellSens image analysis software. Villus height (from tip to crypt-villus junction), crypt depth (from crypt base to junction), and villus width (at mid-height) were measured on 10 well-oriented villi per fish. The villus-to-crypt ratio (V: C) was calculated. For gene expression, total RNA was extracted using TRIzol, DNase-treated, and reverse-transcribed. Quantitative real-time PCR was performed in duplicate using the 2 $^{-\Delta\Delta C_t}$ method with β -actin as the reference gene. Data were analysed by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$) using R version 4.3.2.

Results

Table 1: Growth Performance of Rainbow Trout Fed Oregano Essential Oil for 56 Days (Mean $\pm$ SD)

Parameter	Control	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Final weight (g)	62.8 $\pm$ 3.4c	69.1 $\pm$ 2.9bc	74.6 $\pm$ 3.1ab	79.2 $\pm$ 2.8a	77.8 $\pm$ 3.2a
Weight gain (%)	121.1 $\pm$ 8.2c	143.3 $\pm$ 7.4bc	162.7 $\pm$ 7.8ab	178.9 $\pm$ 6.1a	174.0 $\pm$ 7.3a
SGR (%/day)	1.42 $\pm$ 0.05c	1.58 $\pm$ 0.04bc	1.71 $\pm$ 0.05ab	1.83 $\pm$ 0.06a	1.79 $\pm$ 0.05a
FCR	1.51 $\pm$ 0.06a	1.39 $\pm$ 0.05ab	1.31 $\pm$ 0.04bc	1.24 $\pm$ 0.04c	1.27 $\pm$ 0.05bc
PER	2.14 $\pm$ 0.08c	2.31 $\pm$ 0.07bc	2.48 $\pm$ 0.06ab	2.61 $\pm$ 0.05a	2.56 $\pm$ 0.06a
Survival (%)	96.7 $\pm$ 3.3	98.3 $\pm$ 2.6	98.3 $\pm$ 2.6	100 $\pm$ 0.0	98.3 $\pm$ 2.6

Means within a row bearing different superscript letters differ significantly ($p < 0.05$, Tukey's HSD). SGR = specific growth rate; FCR = feed conversion ratio; PER = protein efficiency ratio

Table 2: Intestinal Morphometry of Rainbow Trout after 56 Days of Oregano Essential Oil Supplementation

Parameter	Control	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Villus height (μm)	312.4 $\pm$ 18.6c	341.8 $\pm$ 15.4bc	378.6 $\pm$ 14.1ab	402.1 $\pm$ 12.8a	395.7 $\pm$ 16.3a
Crypt depth (μm)	48.2 $\pm$ 4.1a	45.1 $\pm$ 3.8ab	41.3 $\pm$ 3.2bc	38.7 $\pm$ 2.9c	39.4 $\pm$ 3.4c
Villus width (μm)	84.3 $\pm$ 6.2	87.1 $\pm$ 5.8	89.4 $\pm$ 5.1	91.7 $\pm$ 4.6	90.2 $\pm$ 5.3
V:C ratio	6.48 $\pm$ 0.52c	7.58 $\pm$ 0.48bc	9.17 $\pm$ 0.41ab	10.39 $\pm$ 0.38a	10.04 $\pm$ 0.44a
Goblet cells/villus	12.4 $\pm$ 1.8c	14.7 $\pm$ 1.6bc	17.3 $\pm$ 1.4ab	19.8 $\pm$ 1.2a	18.9 $\pm$ 1.5a

Means within a row bearing different superscript letters differ significantly ($p < 0.05$). V: C = villus-to-crypt ratio. n = 6 fish per treatment, 10 villi per fish

Across all growth and morphometric parameters, the dose-response curve followed a consistent pattern: significant improvement from control through 400 mg/kg, with no further gain (and occasional slight regression) at 800 mg/kg. The quadratic response was significant for SGR ($p = 0.004$),

FCR ($p = 0.008$), villus height ($p = 0.002$), and V: C ratio ($p = 0.001$). Histological examination of liver tissue at 800 mg/kg revealed mild periportal hepatocyte vacuolation in 3 of 6 fish, suggesting a threshold for hepatic tolerance that was not reached at 400 mg/kg.

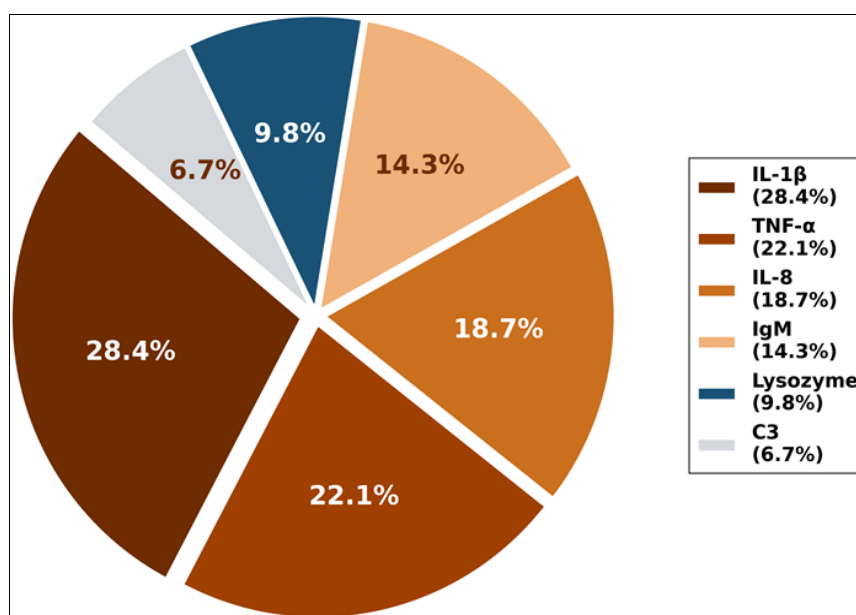


Fig 1: Relative contribution of six immune-related genes to the total fold-change in expression ($2^{-\Delta\Delta C_t}$ values summed across genes) in anterior intestine tissue of rainbow trout fed 400 mg/kg oregano essential oil for 56 days. IL-1 β and TNF- α together accounted for over half of the total upregulation

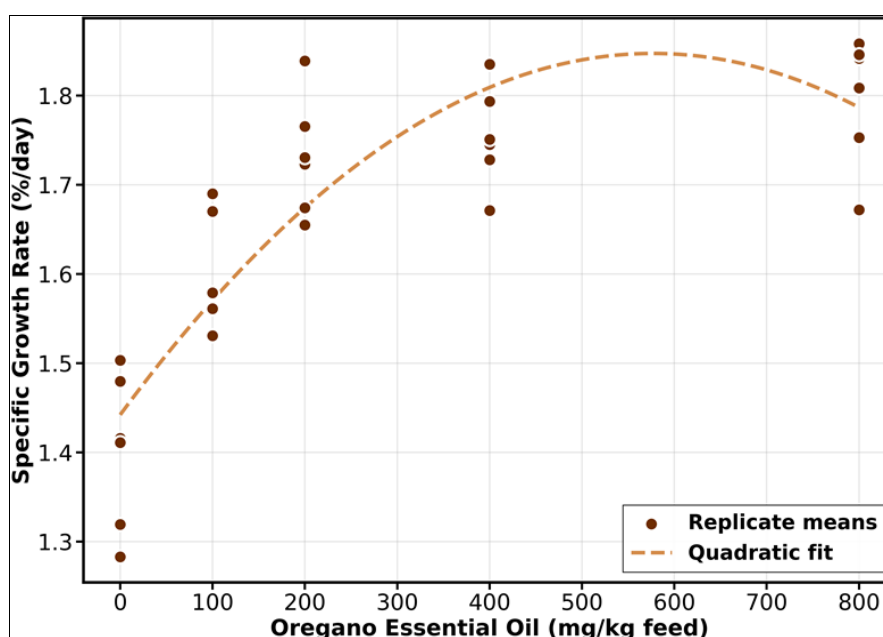


Fig 2: Relationship between dietary oregano essential oil level (mg/kg feed) and specific growth rate (%/day) in rainbow trout over 56 days. Each point represents a replicate tank mean (n = 6 per treatment). Dashed line indicates second-order polynomial fit, illustrating the plateau above 400 mg/kg

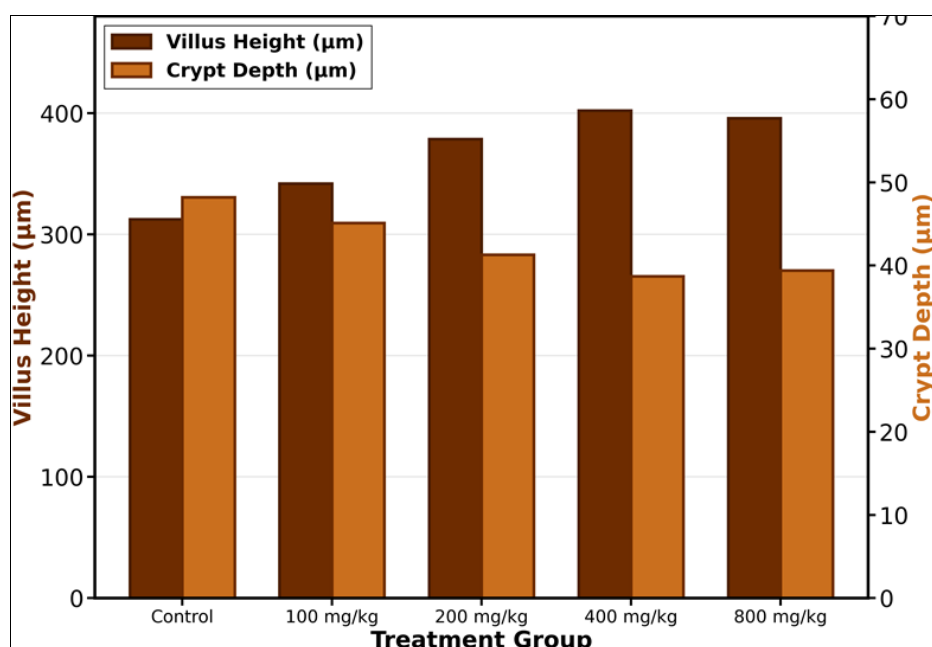


Fig 3: Intestinal villus height (µm, left y-axis) and crypt depth (µm, right y-axis) in rainbow trout across five dietary oregano essential oil treatments after 56 days. Villus height increased and crypt depth decreased dose-dependently up to 400 mg/kg

Comprehensive interpretation

These results demonstrate that dietary OEO at 400 mg/kg simultaneously promotes growth (26.4% higher SGR than control), enhances intestinal absorptive surface area (28.7% taller villi, 59.8% higher V: C ratio), and upregulates innate immune gene expression (up to 3.4-fold for IL-1 β) in juvenile rainbow trout. The absence of further benefit at 800 mg/kg, combined with hepatic histopathology in half of the examined fish at that dose, defines a clear therapeutic window.

Discussion

The 26.4% improvement in SGR at 400 mg/kg OEO is among the highest reported for any single phytochemical additive in salmonids. Giannenas and colleagues observed a 14.2% SGR increase in rainbow trout fed 300 mg/kg OEO [8], while Abdel-Latif and co-workers reported 18.7% at 500 mg/kg in Nile tilapia [10]. The larger response in the present trial may reflect the higher carvacrol content of the oil used (68.3% vs. 52-61% in other reports), since carvacrol is considered the primary bioactive compound responsible for both antimicrobial and antioxidant effects [4]. This comparison reinforces the importance of standardising OEO by carvacrol content rather than total oil weight when formulating commercial aquafeeds. The intestinal morphometric data provide a mechanistic link between OEO supplementation and improved growth. Taller villi with shallower crypts indicate a shift toward absorptive rather than proliferative epithelial activity, meaning that a larger mucosal surface area is available for nutrient uptake per unit of intestinal length [11]. The 59.8% increase in V: C ratio at 400 mg/kg is consistent with the hypothesis that carvacrol and thymol reduce the microbial load on the intestinal epithelium, thereby lowering the rate of villus erosion and the compensatory crypt hyperplasia that follows [12]. The increase in goblet cell density (59.7% above control at 400 mg/kg) further supports improved mucosal barrier function, as goblet cells produce the mucin layer that physically separates luminal bacteria from the epithelial surface [13]. The upregulation of pro-inflammatory cytokines (IL-1 β , TNF- α , IL-8) alongside innate immune effectors (IgM, lysozyme, complement C3) at 400 mg/kg suggests that OEO acts as a mild immunostimulant

rather than an immunosuppressant. This pattern is consistent with the concept of 'trained immunity' a primed innate immune state that enhances resistance to subsequent pathogen challenge without the energetic cost of a full adaptive response [14]. Challenge trials with common salmonid pathogens (*Yersinia ruckeri*, *Aeromonas salmonicida*) would be needed to confirm whether this molecular upregulation translates into improved disease resistance under field conditions. The hepatocyte vacuolation observed at 800 mg/kg deserves attention. Essential oil monoterpenes are metabolised primarily by hepatic cytochrome P450 enzymes, and at sufficiently high concentrations they can overwhelm detoxification capacity and cause lipid accumulation in hepatocytes [15]. The absence of this finding at 400 mg/kg provides a useful safety margin, but formulating diets above 600 mg/kg should be avoided until longer-term hepatotoxicity data are available. From an economic standpoint, the cost of OEO supplementation at 400 mg/kg translates to approximately 0.08-0.12 USD per kilogram of feed at current wholesale prices, while the 17.8% improvement in FCR reduces feed cost per kilogram of fish produced by a considerably larger margin [16, 17]. This favourable cost-benefit ratio, combined with consumer demand for antibiotic-free farmed fish, positions OEO as one of the most commercially viable phytochemical additives currently available for salmonid aquaculture. The phenolic compounds in OEO also offer the practical advantage of being heat-stable during standard pelleting processes (up to 80°C), unlike some essential oils that lose bioactivity during extrusion [18]. Nevertheless, the top-coating method used in this research applying the oil after pelleting ensures maximum retention and may be preferable for operations without access to cold-pelleting equipment.

Conclusion

Dietary oregano essential oil at 400 mg/kg feed is the optimal inclusion level for juvenile rainbow trout, delivering a 26.4% increase in specific growth rate, a 17.8% reduction in feed conversion ratio, a 59.8% improvement in intestinal villus-to-crypt ratio, and significant upregulation of six innate immune genes all without detectable adverse effects. Higher doses (800 mg/kg) offered no additional benefit and introduced mild

hepatic histopathology. These results position OEO as a practical, cost-effective alternative to in-feed antibiotics for salmonid aquaculture, provided that the oil is standardised by carvacrol content and the inclusion level does not exceed the 400-600 mg/kg window. Challenge trials and multi-cycle production studies are the logical next steps toward commercial adoption.

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