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NANO-OREGANO ESSENTIAL OIL IMPROVES RAINBOW TROUT'S (*ONCORHYNCHUS MYKISS*) GROWTH PERFORMANCE, OXIDATIVE STATUS, FATTY ACID PROFILE OF FILLET, AFFECTS GENE EXPRESSION AND SUPPORTS SKIN AND INTESTINAL HISTOMORPHOMETRY

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Abstract

The present study was conducted to investigate the effects of a microencapsulated oregano feed additive containing oregano essential oil named "VISNAT 1" on growth performance, intestinal and skin histomorphometry, serum biochemical parameters, fillet oxidative status and fatty acids (FA) profile of rainbow trout (*Oncorhynchus mykiss*) under conventional farming conditions. Also, changes in the expression levels of both insulin-like growth factor-1 (IGF-1) and growth hormone 1 (GH-1) genes were assessed. A total of 200 sub-adult trouts (52 g±3.2) were randomly allocated into two different treatments with four replicates each. The control group was fed a basal diet, while oregano group was fed diet supplemented with oregano essential oil at the level of 1.0 kg/t of feed. The results indicate that the phytobiotic product beneficially altered feed, fillet and serum oxidative status and improved performance of trouts. Also, oregano essential oil beneficially affected the histopathology and histomorphometry of intestine and skin. Moreover, oregano oil addition in the rainbow trout diet can initiate mitogenic and anti-apoptotic responses in the liver, which is one of the main actions of IGF-1 receptor promoting body growth and health. As a result, oregano essential oil can be used as a supplement on trouts diet but further investigation upon its action should take place.

Key words: rainbow trout, oregano essential oil, performance, antioxidant status, IGF-1

Aquaculture accounts for at least 17% of animal origin proteins consumed in a global scale (FAO, 2018) and has advanced tremendously (Sönmez et al., 2022 b). However, intensive aquaculture systems give rise to a range of interrelated factors, including compromised animal welfare and physiological processes, stress on the cultured species, and environmental degradation (Filogh et al., 2023). Scientific and industrial communities invest on supporting increased production volumes based on precision nutrition, genetic selection and superior management protocols that fortify animal health and efficiency. The use of nontherapeutic antimicrobials could support fish growth (Van Boeckel, 2017). Nonetheless, the pressure on aquaculture to reduce antibacterial failures along with pressure to eliminate feed antibiotics as growth enhancers has initiated the use of natural products to develop eco-friendly methods (Sönmez et al., 2021). This new generation of feed additives includes herbs and their essential oils and extracts (Giannenas et al., 2003; Brenes and Roura, 2010). Essential oils are aromatic and volatile oily lipids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, wood, fruits and roots). These natural products have been reported to promote various functions like growth (Shalaby et al., 2006), immune responses (Bugdayci and Ergun, 2011) and disease resistance (Yılmaz et al., 2012) in aquaculture due to their broad antimicrobial activity spectrum as well as presumed safety and efficacy (Özdemir et al., 2022; Rashidian et al., 2022). Herbs and essential oils have been used traditionally to stimulate the production of endogenous secretions in the small intestinal mucosa, pancreas and liver, and thus aid digestion (Williams and Losa, 2001; Lee et al., 2002; Barreto et al., 2008). Many studies have confirmed that the addition of plants or their extracts in the diets, has a beneficial effect to improve growth parameters and protect from diseases in aquaculture (Salem et al., 2022; Sönmez et al., 2022 a).

Additionally, medicinal plants are main sources of natural antioxidants. Generally, fish fat contains higher levels of polyunsaturated fatty acids (PUFA). This is due to the relatively high content of phospholipids in the membrane structure of the muscle cells (Bystrický and Dičáková, 1998). Even a slight increase in the unsaturation of fatty acids in muscle membranes is related to increasing of their susceptibility to oxidation of meat and meat products (Marcinčák et al., 2010). Lipid oxidation is one of the primary mechanisms of quality deterioration in food, especially in meat products (Guo et al., 2021). Therefore, there is a need to increase the antioxidant capacity of muscles that can be achieved by feeding of antioxidant active substances. Using of natural antioxidants in fish diets is a simple method to achieve higher antioxidant stability, improve sensory properties and prolong the storage of fish. There is a significant lack of information available between the main effects of oregano essential oil and its responses in terms of gut tissue morphology. In this paper, intestinal morphology will be evaluated.

It is now well established that insulin-like growth factors (IGF-I and -II) are key molecules with an important role in the regulation of numerous cellular processes, such as proliferation, survival, differentiation, and migration. The biological actions of IGFs are mainly mediated through type-1 (IGF-1) receptor on the cell surface, resulting in tyrosine phosphorylation of the beta-domain of the receptor, leading to signaling cascades such as the mitogen activated protein kinase (MAPK), phosphoinositol-3-kinase and protein kinase B (Akt) signaling pathways (Le Roith et al., 2001; Nakae et al., 2001; Aksakal et al., 2010).

Oregano essential oil (EO) is known for its antioxidant activity. Evidence suggests that dietary inclusion of oregano EO improved growth performance, immune and antioxidative responses in aquaculture species (Magouz et al., 2022). Since oregano EO contains volatile and easily oxidized active ingredients, protection from environmental factors is important for retaining its activity in the marketed product. This problem may be tackled by spray drying, which is an industrially established continuous process that in combination with emulsification offers a method for EO microencapsulation (Reineccius, 2004; Da Costa et al., 2013; Bakry et al., 2016; Baranauskaite et al., 2017). Microencapsulated products are protected from humidity and light that normally lead to oxidation of alcohols, reduced antimicrobial activity and even inactivation (Reineccius, 2004; Fernandes et al., 2008; Jafari et al., 2008; De Barros Fernandes et al., 2013). Moreover, microencapsulation is expected to increase the solubility of the EO by facilitating wetting of the wall materials and re-emulsification of sprayed dried product (EOSD), a process that possibly occurs when in contact with gastric fluids (Deshmukh et al., 2016).

The aim of the present study was to evaluate the effects of oregano essential oil on rainbow trout growth performance, gut and skin histomorphometry, hematological parameters, fillet oxidative status and fatty acid profile, under conventional farming conditions. Supplementary, a second objective of this study was to investigate the changes detected on the expression levels of both IGF-1 and GH-1 genes, following a diet with an oregano oil feed additive in the liver of rainbow trout.

Material and methods

Ethics and procedures

Fish breeding, sampling, euthanasia, experimental procedures and biosecurity precautions were conducted in accordance with Greek legislation governing experimental animals and were approved by the Fishery Division of the Ministry of Rural Development and Food (Reg. 489181(3254)/07.02.2018) in research experimental facilities and conducted under the approved research study AUTh Research Committee No 72887.

Environmental setting

The experiments were carried out at Hydrobiological Station of Ioannina in Louros River, Ioannina, Epirus, in Vouliasta, Greece, an important freshwater ecosystem of Western Greece (Figure 1). Louros river springs from Mount Tomaros, near Dodoni Oracle (Prefecture Ioannina). It flows southwards for about 73 km, having a mean annual discharge of 19–28.6 m^3s^{-1}. The catchment area is 780 km^2 of which 332 km^2 belong to the highlands and 448 km^2 to the lowlands. The river drains from 1400 m over the sea level to Amvrakikos Gulf which is a RAMSAR-protected ecosystem. The underlying geology in the wider area is dominated by alluvial geological formations, calcitic and silicate tertiary deposits as well as hard limestones. The annual average catchment precipitation was 90.7 mm with the minimum in August (no rainfall) and the maximum in February (335.9 mm). The annual mean temperature during the study period was 17.9°C and extreme values of 7.8 and 28°C were registered in February and August, respectively.



Figure 1. Study area and fish hatchery facilities

Table 1. Temperature, dissolved oxygen, and pH mean values for the time period September to November 2021

Environmental indexes	Growth tank		Culture tank	
	control	oregano	control	oregano
T (°C)	13.2	13.2	12.4	12.4
O_2 (mg/l)	9.6	9.6	9.8	9.9
pH	7.6	7.6	7.5	7.5

Table 2. Composition of basal diet, g/kg (form: pellets 3.5 mm)

Ingredients	Control diet	Chemical analysis[1]	Content
Wheat grains, extruded (134)[2]	300.0 g/kg	Dry matter	854.1 g/kg
Soybean meal, extruded	70.0 g/kg	Crude protein	441.5 g/kg
Fish meal (720)	340.0 g/kg	Crude fat	170.0 g/kg
Sunflower meal (420)	75.0 g/kg	Crude fiber	19.5 g/kg
Fish oil	30.0 g/kg	Ash	98.8 g/kg
Soy oil	21.1 g/kg	Starch	126.5 g/kg
Gluten meal (595)	97.5 g/kg	Sugars	3.77 g/kg
Wheat bran meal (155)	7.00 g/kg	Calculated analysis	
Binding (high protein) (821)	25.0 g/kg	Ca	18.3 g/kg
Limestone	21.0 g/kg	P (total)	12.0 g/kg
Monocalcium phosphate	3.0 g/kg	Lysine total	31.5 g/kg
L-Lysine HCL (750)	0.2 g/kg	Methionine Cystine	19.2 g/kg
DL-Methionine (990)	3.5 g/kg	Threonine	17.3 g/kg
Vitamin A 1000	0.011 g/kg	Tryptophan	4.78 g/kg
Vitamin D_3 100	0.015 g/kg	Vitamin A	11000 IU
Vitamin E 50% DL-α-tocopheryl acetate	0.5 g/kg	Vitamin D_3	1500 IU
Vitamin K_3 50% menadione	0.6 g/kg	Vitamin E	300 mg
Vitamin B_1	0.05 g/kg	Vitamin K_3	20 mg
Vitamin B_2 80%	0.05 g/kg	Vitamin B_1	49 mg
Vitamin B_6	0.03 g/kg	Vitamin B_2	40 mg
Vitamin B_{12} 1%	0.01 g/kg	Vitamin B_6	30 mg
Niacin	0.350 g/kg	Vitamin B_{12}	0.1 mg
Biotin 2%	0.075 g/kg	Vitamin C	250 mg
Pantothenic acid 90%	0.15 g/kg	Niacin	350 mg
Folic acid 99%	0.008 g/kg	Biotin	1.5 mg
Inositol	0.2 g/kg	Pantothenic acid	147 mg
Choline 50%	4 g/kg	Folic acid	7.6 mg
STAY C35 (vitamin C)	0.704 g/kg	Inositol	200 mg
Manganese oxide 62%	0.113 g/kg	Choline	2000 mg
Zinc oxide 78%	0.09 g/kg	Iron	50 mg
Copper sulphate 25%	0.02 g/kg	Manganese	70 mg
Cobalt sulphate 20%	0.005 g/kg	Zinc	70 mg
Selenium salt 1%	0.01 g/kg	Copper	5 mg
Iodine salt	0.004 g/kg	Cobalt	1 mg
Iron sulphate 30%	0.170 g/kg	Selenium	0.1 mg
Endox (antioxidant)[3]	0.1 g/kg	Iodine	2.5 mg

[1]According to AOAC International (1995).

[2]Values in parentheses are the crude protein (N×6.25) or pure amino acid content, in g/kg.

[3]Containing calcium carbonate, ground corn cob, amorphous silicon dioxide, citric acid, soybean oil, ethoxyquin (a preservative up to 3.0%), butylated hydroxytoluene, phosphoric acid, butylated hydroxyanisole and mono and diglycerides.

Oregano oil was provided in micro encapsulated form. The methodology used for this purpose is described by Partheniadis et al. (2019). Initially an EO in water emulsion was prepared and the EO droplets were stabilized by a mixture of gums and carbohydrates (Ré, 1998; Botrel et al., 2012). The emulsion is then spray-dried under appropriate conditions of temperature and feed rate, and a powder product consisting of single or agglomerated particles is obtained where the EO is encapsulated.

Experimental design, diet and performance

Two hundred sub-adult trouts (52 g±3.2) were randomly allocated into 2 different treatments with four replicates each. All subgroups were housed in separate tanks containing aerated recirculated freshwater and reared at 13.2±0.1°C with a photoperiod of 12:12 h light: dark. Water pH fluctuated between 7.5 and 7.6, and dissolved oxygen between 9.6 and 9.9 mg/l (Table 1). A complete basal diet was formulated to meet trouts' nutrient requirements, discharged of any supplemental antimicrobial or other feed additives. The diet was in pelleted form and analyzed according to the Weende system (Table 2). The control group was fed the basal diet, while the oregano group was fed with the basal diet further supplemented with an oregano essential oil product (visNaturalis) at the level of 1 g/kg feed. Fish were fed to apparent satiation three times a day for the entire experimental period that lasted for 90 days.

Sampling

To determine the effects of the different dietary treatments on several parameters 24 fish per treatment were collected at the end of the experimental period. Fillet samples of six fish per sub-group were assessed to determine the malondialdehyde values evaluated on days 0, 4 and 7 under refrigerated storage according to Giannenas et al. (2011). Also, 6 dorsal fish fillets from each group were stored for the analysis of fatty acid profile. Blood samples from six fish per replicate were collected from the caudal vein. Fish were anesthetized with phenoxyethanol (0.2 ml/l). Blood samples were left to clot for 12 h at 4°C. Following centrifugation at 700 g for 30 min at 4°C, the serum was collected, aliquoted, and stored at −20°C until use for the detection of lysozyme, reactive nitrogen intermediates, total complement, and catalase activity. Glutathione peroxidase, malondialdehyde and IgM serum levels were also detected. After euthanasia, 12 fish per treatment were necropsied. From each fish, skin, liver, and intestinal samples were taken and treated properly for further analysis.

Feed profile

Total phenolic content (TPC)

The total phenolic content of fish feed was assessed by the Folin-Ciocalteu method (Jang et al., 2008). The total phenolic content was expressed in mg gallic acid equivalents (GAE) per L of extract.

Determination of radical scavenging activity (DPPH)

For the determination of radical scavenging activity, the method described by Vasilopoulos et al. (2022) was used. The results of each sample are expressed as % radical scavenging activity (% RSA) and are calculated based on the formula:

$$\frac{Ablank - Asample}{Ablank} \times 100$$

Fillet profile

Performance parameters

All trouts from each treatment group were individually weighed at the time of their placing into the water tank (day 0) and on days 30, 60 and 90 of age. Twelve hours prior to fish weighing, diets were removed and feed consumption within each subgroup was determined. Feed intake was recorded daily. Feed conversion ratio (FCR) values were calculated as the ratio of feed intake to weight gain on days 30, 60 and 90 of age. Body weight (BW) of all fish was measured at the end of the trial. Mortality was recorded daily in each water tank.

Lipid and protein oxidation

To assess the effect of dietary treatment on lipid oxidation of raw trout fillet during refrigerated storage, samples were thawed, wrapped in transparent oxygen-permeable polyvinyl chloride film (6000–8000 $cm^3/m^2 \times 24$ h), and placed in a non-illuminated refrigerated cabinet at 4°C for 7 days, and submitted to the determination of lipid oxidation at 0, 4 and 7 days. The excised tissues were assayed for the levels of malondialdehyde (MDA), according to Ahn et al. (1999), with minor modifications.

For the determination of protein carbonyls, the method of Patsoukis et al. (2004) was applied to fish fillet samples. Calculation of protein carbonyl concentration was based on the molar extinction coefficient of DNPH and was expressed as nmol/mg.

Fatty acid profile

The determination of fatty acids on fish fillets was carried out according to O'Fallon et al. (2007) and with the assistance of gas chromatography. All gas chromatography parameters are described by Lioliopoulou et al. (2023).

Blood serum profile

Determination of total proteins, immunoglobulin M (IgM), malondialdehyde (MDA) and glutathione peroxidase (GSH-PX) levels

For the total protein detection and quantification in the serum samples, a copper-based total protein quantification method was used. More specifically, the BCA Protein Assay (Thermo Fisher Scientific) which is a popular method for colorimetric detection and quantitation of total protein, was used. Bovine serum albumin (BSA, Sigma-Aldrich) served as standard.

Total serum IgM levels were determined by ELISA (Enzyme Linked Immunosorbent Assay) using Fish Immunoglobulin M (IgM) ELISA Kit (CUSABIO) and following manufacturer's protocol. The assay employs the competitive inhibition enzyme immunoassay technique, thus, there is a pre-coated microtiter plate with goat-anti-rabbit antibody.

Serum glutathione peroxidase (GSH-PX) levels were determined by ELISA (Enzyme Linked Immunosorbent Assay) using Fish Glutathione Peroxidase (GSH-PX) ELISA Kit (CUSABIO) and following manufacturer's protocol.

Malondialdehyde (MDA) was used as a marker of lipid peroxidation in the serum, thus the MDA levels in the serum were determined by ELISA (Enzyme Linked Immunosorbent Assay) using MDA (Malondialdehyde) ELISA Kit (Fine Test) and following manufacturer's protocol.

Histopathology and histomorphometry of intestine and skin

The consistency of our sampling was achieved by sampling only the middle part of the proximal intestine adjacent to annexed pyloric caeca and the dorsal skin, respectively. Samples were immediately fixed in 10% neutral buffer formalin. After fixation, dewaxing and rehydration according to the standard histological procedure, 4–5 μm thick sections were stained with hematoxylin/eosin (HE) in order to evaluate the morphological features. In the fish intestine, most goblet cells have a low affinity for Periodic acid–Schiff staining but a high affinity for Alcian Blue pH 2.5. Therefore, in order to have a more detailed qualitative analysis of the overall complex carbohydrates, a combined Alcian Blue pH 2.5 (AB) and Periodic acid–Schiff staining (PAS) (staining kit Bio-Optica, 04-163802A) was also performed. The Image J image processing and analysis program (NIH, Bethesda, MD) was used to analyze the anterior intestine and skin morphometrical features. The following parameters were measured or counted in favorably orientated anterior intestinal sections: villi length measured from the apex of the villus to the basal lamina of the enterocytes (10 villi/fish) and the total number of goblet cells per villus (5 villi/fish). Also, the presence or absence of leucocyte infiltrations in the lamina propria and muscularis mucosae and villi fusion were examined.

Skin samples were also stained with a combined Alcian Blue pH 2.5–PAS to visualize and evaluate the mucoid cells (MCs). Ten microscopic images of skin (×100) were captured where the epithelium was intact. The number of MCs was counted, and the length of the epidermis was measured in 5 randomly selected images.

Changes in gene expression levels and quantitative real-time PCR analysis

Total RNA was isolated from trout liver stored at –80°C. Initially, the tissues were ground to a fine powder and the RNA was extracted using the Total RNA Isolation (TRI) Reagent (Ambion), according to the manufacturer's instructions. The preliminary quantity and purity of the extracted RNA were determined using the BioPhotometer (Eppendorf) at 260 and 280 nm, and RNA integrity was confirmed using agarose gel electrophoresis and visualization of the 28S and 18S ribosomal RNA. RNase inhibitor (Invitrogen) was added to each sample (1 unit per μg of RNA) before storage at –80°C to reduce degradation. Before reverse transcription (RT), all samples were pretreated with DNAse (Fermentas) at a concentration of 1 unit per μg of RNA. One μg of total RNA was reverse transcribed to cDNA according to the manufacturer's instructions using the Invitrogen SuperScript II Reverse Transcriptase kit.

Quantitative expression analysis of the genes was performed with real-time PCR, using a LightCycler real-time PCR machine (Roche Molecular Biochemicals), as previously described (Michailidis et al., 2010), using the primers illustrated in Table 2. PCR was performed using the KAPA SYBR FAST qPCR kit (Kapa Biosystems) and 0.2 pmol of each primer in a final volume of 20 μl using as template 1/10 of the initial cDNA synthesis reaction. The levels of gene expression were measured using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard for cDNA normalization. Incubation at 95°C for 10 minutes was followed by 45 cycles of incubation at 95°C for 10 seconds, 56°C for 8 seconds, 72°C for 8 seconds, and reading at 60°C. A melting curve from 65 to 95°C with read every 0.2°C and 5 sec hold between reads is used to identify the PCR products. All of the reactions were carried out six times with cDNA synthesized from RNA extracted from the liver of different trouts. The threshold cycle (Ct) values of the PCRs were averaged, and the comparative Ct method was used to calculate the relative transcript levels (Livak and Schmittgen, 2001). The relative level of each mRNA in each sample was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and expressed as a ratio relative to the housekeeping gene GAPDH. The changes in the gene transcription levels were expressed as relative values to the control samples.

Statistical analysis

Experimental data were analyzed as a randomized block design. Performance data were analyzed by a one-way ANOVA with initial body weight used as a covariate and the pen being the experimental unit. For data on antioxidant activity, bacteria loads and nonspecific individual samples were considered to be nested within pens, and data were analyzed by a nested ANOVA; in addition, data on antioxidant activity were analyzed by a two-way nested ANOVA with time and treatment being the experimental factors. As bacterial numbers were not normally distributed, they were log-transformed to create a normal distribution prior to analysis. Bacteria load means are presented on a transformed basis. Levene's test was performed to check the homogeneity of variances and Tukey's test was carried out to assess any significant differences at a probability level of 0·05 among the experimental treatments. All data were subjected to analysis using the statistical package of SPSS version 17.00 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

Feed profile

Total phenolic content (TPC)

As shown in Table 3, the oregano group contains higher amounts of total phenolics compared to the control group.

Table 3. Total phenolic content of the experimental groups

	Control	Oregano
mg GAE/L extract	10.35±0.78	43.24±2.84

DPPH (Radical scavenging activity)

The antioxidant activity (RSA) was determined by the DPPH assay which measures the sample's capacity to scavenge free radicals. The % scavenging activity of oregano group is significantly increased compared to that of the control diet in 20 min measurements (% RSA: 103.68±1.69 and 59.62±0.73 respectively) and in 60 min measurements (% RSA: 101.90±1.78 and 66.37±0.24 respectively) (Table 4).

Table 4. Radical scavenging activity of the two experimental groups in 20 and 60 minutes

	% RSA (20 min)	% RSA (60 min)
Control	59.62±0.73	66.37±0.24
Oregano	103.68±1.69	101.90±1.78

Fillet profile

Performance parameters

The levels of survival in both groups at the end of the experiment were high (≈97%) while no significant differences were noted between the control and the oregano group. Body weights and feed intake of trouts after 90 days of feeding on experimental diets are presented in Table 5.

High growth performance was observed in both groups; fish biomass increased by over 100%. Until day 30, there were no significant differences in weight gain between fish fed the oregano supplements and the control diet, yet on day 90 the oregano groups presented higher body weight than the control group ($P<0.002$) (Table 5). FCRs are typically 1.0–2.4 for farmed fish. In our study, FCR in both groups presented a decrease from 30th to 90th day. However, the oregano group presented lower total FCR compared to the control group indicating higher efficiency (Table 5).

Lipid and protein oxidation

Fish in the oregano group displayed decreased concentrations of MDA on days 0, 4 and 7 (Table 6). Consequently, there is a strong indication that oregano essential oil has influenced trout fillet antioxidant status.

Moreover, a decrease in the concentration of protein carbonyls was observed in the oregano group (Table 6). Thus, it is considered that oregano essential oil also affected trout's fillet protein oxidation.

Fatty acids profile

The results of the fatty acid profile for the experimental groups are shown in Table 7. Docosahexaenoic acid (DHA) is an omega-3 fatty acid found very likely in fish. Oregano group possesses a higher amount of DHA, compared to the control group, which can be justified by the supplementation of oregano in trout's diet, a phytobiotic rich in n3 fatty acids. In addition, an increase in total polyunsaturated fatty acids (Σ PUFA) was observed in oregano group.

Table 5. Effect of diet supplementation with oregano essential oil on trout body weight (BW) and feed conversion ratio (FCR)

BW (g)	Control	Oregano	SEM[1]	P
Day 1	53.7	52	0.14	0.123
Day 30	67	64	0.65	0.215
Day 60	122.8	128.7	0.42	0.245
Day 90	170 b	220 a	0.68	0.002
FCR	Control	Oregano	SEM	P
Days 1–30	2.44	2.70	0.17	0.289
Days 31–60	1.18	1.01	0.05	0.367
Days 61–90	2.06 a	1.06 b	0.21	0.003
Days 1–90	1.68 a	1.16 b	0.11	0.005

[1]SEM: Standard error of mean. a, b – values in the same line with the same letters do not differ significantly ($P\leq0.05$).

Table 6. Effect of dietary supplementation with oregano oil on trout fillet oxidative status (MDA, nmol/g) and protein carbonyls (nmol/mg)

MDA values	Control	Oregano	SEM[1]	P
Day 0	53.798 a	39.224 b	0.02	<0.001
Day 4	71.541 a	45.244 b	0.12	<0.001
Day 7	88.65 a	51.897 b	0.06	<0.001
Protein carbonyls	Control	Oregano	SEM	P
	36.36 a	10.00 b	0.23	<0.001

[1]SEM: Standard error of mean. a, b – values in the same line with the same letters do not differ significantly ($P\leq0.05$).

Table 7. Effect of dietary supplementation with oregano oil on trout fillet's fatty acid profile

Fatty acid	Control	Oregano
C14:0 (Myristic)	2.85±0.19	2.42±0.38
C14:1 (Myristoleic)	0.04±0.01	0.03±0.35
C15:0 (Pentadecanoic)	0.25±0.04	0.21±0.04
C15:1 (cis-10-Pentadecenoic)	0.02±0.00	0.06±0.04
C16:0 (Palmitic)	17.17±0.33	16.55±0.88
C16:1 (Palmitoleic)	3.83±0.39	3.18±0.53
C17:0 (Heptadecanoic)	0.40±0.02	0.39±0.03
C17:1 (cis-10-Heptadecenoic)	0.43±0.02	0.34±0.07
C18:0 (Stearic)	3.44±0.09	3.43±0.52
C18:1n9t (Elaidic)	0.11±0.04	0.06±0.02
C18:1n9c (Oleic)	43.24±0.36	43.38±1.07
C18:2n6t (Linolelaidic)	0.11±0.01	0.07±0.04
C18:2n6c (Linoleic)	16.67±0.11	17.10±0.85
C18:3n6 (γ-Linolenic)	0.38±0.10	0.46±0.11
C20:0 (Arachidic)	0.20±0.02	0.22±0.04
C18:3n3 (a-Linolenic)	3.90±0.22	3.74±0.3
C20:1n9c (cis-11-Eicosenoic)	2.16±0.17	2.03±0.21
C21:0 (Henicosanoic)	0.29±0.01 a	0.26±0.0 b
C20:2 (cis-11,14-Eicossadienoic)	0.74±0.07	0.66±0.1
C20:3n3 (cis11,14,17-Eicosatrienoic)	0.45±0.02	0.45±0.03
C22:0 (Behenic)	0.06±0.01	0.07±0.02
C20:4n6 (Arachidonic)	1.30±0.11	1.44±0.25
C22:1n9 (Erucic)	0.16±0.02	0.16±0.03
C22:2 (cis-13,16-Docosadienoic)	1.69±0.15	1.96±0.41
C24:1n9 (Nervonic)	0.10±0.01	0.08±0.02
C22:6n3 (cis-4,7,10,13,16,19-Docosahexaenoic)	4.82±0.32	5.47±1.71
ΣSFA (Total Saturated)	24.66±0.34	23.55±1.08
ΣMUFA (Total Monounsaturated)	50.09±0.05	50.57±1.18
Σ PUFA (Total Polyunsaturated)	30.06±0.63	31.35±1.33

a – each value is the mean of triplicate determinations ± s.d.
b – different letters within the same row indicate significant differences according to Tukey's test (P≤0.05).

Blood/serum parameters

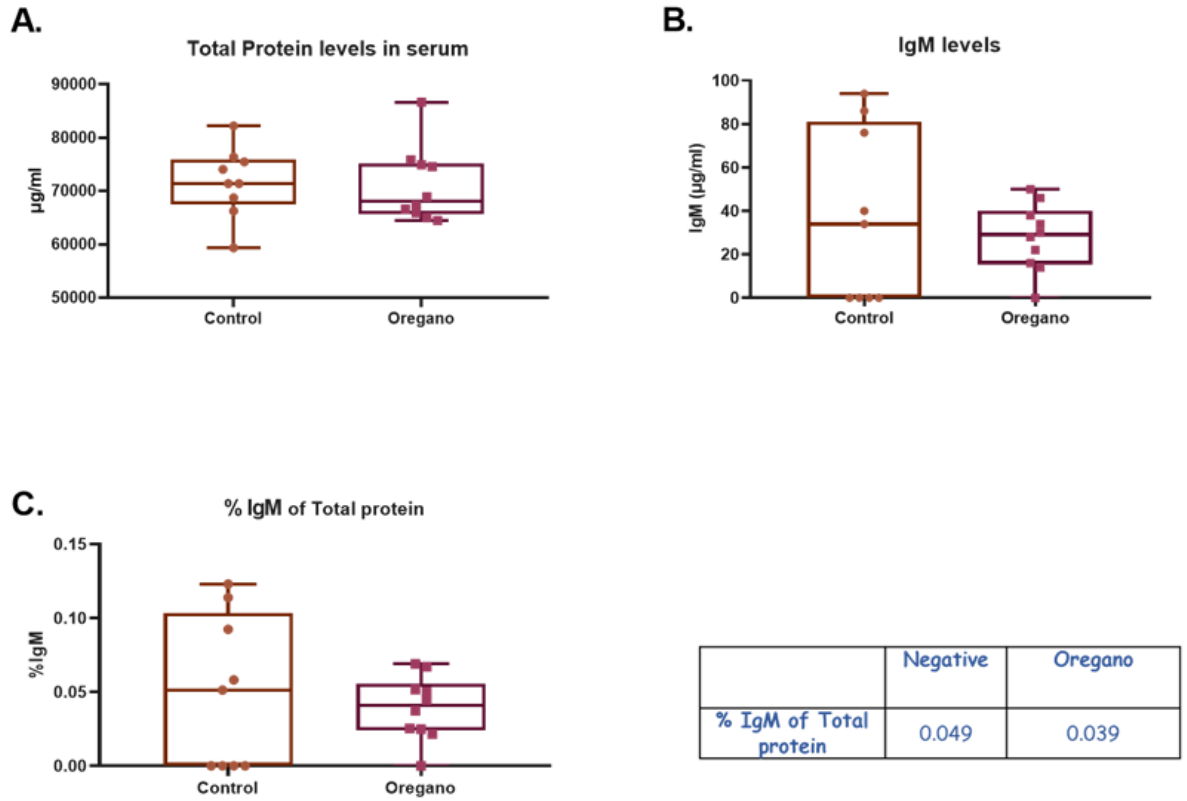
Total proteins, immunoglobulin M (IgM), malondialdehyde (MDA) and glutathione peroxidase (GSH-PX) levels

The total proteins' concentration and the IgM levels in the serum, were measured in both control samples and in oregano group. No significant change of the total protein concentration was observed between the two groups (Average protein concentration: control group = 71676 μg/ml, oregano group = 71009 μg/ml) (Figure 2 A). Moreover, IgM levels were not significantly altered after dietary administration of oregano oil, with respect to control fish (Figure 2 B). Thus, as shown in Figure 2 C, the % IgM levels in respect of the total protein concentration remained constant in fish fed with basal diet, and in fish fed with the basal diet supplemented with the oregano oil product.

In Figure 3 A, it is displayed that there is a statistically significant increase of the MDA serum levels in the oregano group (20596 ng/ml) compared to fish fed with the basal diet (12978 ng/ml, P-value = 0.0002). However, no such difference is observed in GSH-PX levels in the serum of both groups (Figure 3 B). Moreover, there seems to be no correlation between MDA and GSH-PX serum levels in the samples of the control group (Pearson r = –0.218, Figure 3 C) and of the oregano group (Pearson r = –0.265, Figure 3 D).

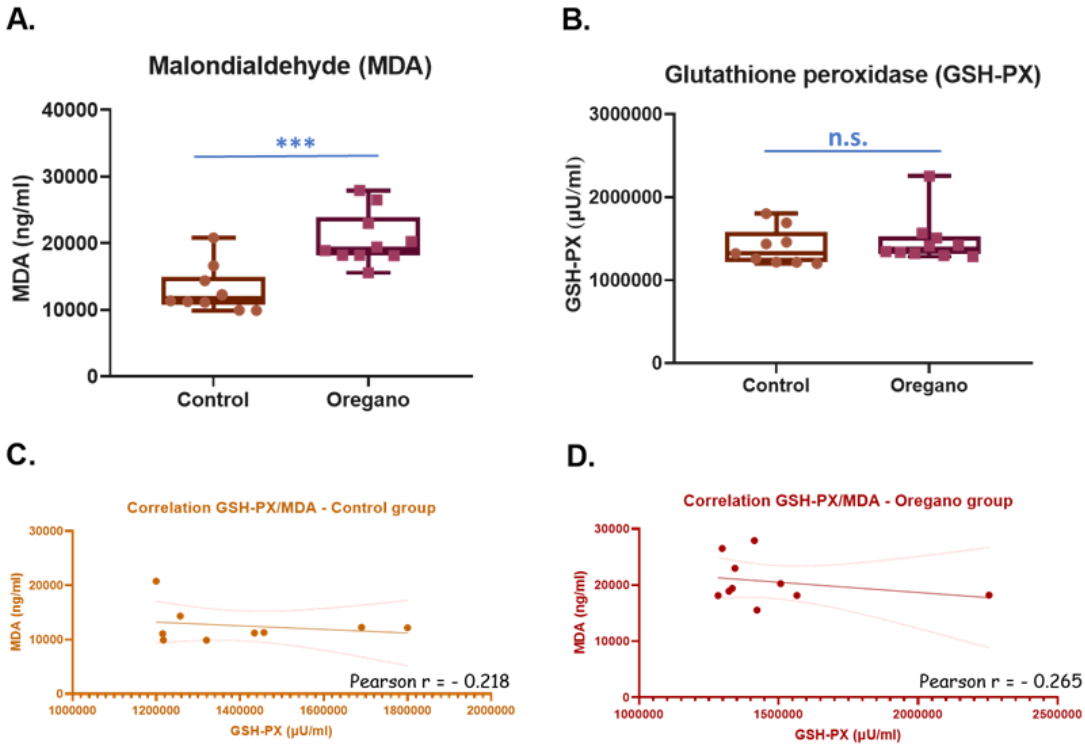
Histopathology and histomorphometry

The Alcian Blue pH 2.5–PAS combined staining revealed a range of blue stain intensity in all investigated tracts indicating a heterogeneous production of complex carbohydrates. A few cells produced only neutral mucus, some produced acid mucus, while the majority secreted a mixed combination of acid and neutral mucus. Oregano group showed higher villi length and the number of goblet cells per villi in the anterior intestine was also bigger (Figure 4). No changes were detected regarding villi fusion and leucocytic infiltration in the lamina propria and/or muscularis mucosae of the intestinal sections. Skin MC number and size were significantly higher in the oregano group (Table 8).



	Negative	Oregano
% IgM of Total protein	0.049	0.039

Figure 2. A) Total protein concentration levels in the serum (μg/ml) of control and oregano groups. B) IgM levels in the serum (μg/ml) of control and oregano groups. C) Figure displaying the percentage of IgM in respect of the total protein concentration in both control and oregano groups and a table displaying the average percentage of IgM in respect of the total protein concentration in both groups



***P-value = 0.0002, n.s. (non-significant): P-value>0.05, Pearson r = Pearson correlation coefficient.

Figure 3. A) Malondialdehyde (MDA) serum levels (ng/ml) in control and oregano groups. B) Glutathione peroxidase (GSH-PX) serum levels (μU/ml) in control and oregano groups. C) Correlation figure of MDA levels (ng/ml) and GSH-PX levels (μU/ml) in samples of the control group. D) Correlation figure of MDA levels (ng/ml) and GSH-PX levels (μU/ml) in samples of the oregano group

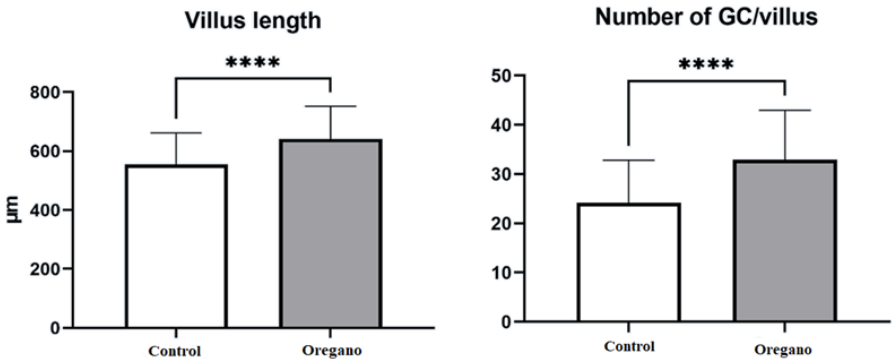


Figure 4. Effect of dietary supplementation with oregano oil on trout villus length (left) and number of goblet cells/villus (right). Mann–Whitney analysis

Table 8. Effect of dietary supplementation with oregano oil on trout number of skin mucoid cells, mucoid cells minimum Feret's diameter and length of the epidermis

	Control	Oregano	P
Number of MCs[1]	90.12±15.3 7 b	122.79±39.41 a	<0.001
Size of MCs	16.70±2.31 b	17.76±2.05 a	<0.001
Thickness of the outer epidermis (µm)	351.90±93.98	387.00±120.14	NS

[1]MCs: Mucoid cells.

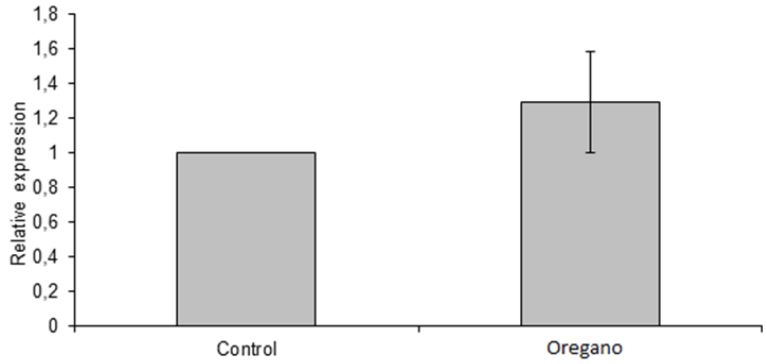


Figure 5. Changes in the expression of GH-1 gene in the trout liver following feed addition with "VISNAT 1". The relative abundance of mRNA was examined using quantitative real-time PCR analysis. Values represent the mean ± SEM (n = 6)

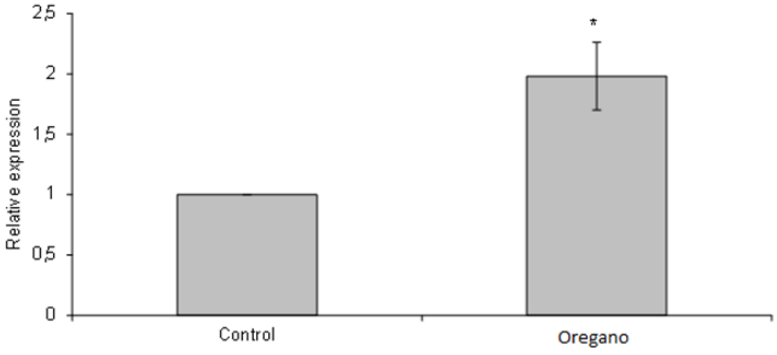


Figure 6. Changes in the expression of IGF-1 gene in the trout liver following feed addition with "VISNAT 1". The relative abundance of mRNA was examined using quantitative real-time PCR analysis. Values represent the mean ± SEM (n = 6). Asterisk indicates that the difference in the expression levels is significantly different (P<0.05)

Changes in gene expression levels and quantitative real-time PCR analysis

After normalization to GAPDH expression, quantitative real-time PCR analysis revealed a significant up-regulation ($P<0.05$) in the expression levels of IGF-1 gene in the liver of the treated group (Figure 5). No statistically significant changes ($P>0.05$) were observed for the GH1 gene in the treated group compared to the controls (Figure 6).

Discussion

In recent years, a lot of research has been done to investigate various plants and their components, oils, extracts, and active compounds as functional food additives. Oregano essential oil has been validated in several aquatic animal species due to its beneficial effects in growth and health. This study focused on the incorporation of oregano essential oil in trout's diet and the evaluation of some parameters such as growth, performance, antioxidant status, fatty acid profile and intestinal histomorphometry.

In this research, the experimental group fed with oregano oil had displayed better performance than the control group. Specifically, there was an increase in trout's body weight on oregano group. Another important fact is that the oregano group presented lower total FCR compared to the control group. There are a lot of studies that present the use of plant extracts as growth promoters. This effect may be affected by many factors such as dose, species, feeding duration and physiological conditions. Awad et al. (2012) also suspected that fish growth performance can also be enhanced by increased digestive enzyme activity. Thus, it can be deduced from this that the phenolic compounds contained in oregano essential oil can promote fish growth (Bili, 2023).

Moreover, one of our research targets was to investigate if long-term consumption of oregano feed additive affected the antioxidant status of trout. In general, the literature is replete with evidence of plant extracts' *in vitro* antioxidant activity as a result of their ability to scavenge free radicals via single-electron transfer *in vitro* (Amoush et al., 2022; Bili, 2023). Herein, the lipid oxidative stability as well as the protein oxidation was significantly improved in the oregano group. Although there was 58.7% increase of the serum MDA levels in the fish fed with oregano essential oil supplemented diet disclosing presence of lipid peroxidation in the group's serum, there was no correlated increase in the in GSH-PX levels in the serum (Figure 5). At the same time, MDA levels in the raw trout fillet during refrigerated storage (0, 4 and 7 days) were decreased in the oregano group compared with the control group (27%, 36.8% and 41.5% respectively) implying that oregano essential oil has a positive influence in the trout fillet's antioxidant status (Table 6). Although MDA increase in the serum normally plays a role as a biomarker of cellular oxidative stress, it does not always correlate to lipid peroxidation damage in the tissue and vice versa. For example, it has been shown that ethanol consumption causes in humans and in rats elevated MDA levels in the serum without any simultaneous evidence of liver damage (Clot et al., 1994; Lecomte et al., 1994; Koch et al., 1991). Moreover, Marnett showed that MDA is also produced normally during the process of prostaglandin synthesis (Marnett, 2002) which in fish play a crucial role in immune response's development (Gómez-Abellán et al., 2016). In our study, the MDA levels in the serum are statistically significantly elevated but if we observe the actual arithmetical values, the mean MDA level of the oregano group in the serum (21 μg/ml) is comparable to several individual MDA levels observed in the control group (17–21 μg/ml). Thus, herein the MDA levels increase in the serum but that does not seem to affect in depth or to reflect in the antioxidant benefits that the oregano oil supplemented diet, adds in the raw trout fillet. This suggests that the MDA level's increase in the serum can be attributed to one of the main components of essential oregano oil by affecting the normal prostaglandin synthesis process (Gorelik et al., 2008). In any case, further studies are needed in order to investigate whether the above hypothesis is correct or the slightly increased MDA levels are due to oxidative stress which is eventually compensated by other defense mechanisms before the damage is incorporated in the liver tissue. Furthermore, the antioxidant positive influence of the raw trout fillet was accompanied with positive influence in the fillet's protein oxidation with a 72.5% decrease in the group's protein (Table 6). The significant *in vitro* antioxidant activity of the oregano oil diet in trout's fillet was also well correlated with the high total phenolic content (Table 3).

Dietary fats are well known to influence fatty acid composition of fish fillet. Polyunsaturated fatty acids (PUFA) are limited but important nutrients for fish health. There is a need for better understanding of the processes that influence PUFA levels in fish due to the increasing human demand as they beneficially affect health (Sawyer et al., 2016)process-based mass balance model for freshwater fish that estimates concentrations of n-3 (α-linolenic acid: ALA, eicosapentaenoic acid: EPA and docosahexaenoic acid: DHA. Terenina et al. (2011) showed that oregano oil is a natural antioxidant which inhibits the oxidation process of higher fatty acids. In our research, the addition of oregano essential oil had no significant effect on the total saturated and unsaturated fatty acids. However, the slight increase in total polyunsaturated fatty acids of oregano group can perhaps be explained by the action of oregano oil addition on trout's diet. However, further investigation on trout's fatty acid profile is required.

It has been demonstrated that the intestinal and skin histomorphometry is sensitive to dietary ingredients (Nimalan et al., 2022). The length and density of the intestinal villus are related to absorption capacity (Abdel-Tawwab et al., 2021). Our findings suggest that adding an oregano essential oil feed supplement affects intestinal and skin histomorphometry. Specifically, there was an increase in villus length and density. These findings indi-

cate that oregano essential oil can improve villus development, increase nutrient absorption efficiency and enhance growth. Finally, in the present study, we also investigated the effects of the addition of oregano oil in trouts' diet, in the expression levels of insulin-like growth factor-1 and growth hormone-1 genes in the liver of rainbow trout. Growth hormone (GH) is a pleiotropic endocrine factor that is fundamental for somatic growth, energy mobilization, gonadal development, and behavior in many vertebrate species, including fish (Forsyth et al., 2002; Aksakal et al., 2010). The regulatory network for GH is very complicated, including many endocrine and environmental factors and its actions are initiated by binding to membrane-bound GH receptors (GHRs) on target tissues (Daughaday, 2000). In vertebrate species, the major target of GH action is the liver and in fish, GHRs have been identified in several salmonids and share many characteristics with the mammalian GHRs, showing strong conservation between fish and mammals (Robertson et al., 2006; Vergani et al., 2009). There are two genes for the growth hormone (GH-I and GH-II) in rainbow trout which have minor differences, and, in both trout and salmon, GH-1 mRNA is predominant (Rentier-Delrue et al., 1989).

During the last years, many studies have reported that essential oils from *Origanum* species can promote growth, develop fish health and raise the resistance to disease in fish, as a result of their addition to fish feed (Diler et al., 2017). Furthermore, the investigation of the modulations in expression levels of both IFG-1 and GH-1 genes has become of particular interest for many researchers, due to the important roles of both genes in various biological processes (Aksakal et al., 2010). The data presented in this study revealed that addition of oregano oil to the diet of rainbow trout resulted in a significant increase in the expression levels of IGF-1 gene in the liver, while no significant changes were observed regarding the expression levels of GH-1 gene. These data suggest that oregano oil addition in the rainbow trout can initiate mitogenic and anti-apoptotic responses in the liver, which is one of the main actions of IGF-1 receptor.

Conclusion

Our studies showed that the supplementation of oregano oil in fish feed can positively affect total phenolic content and improve radical scavenging activity due to the antioxidants present in oregano. The present study found that oregano essential oil as feed additive also improved trout's growth performance and reduced MDA on trout's fillet. Also, FCR values were lower in fish fed oregano supplemented diet compared to control, while feed intake was unaffected, indicating growth promoting activity. Our findings suggest that adding an oregano essential oil feed supplement has a beneficial impact on intestinal and skin histomorphometry. Finally, quantitative real-time PCR analysis showed that oregano essential oil feed supplement affects the main actions of IGF-1 receptor. However, more research with different important aquaculture species and longer time scales is needed to fully evaluate the value of including oregano in trout diet.

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