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# Dietary oregano essential oil improved the immune response, activity of digestive enzymes, and intestinal microbiota of the koi carp, *Cyprinus carpio*

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**Running Head**: Effect of oregano essential oil on gut microbiota of koi carp

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## ABSTRACT

The objective of this research was to evaluate the effects of dietary oregano essential oil (OEO) on the non-specific immunity and intestinal bacterial community of *Cyprinus carpio* (*C. carpio*). We randomly assigned 300 fish to one of the four treatments: basal diet (0 mg/kg OEO; control), OEO-L (basal diet plus 500 mg/kg OEO), OEO-M (basal diet plus 1500 mg/kg OEO), and OEO-H (basal diet plus 4500 mg/kg OEO). Blood and intestinal samples were collected at the end of the experiment to investigate the immune response, digestive enzyme activity levels, and intestinal microbiota. Dietary supplementation with OEO significantly increased the levels of lysozyme ($P < 0.0001$), complement C3 ($P < 0.0001$), complement C4 ($P < 0.0001$), superoxide dismutase activity (SOD; $P < 0.0001$), and glutathione peroxidase (GPx; $P < 0.0001$), but significantly decreased malonaldehyde (MDA) levels ($P < 0.0001$). OEO supplementation also significantly increased the activity levels of protease ($P < 0.0001$), lipase ($P < 0.0001$), and amylase ($P = 0.0071$), but significantly downregulated the relative expression of tumor necrosis factor α (*TNF-α*) ($P = 0.0002$) and transforming growth factor β (*TGF-β*) ($P = 0.0064$). After *Aeromonas hydrophila* (*A. hydrophila*) injection, the 7-d cumulative survival rate of *C. carpio* was significantly increased by oral OEO administration ($P = 0.005$). Bacterial community composition in the OEO-H group was distinct from that in the control (ANOSIM, $R = 0.3165$, $P = 0.011$). We identified 22 taxa, which were differentially abundant between the OEO-H group and the control, as potential biomarkers. The genera *Propionibacterium*, *Brevinema*, and *Corynebacterium_1* were enriched in the OEO-H group, whereas *Vibrio* was enriched in the control. Thus, in *C. carpio*, dietary OEO increased digestive enzyme activity and antioxidant capability, stimulated immunomodulatory effects, and enhanced

disease resistance. These beneficial effects were probably due to OEO-mediated alternations in the structure of the *C. carpio* gut microbiota.

**Keywords**: non-specific immunity; antioxidant; disease resistance; digestive enzyme; intestinal bacterial community

## INTRODUCTION

Oregano essential oil (OEO), which is extracted from natural plant products, is characterized by a high phenolic content. The main components of OEO are carvacrol and thymol; together, these constitute about 780-820 g/kg of the total oil (Sivropoulou et al., 1996). The remaining fraction of the total oil contains other minor components, including the carvacrol precursor (ρ-cymene; about 50 g/kg of the total oil) and the thymol precursor (γ-terpinene; about 70 g/kg of the total oil) (Adam et al., 1998). OEO has antimicrobial (Hammer et al., 1999; Lambert et al., 2001; Burt et al., 2005), anti-inflammatory (Lima et al., 2013; Ocaña-Fuentes et al., 2010), and antioxidant properties (Young et al., 2003; Botsoglou et al., 2002). Thus, OEO is regarded as a promising alternative to antibiotics and is routinely used in industrial feeds.

In fish, dietary supplementation with OEO improves growth performance (Zheng et al., 2009; De Moraes França Ferreira et al., 2014; Diler et al., 2016), the immunological responses (Diler et al., 2016; Ran et al., 2016; Mabrok and Wahdan, 2018), and pathogens/disease resistance (Zheng et al., 2009; Diler et al., 2016; Espirito Santo et al., 2019). However, few studies to date have investigate the effects of OEO on the gastrointestinal (GI) tract microbiotas of aquatic species. The GI tract supports a vast population of microorganisms that play important roles in host growth, development, and health (Rawls et al., 2004; Nicholson et al., 2005; Nayak, 2010). Studies of swine and

poultry have shown that OEO improves host health by inhibiting harmful intestinal bacteria while promoting the growth of beneficial intestinal bacteria (Cho et al., 2014; Zou et al., 2016; Mitsch et al., 2004). Dietary supplementation with thymol and carvacrol can change the bacterial community composition of tilapia (Ran et al., 2016). We hypothesized that the beneficial effects of OEO on host animals might be closely associated with the host intestinal microbiota. Thus, in this study, we assessed the effects of dietary OEO on both the host immune response and the host gut bacterial community, in order to identify interactions between the host and the gut microbiota.

## MATERIALS AND METHODS

### *Diet preparation*

The basal diet was composed of soybean meal, fish meal, wheat flour, wheat bran, fish oil, and premix (Table 1). We added 0 mg/kg, 500 mg/kg, 1500 mg/kg, and 4500 mg/kg OEO (purity of 80 g/kg; extracted from *Origanum vulgare* L.) to the diet of the control, OEO-L, OEO-M, and OEO-H groups, respectively. All ingredients were finely grounded, thoroughly mixed, and pelleted through a 2.0 mm diameter die. The feed pellets were dried at 60ºC for 4 h.

### *Fish and culture conditions*

Koi carp (*Cyprinus carpio*) were acquired from a commercial hatchery and acclimated to experimental conditions for 4 weeks. We randomly allocated 300 fish (average initial weight:15.6 ± 3.3 g) to one of four diets (Control, OEO-L, OEO-M, or OEO-H). Each treatment was performed in triplicate. Fish were fed 20 g/kg of initial body weight twice

daily (at 9:00 and 16:00). Fish were kept in a recirculating culture system, which consisted of three 200-L tanks per treatment, for eight weeks. During the experimental period, one fifth of the water volume was exchanged daily with fully aerated tap water. Water temperature was maintained at 21ºC ± 1ºC. Water quality was maintained as follows: dissolved oxygen 8.0-8.5 mg/L, $NH_4^+$-N < 1.0 mg/L, $NO_2$-N < 0.05 mg/L. The experiments were carried out at an aquaculture farm at the Beijing Fisheries Research Institute, Beijing, China. The experimental protocol was approved by the Animal Ethics Committee of the Beijing Fisheries Research Institute, Beijing Academy of Agriculture and Forestry Sciences (Beijing, China).

***Sampling***

At the end of the experiment, 36 fish (n = 9 per treatment) were anaesthetized by immersion in 0.1 g/L of MS-222. Blood was taken from the caudal veins of the anesthetized fish, and centrifuged at 1200 × g for 20 min at 4ºC. The serum from each sample (n = 9 per treatment) was decanted and stored at -20ºC for further analysis of lysozyme, complement C3 and C4, SOD, GPx, and MDA. After blood sampling, fish were incised ventrally, and the intestinal tracts were carefully removed. Intestinal tracts were then dissected into segments. Different segments were used for the investigation of digestive enzymes, bacterial community composition, and cytokine mRNA expression (n = 9 per treatment).

***Challenge test with A. hydrophila***

The remaining fish (n = 66 per treatment) were injected intraperitoneally with 200 μL of phosphate buffered saline containing $10^7$ colony forming unit (cfu) /mL of *A. hydrophila*. The strain of *A. hydrophila* used in this study was originally isolated from infected koi carp, and was provided by Shanghai Ocean University (China). We then recorded cumulative

survival across all injected fish for 7 days following infection.

**Measurements**

***Blood parameters***

Lysozyme activity was determined by measuring the decrease in turbidity after lysis of the gram-positive bacterium *Micrococcus lysodeikticus* (Lysozyme kit, Nanjing Jiancheng Biological Engineering Research Institute, Nanjing, China) (Demers and Bayne, 1997). Complement C3 or C4 was assayed by measuring the increase in turbidity after immune response of C3 or C4, and the increase in C3 or C4 antibodies (C3 or C4 kit, Weiyi Biotechnology Co., Ltd. Zhejiang, China) (Thomas, 1998; Vibertigc, 1990). SOD activity was measured by pyrogallol autoxidation method (HY-60001 kit, Beijing Sino-uk institute of biological technology, Beijing, China) (Marklund and Marklund, 1974). GPx activity was measured on the detection of non-enzymatic utilization of GSH as the reacting substrate after incubation with dithiobinitrobenzoic acid (DTNB) (HY-60005 kit, Beijing Sino-uk institute of biological technology, Beijing, China) (Moin, 1986). MDA was measured by thiobarbituric acid (TBA) method (HY-60003 kit, Beijing Sino-uk institute of biological technology, Beijing, China) (Choi and Oris, 2000).

***Digestive enzyme activities***

Intestinal samples were thoroughly homogenized in phosphate buffered saline (pH7.5, PBS), and centrifuged at 10000 × g for 20 min at 4 °C. The supernatant was used to determine the total protein content as previously reported (Bradford, 1976). Protease activity was measured by casein hydrolysis method (HY-50038 kit, Beijing Sino-uk institute of biological technology, Beijing, China). Lipase activity was measured by

p-nitrophenylphosphate (p-NPP) hydrolysis method (HY-60115 kit, Beijing Sino-uk institute of biological technology, Beijing, China). Amylase activity was measured by 2-chloro-4-nitrophenol-α-D-maltotrioside (CNPG3) hydrolysis method (Amylase kit, BioSino Bio-Technology & Science Inc. Beijing, China ).

***Quantitative real-time PCR***

Total RNA was extracted from each sample using TRIzol® (Invitrogen, CA, USA), and DNA was removed using DNase I (TaKaRa) for 30 min at 37 °C. RNA degradation and contamination was monitored on 1% agarose gels. RNA quality was determined using the 2100 Bioanalyzer (Agilent) and quantified using the ND-2000 (NanoDrop Technologies). Total RNA was reverse transcribed to the first-strand cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), and the qPCR was performed using 1 μL of cDNA, 2× SYBR Green PCR Master Mix (Applied Biosystems), with 200 nM of forward and reverse primers. The cycling conditions were as follows: 10 min at 95°C; 40 cycles of 95°C for 15 s; and 1 min at 60°C. Each assay was run on an Applied Biosystems 7300 Real-Time PCR system in triplicates. The relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method. The gene specific primers were listed in Table S1.

***Gut microbiota***

DNA extraction and preparation of amplicons for high-throughput sequencing. Bacterial DNA was extracted from 0.2 mg of intestinal tissue in each replicate using TIANamp Stool DNA kit (Tiangen, China). Amplification by PCR was conducted with the 341f/806r primer set that amplifies the V3-V4 region of the 16S rRNA gene (341F:

5′-CCTAYGGGRBGCASCAG-3′; 806R 5′-GGACTACNNGGGTATCTAAT-3′). PCR amplification were performed in 30 μL mixture containing 15 μL of 2× Phusion High-Fidelity PCR Master Mix (New England Biolabs, USA), 10 μL of 1 ng/μL DNA template, 3 μL of 2 μM forward and reverse primers, and 2 μL HPLC-grade water. The amplification program consisted of an initial cycle of 98°C for 1 min, followed by 30 cycles of 98°C for 10 s, 50°C for 30 s, and 72°C for 30 s, and a final cycle of 72°C for 5 min. The PCR products were excised from 2% (w/v) agarose gel and purified using GeneJET Gel Extraction kit (Thermo Scientific, Germany). Libraries for sequencing were constructed using Ion Plus Fragment Library Kit (Life Technologies, USA), and quantified using the Ion Library Quantitation Kit (Life Technologies). Sequencing of amplified bacterial 16S rRNA gene fragments was performed using an Ion S5 XL platform by Novogene Bioinformatics Technology Company (China). The sequence reads have been submitted to the Sequence Read Archive (SRA) under the accession number PRJNA579875.

***Bioinformatic analysis***

Single-end reads were assigned to samples based on their unique barcodes, and truncated by removing the barcode and primer sequences. Raw reads were quality filtered following the Cutadapt quality control process to obtain high-quality clean reads (Caporaso et al., 2010). Chimeric sequences were identified by comparison with those in the SILVA database (https://www.arb-silva.de/), and then removed using UCHIME (Edgar et al., 2011). Sequences were then assigned to operational taxonomic units (OTUs) at the 97% similarity level using UPARSE (Edgar, 2013). Representative OTUs were assigned to bacterial taxa using the SILVA SSURef database and the RDP classifier in QIIME (Quast et al., 2012). Alpha diversity was analyzed using QIIME. Principal coordinate analysis (PCoA), based on

weighted UniFrac distance, was performed using QIIME. Linear discriminant analysis of the effect size (LEfSe) was performed to estimate bacterial taxa that were differentially represented between groups, with the logarithmic linear discriminant (LDA) analysis threshold score set to 4.0 (Segata et al., 2011).

***Statistical analysis***

Digestive enzymes data, blood parameters, gene expression levels and survival rates were analyzed using the general linear model in SAS (SAS Version 8.01, SAS Institute, Inc., USA). Duncan's multiple range test was conducted when a significant difference was detected among means. Significant differences in alpha diversity indices between the OEO-treated groups and the control were identified using Student's *t*-test. An analysis of similarity randomization test (ANOSIM) was performed to determine if bacterial composition differed between the treatment groups and the control. A value of $P < 0.05$ was considered statistically significant. LEfSe analysis was performed using the Kruskal-Wallis test, and logarithmic LDA threshold score was 4.0.



## RESULTS

***Digestive enzymes***

Protease and lipase activity levels were significantly greater in the OEO-M and OEO-H groups than in the control ($P < 0.0001$) and OEO-L groups ($P < 0.0001$). Amylase activity level was significantly greater in the OEO-H group than in the control or OEO-L group ($P = 0.0071$; Fig. 1).

***Blood parameters***

Dietary supplementation with OEO significantly increased the levels of lysozyme ($P < 0.0001$), complement C3 ($P < 0.0001$), and complement C4 ($P < 0.0001$). The greatest levels of C3 and C4 were detected in the OEO-L group (Fig. 2). SOD and GPx activity levels were significantly greater in the OEO-treated groups as compared to the control (SOD: $P < 0.0001$; GPx: $P < 0.0001$). SOD and GPx activity levels were highest in the OEO-H group. However, the MDA level decreased significantly in all OEO-treated groups as compared to the control ($P < 0.0001$; Fig. 3).

***Intestinal gene expression***

Dietary supplementation with OEO significantly decreased *TNF-α* expression in the OEO-L group ($P = 0.0002$), while *TGF-β* expression was significantly decreased in all OEO-treated groups ($P = 0.0064$). No differences in interleukin 1β (*IL-1β*) expression were observed among groups (Fig. 4).

***Cumulative survival***

Dietary supplementation with OEO improved the cumulative survival of *C. carpio* following *A. hydrophila* infection. The 7-d cumulative survival rate increased significantly in the OEO-supplemented groups ($P = 0.005$) from 56.1% in the control group to 83.3% in the OEO-L group, 73.7% in the OEO-M group, and 89.0% in the OEO-H group (Fig. 5).

***Bacterial community***

A total of 2,807,698 high-quality DNA sequences were obtained from the 36 samples. Seqeunces were assigned to 2,838 OTUs, with 97% nucleotide sequence identity among reads within each OTU (Table S2). Fusobacteria were the most abundant phylum across all samples (26.84%), followed by Proteobacteria (20.44%), Actinobacteria (18.76%), Firmicutes (16.50%), Bacteroidetes (9.91%), Spirochaetes (1.16%), Deinococcus-Thermus

(0.36%), Cyanobacteria (0.3%), Chloroflexi (0.15%), and Verrucomicrobia (0.11%); 5.48% of all bacteria fell into other phyla. In the OEO-H group, Actinobacteria were significantly more abundant relative to the control ($P$ = 0.0052), while Bacteroidetes were significantly less abundant ($P$ = 0.0026; Fig. 6).

Dietary supplementation with OEO had no effect on alpha diversity, as indicated by the observed species, and the Chao 1, Shannon, and Simpson indices (Fig. S1). The PCoA, based on the weighted UniFrac distances, indicated that the bacterial community of the OEO-H group was distinct from that of control group (ANOSIM, R= 0.3165 , $P$ = 0.011). However, no differences were observed between the OEO-L group and the control group (ANOSIM, R = -0.0091, $P$ = 0.447) or between the OEO-M group and the control group (ANOSIM, R = -0.0076, $P$ = 0.402; Fig. 7). By LEfSe analysis, we identified 22 differentially abundant taxa that potentially discriminated between the OEO-H group and the control group. These taxa were considered potential biomarkers. The genera *Propionibacterium*, *Brevinema* and *Corynebacterium_1* were enriched in the OEO-H group, while *Vibrio* was enriched in the control group (Fig. 8). Correlation analysis suggested that *Brevinema* was positively correlated with lysozyme and GPx; *Corynebacterium_1* was positively correlated with IL-6 and SOD; and *Vibrio* was negatively correlated with TNF-α and IL-1β (Fig. 9).

## DISCUSSION

OEO has been identified as one of the most promising alternatives to dietary antibiotics, primarily due to its useful biological properties. Several studies have shown that, in fish, OEO supplementation improves growth rate and feed utilization (Zheng et al., 2009; De

Moraes França Ferreira et al., 2014; Diler et al., 2016). The use of carvacrol and thymol also acts as a growth promoter in the rainbow trout, *Oncorhynchus mykiss* (Ahmadifar et al., 2011). One of the possible reason would be the activation of digestive enzymes. Dietary supplementation with OEO had positive effects on the digestive enzyme secretions in poultry (Lee et al., 2003; Jang et al., 2007). Our results demonstrated that OEO supplementation stimulates fish digestive function by increasing the activity levels of protease, lipase, and amylase. Additionally, dietary supplementation with OEO stimulate appetite in fish (Abdel-Latif and Khalil, 2014), which contributes to the enhanced growth rate.

The antioxidant properties of OEO have been attributed to its ability to scavenge free radicals, chelate transition-metal ions, and decompose peroxides (Yanishlieva et al., 1999; Su et al., 2007; Embuscado, 2015). The antioxidant activity of major components in thyme essential oil have been studied separately and ranked as: thymol > carvacrol > γ-terpinene > myrcene > linalool > ρ-cymene > limonene > 1,8-cineole > α-pinene (Youdim et al., 2002). The antioxidant activity is primarily due to the presence of aromatic rings and the number and arrangement of the hydroxyl groups (Farag et al., 1989; Brewer, 2011). Oral administration of OEO in poultry feed effectively delayed lipid oxidation and improved the oxidative stability of the meat (Botsoglou et al., 2002; Marcinčák et al., 2008). Similarly, dietary supplementation with OEO enhanced serum antioxidant activity levels in channel catfish (Zheng et al., 2009), rainbow trout (Giannenas et al., 2012), and Nile tilapia (Abdel-Latif and Khalil, 2014). This was consistent with our results, which showed that after OEO supplementation, serum SOD and GPx increased, while MDA decreased. Dietary supplementation with OEO in its natural form has a pronounced effect on fish

innate immunity. The increased level of lysozyme activity observed after OEO supplementation in this study was consistent with several previous studies, which showed that OEO played an important role in the enhancement of non-specific immune responses (Zheng et al., 2009; Diler et al., 2016; Mabrok and Wahdan, 2018). Tilapia treated with a mixture of carvacrol and thymol exhibited increased phagocytic activity in the head kidney macrophages and increased lysozyme activity in the plasma (Ran et al., 2016). However, the immunomodulatory effects of either carvacrol or thymol alone have been debated. Giannenas et al. (2012) indicated that dietary carvacrol increased serum lysozyme activity in rainbow trout (Giannenas et al., 2012), whereas Volpatti et al. (2012) reported that dietary carvacrol reduced serum proteins, immunoglobulins, and lysozyme activity in sea bass. In channel catfish, no significant difference was observed in lysozyme activity after dietary supplementation with carvacrol or thymol alone (Zheng et al., 2009).

Ran et al. (2016) inoculated the gut microbiota of tilapia fed thymol and carvacrol into germ-free zebrafish and found that the genes *SAA*, *IL-1β*, and *IL-8* were downregulated in the zebrafish. However, in the same study, *IL-1β* was upregulated in germ-free zebrafish one day after the direct administration of carvacrol and thymol. This suggests that carvacrol and thymol affect host immunity via two different mechanisms: directly in host tissues, or meditated by the gut microbiota. Here, the expression levels of pro-inflammatory genes in the gut, including *TNF-α* and T*GF-β*, were downregulated in response to OEO administration after 8-w OEO administration. This effect might have been associated with an alternation of the gut microbiota.

Consistent with our results, dietary supplemented with a mixture of carvacrol and thymol altered the gut bacterial communities of piglets, but no difference were observed in bacterial

richness or diversity (Li et al., 2018). Similarly, cluster analyses indicated that dietary thymol and carvacrol substantially altered the gut microbial communities of tilapia (Ran et al., 2016). Dietary supplementation with OEO also inhibited the growth of three species of *Vibrio* bacteria in shrimp tissues (Gracia-Valenzuela et al., 2014), which might explain the decreased abundance of *Vibrio* observed here. Notably, correlation analysis suggested *Vibrio* was negatively correlated with serum TNF-α and IL-1β. Thus, the decreased *Vibrio* might also contribute to the anti-inflammatory activity of OEO.

Furthermore, dietary supplementation with carvacrol and thymol in tilapia upregulated the genes encoding intestinal-tightening TJ proteins and increased the proliferation of goblet cells; this suggested that treatment with these phenols improved the mucosal and epithelial barrier of the fish intestine, possibly increasing pathogen resistance (Ran et al., 2016). This was consistent with our results, which showed that oral administration of OEO significantly enhanced the resistant of fish to *A. hydrophila*. Similar observations have been made in channel catfish (Zheng et al., 2009), rainbow trout (Diler et al., 2016), and tilapia (Espirito Santo et al., 2019; Rattanachaikunsopon and Phumkhachorn, 2010).

OEO and its principal components, carvacrol and thymol, have important antimicrobial properties (Lambert et al., 2001; Kim et al., 1995; Ultee et al., 1998). The antimicrobial effects of OEO and its constituents have been attributed to their lipophilic properties (Conner et al., 1993), as well as to the presence of a phenolic-OH group; the latter easily reacts with enzyme active sites to form hydrogen bonds (Farag et al., 1989). These components also disrupt membrane integrity, affecting pH homeostasis, inorganic ion equilibrium, and the release of outer membrane-associated materials from the cell to the external medium (Lambert et al., 2001; Helander et al., 1998).

Lambert et al. (2001) reported that certain mixtures of carvacrol and thymol have inhibitory effects, similar to those of OEO. Thymol and carvacrol together were 96% as effective as OEO with respect to *Pseudomonas aeruginosa* inhibition. However, other studies have indicated that natural OEO more effectively inhibits bacterial viability than carvacrol plus thymol, suggesting that the minor components of OEO are critical for bacterial inhibition or that synergistic effects exist among OEO components (Lattaoui and Tantaoui-Elaraki, 1994). Compared to carvacrol, thymol, and carvacrol plus thymol, natural OEO more strongly increased growth, antioxidant activity, and resistance to *A. hydrophila* in channel catfish (Zheng et al., 2009). A wide variety of active compounds are found in OEO at low concentrations, including ρ-cymene, γ-terpinene, borneol, linalool, and α-terpinene (Karousou and Kokkini, 2003; Baydar et al., 2004; Bayramoglu et al., 2008). Carvacrol and ρ-cymene have synergistic effects against *Bacillus cereus* (Ultee et al., 2000), *Escherichia coli* O157:H7 (Kiskó and Roller, 2005), and *Edwardsiella tarda* (Rattanachaikunsopon and Phumkhachorn, 2010). This may be because ρ-cymene swells bacterial cell membranes, allowing carvacrol to be more easily transported into the cell (Ultee et al., 2002).

In conclusion, our results indicated that, in fish, dietary supplementation with OEO increased digestive enzyme activity and antioxidant capability. OEO also stimulated immunomodulatory effects and enhanced disease resistance. Oral administration of OEO at the concentration of 4,500 mg/kg diet altered the gut bacterial community composition of fish. The beneficial effects of OEO were probably due to alternations in the structure of the fish gut microbiota.

## ACKNOWLEDGMENTS

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Table 1: Composition and chemical analysis of the basal diet.

| Ingredients | g/kg |
|---|---|
| Soybean meal | 300 |
| Fish meal | 317 |
| Wheat flour | 200 |
| Wheat bran | 150 |
| Fish oil | 23 |
| Premix[1] | 10 |
| **Nutrient[2]** | **g/kg** |
| Dry Matter | 895.0 |
| Crude Protein | 400.2 |
| Crude Fat | 63.4 |
| Crude Ash | 33.7 |
| Calcium | 12.8 |
| Total Phosphate | 11.1 |
| Salt | 10.9 |

[1] provided per kg of basal diet: 2,700 IU vitamin A, 75 IU vitamin D, 65 IU vitamin E, 2.30 mg vitamin B1, 6.80 mg vitamin B2, 6.90 mg vitamin B6, 6.90 [illegible] vitamin K, 2.65 mg folic acid, 0.50 mg biotin, 29.7 mg niacin, 21.0 mg D-pantothenic acid, 5.6 mg Cu, 75 mg Fe, 100 mg Zn, 10 mg Mn, 1.15mg I, and 0.2 mg Se.
[2] Nutrients of the basal diet were analyzed according to AOAC (1995) protocols.

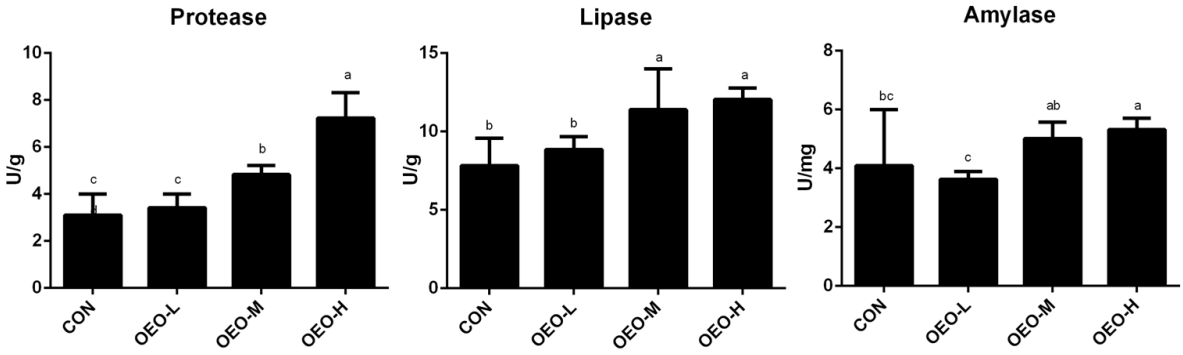


Fig. 1. Effect of dietary oregano essential oil on the activity levels of protease, lipase and amylase in the intestine (n = 9 per treatment).

Data are expressed as means ± standard error of mean. [ab]Mean values with different superscripts are different at $P < 0.05$. OEO-L, OEO-M, OEO-H: Oregano essential oil was supplemented at a concentration of 500, 1,500 or 4,500 mg/kg of basal diet, respectively.

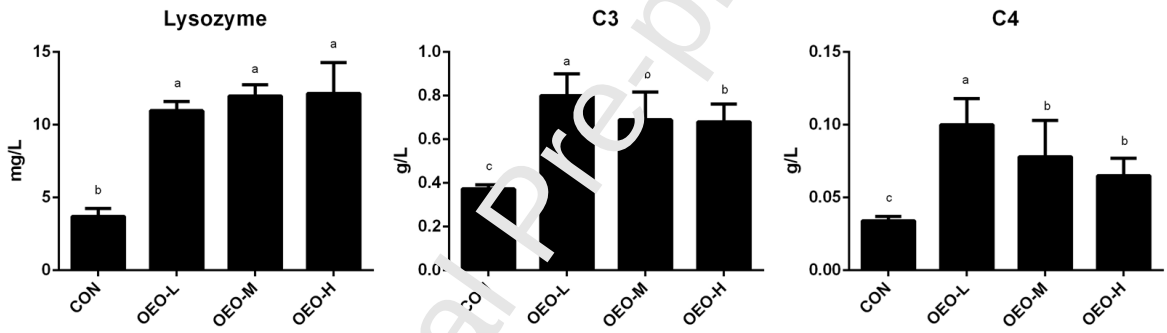


Fig. 2. Effect of dietary oregano essential oil on the concentrations of lysozyme, complement C3 and C4 in serum (n = 9 per treatment).

Data are expressed as means ± standard error of mean. [ab]Mean values with different superscripts are different at $P < 0.05$. OEO-L, OEO-M, OEO-H: Oregano essential oil was supplemented at a concentration of 500, 1,500 or 4,500 mg/kg of basal diet, respectively.

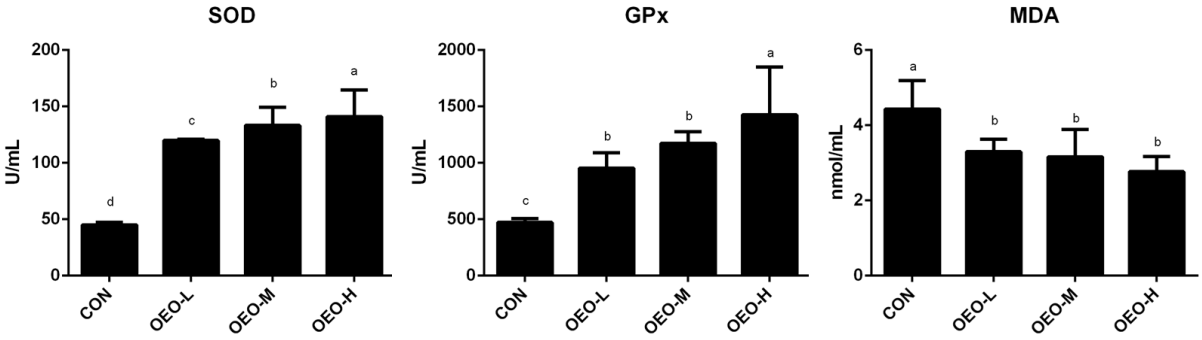


Fig. 3. Effect of dietary oregano essential oil on the concentrations of SOD, GPx and MDA in serum.

Data are expressed as means ± standard error of mean. [ab]Mean values with different superscripts are different at $P < 0.05$. SOD: superoxide dismutase activity, GPx: glutathione peroxidase; MDA: malondialdehyde. OEO-L, OEO-M, OEO-H: Oregano essential oil was supplemented at a concentration of 500, 1,500 or 4,500 mg/kg of basal diet, respectively.

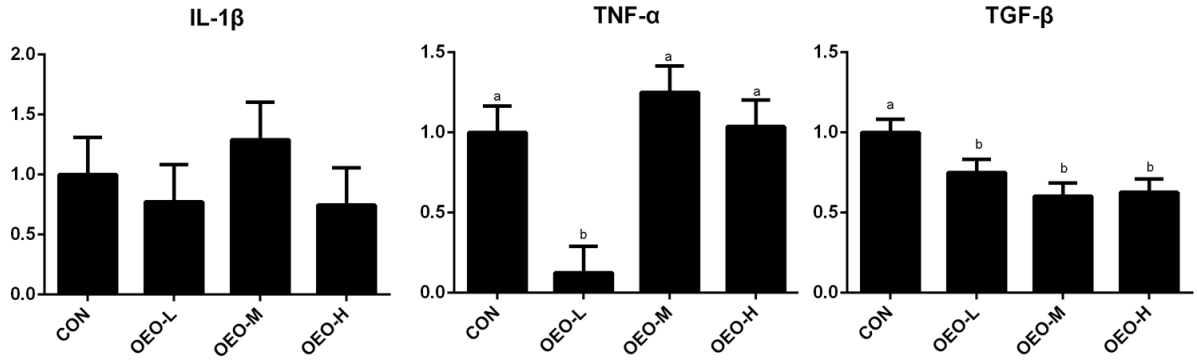


Fig. 4. Effect of dietary oregano essential oil on relative expression of *IL-1β*, *TNF-α* and *TGF-β* genes in the intestine (n = 9 per treatment).

Data are expressed as means ± standard error of mean. [ab]Mean values with different superscripts are different at $P < 0.05$. *TNF-α*: tumor necrosis factor α, *IL-1β*: interleukin 1β, *TGF-β* : transforming growth factor β; OEO-L, OEO-M, OEO-H: Oregano essential oil was supplemented at a concentration of 500, 1,500 or 4,500 mg/kg of basal diet, respectively.

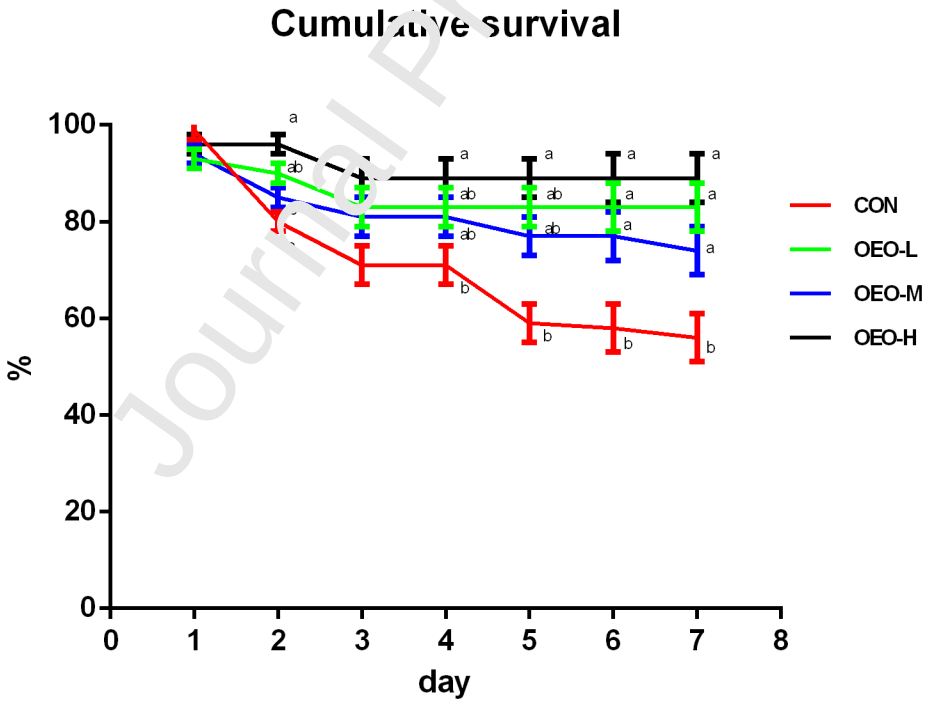


Fig. 5. Effect of dietary oregano essential oil on the cumulative survival rates of fish challenged with *A. hydrophila* (n = 66 per treatment).

Data are expressed as means ± standard error of mean. [ab]Mean values with different superscripts are different at $P < 0.05$. OEO-L,OEO-M, OEO-H: Oregano essential oil was supplemented at a concentration of 500, 1,500 or 4,500 mg/kg of basal diet, respectively.

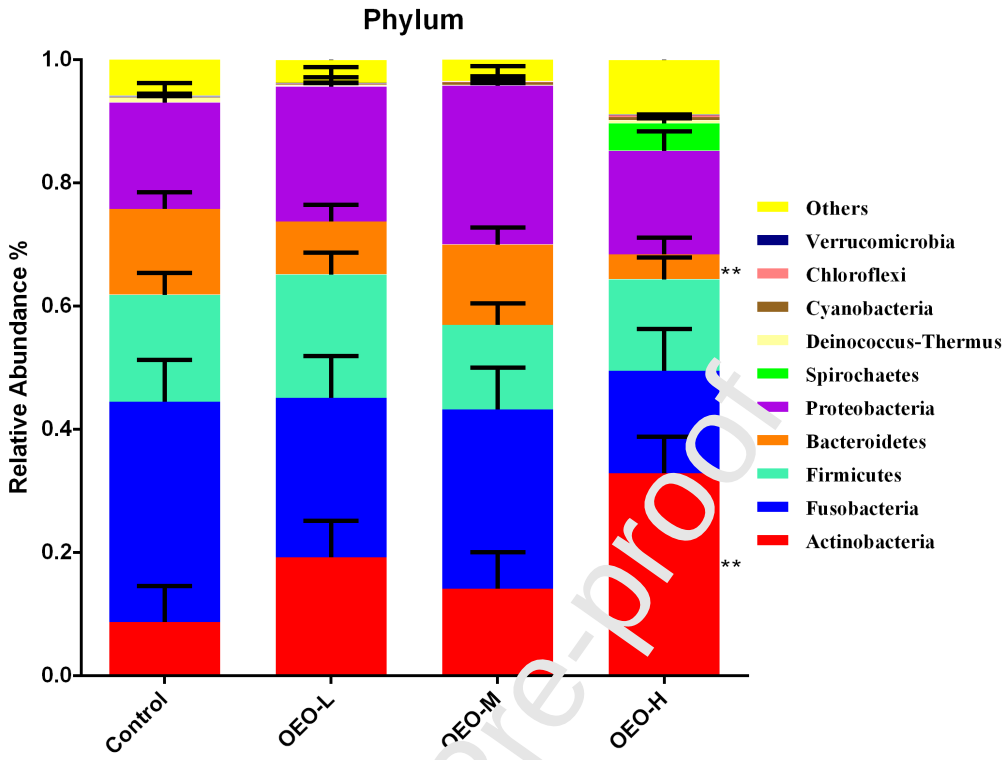


Fig. 6. Relative abundance of intestinal bacteria at phylum levels (n = 9 per treatment). The 10 most abundant phyla and genera are presented. Significant difference was detected by Student's *t*-test between control and OEO-treated groups at *, **, *** at $P < 0.05$, $P < 0.001$, $P < 0.0001$, respectively. OEO-L,OEO-M, OEO-H: Oregano essential oil was supplemented at a concentration of 500, 1,500 or 4,500 mg/kg of basal diet, respectively.

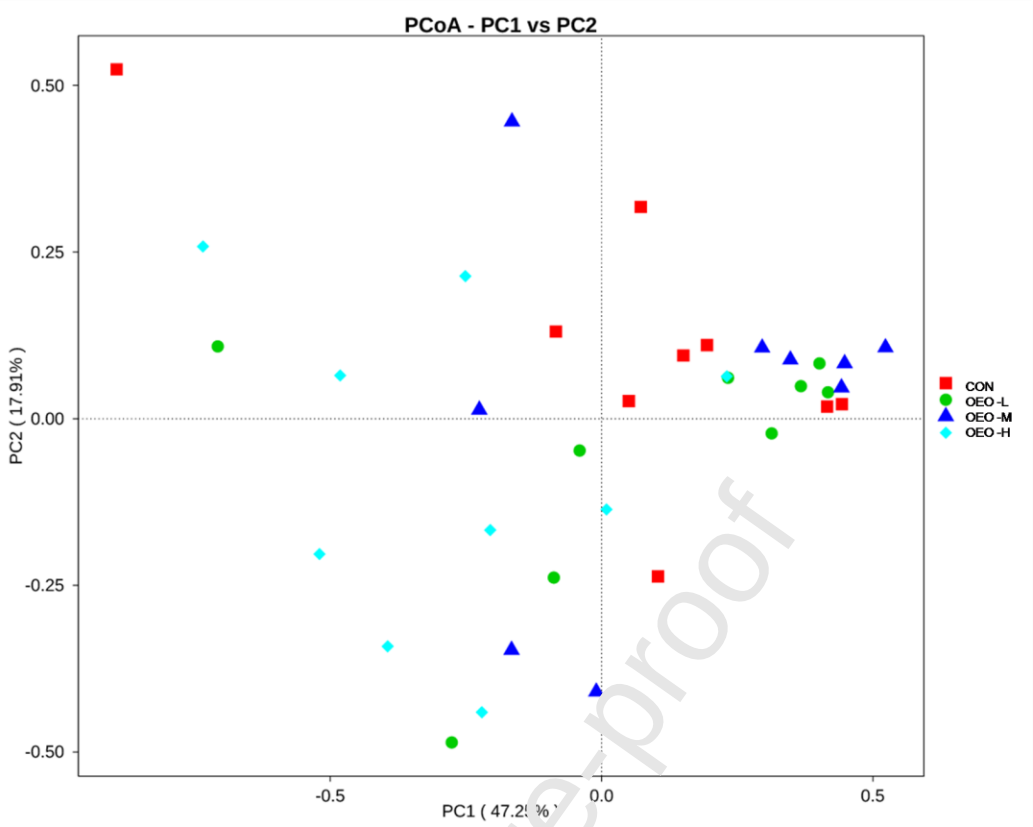


Fig. 7. Intestinal bacterial community by principal coordinate analysis based on weighted UniFrac distance (n = 9 per treatment).

OEO-L,OEO-M, OEO-H: Oregano essential oil was supplemented at a concentration of 500, 1,500 or 4,500 mg/kg of basal diet, respectively.

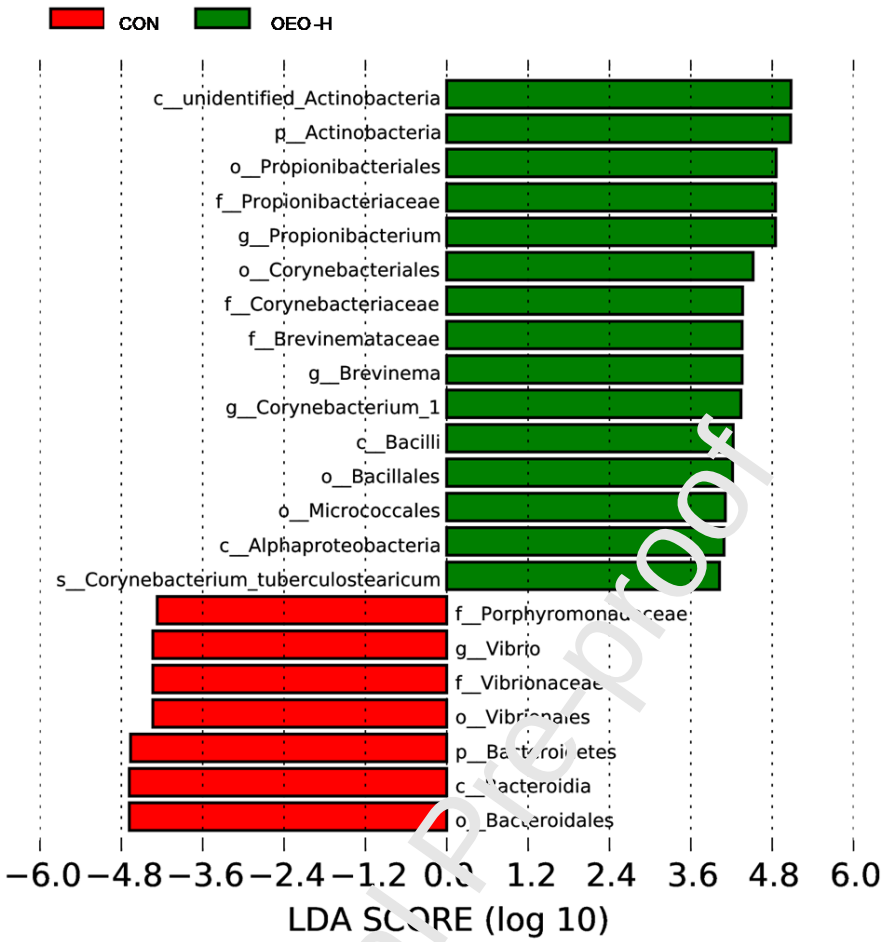


Fig. 8. Taxonomic comparisons of the bacterial communities between the OEO-H group and control using Linear discriminant analysis coupled with effect size (LEfSe) (n = 9 per treatment).
The threshold of the logarithmic LDA score was 4.0. The relative abundance of bacterial taxa was indicated by the size of circle, and treatment groups were identified by the color of circle. OEO-H: Oregano essential oil was supplemented at a concentration of 4,500 mg/kg of basal diet.

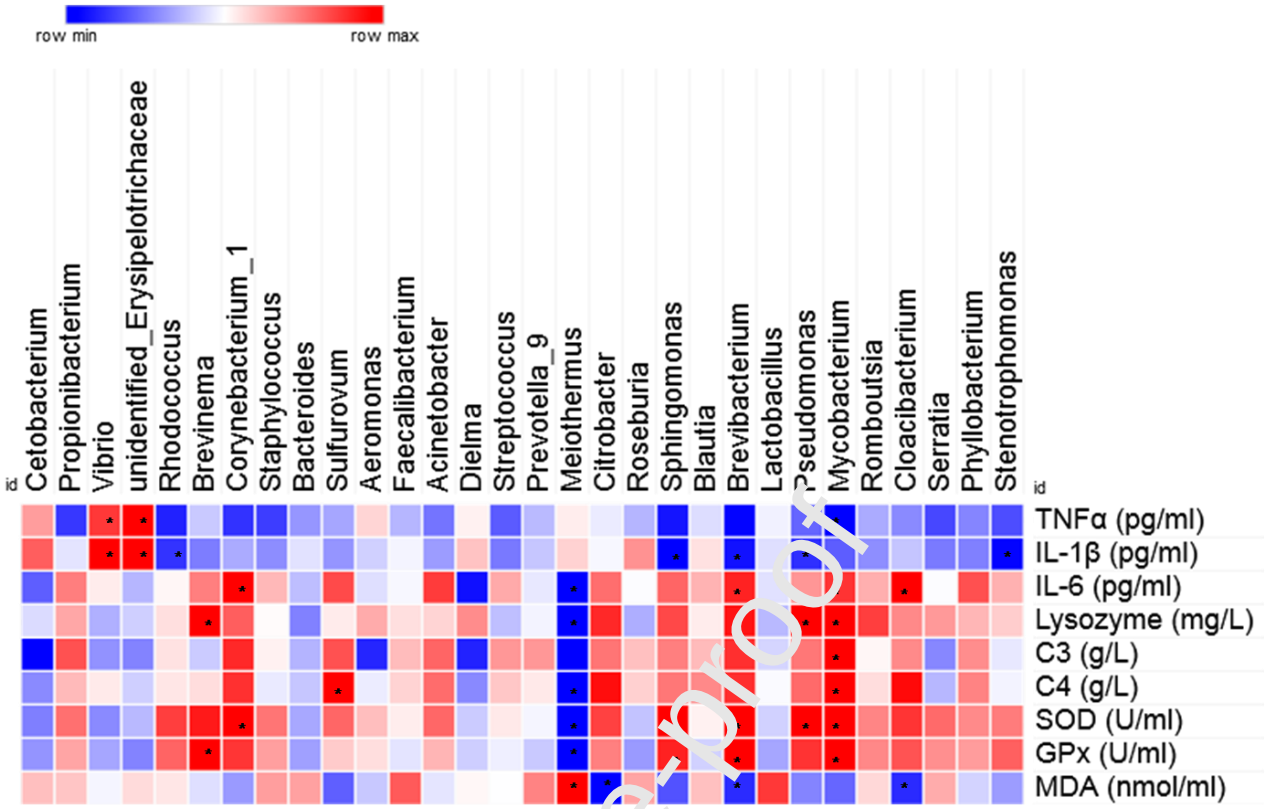


Fig. 9. Spearman's rank correlations between blood indices and relative abundance of bacteria at genus level. The 30 most abundant genera are included in the correlation analysis. Blue and red represent negative and positive correlations, respectively. Significant difference was marked as * at $P < 0.01$. TNF-α: tumor necrosis factor α, IL-1β: interleukin 1β, IL-6: interleukin 6. SOD: superoxide dismutase activity, GPx: glutathione peroxidase, MDA: malonaldehyde.

## Conflict of Interest

The authors declare there is no conflicts of interest regarding the publication of this paper.



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**Highlights:**

- Oregano essential oil increased digestive enzyme activity and antioxidant capability.
- Oregano essential oil enhanced non-specific immunity and disease resistance.
- The beneficial effects of OEO are probably due to alternations in the structure of gut microbiota.