

ARTICLE

Comparative In Vitro and In Vivo Effects of Feed Additives on Rainbow Trout Response to *Ichthyophthirius multifiliis*

Heidi Mathiessen, * Rzgar Mohammad Jaafar,  Azmi Al-Jubury, 

Louise von Gersdorff Jørgensen,  Per Walter Kania,  and Kurt Buchmann 

Laboratory of Aquatic Pathobiology, Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, 1870 Frederiksberg C, Denmark

Abstract

Control of the skin parasitic ciliate *Ichthyophthirius multifiliis* is currently based on laborious chemical and mechanical approaches, thus advocating for alternative control measures. Here, we show that the early development of trophonts (feeding stage residing in the epidermis) in the Rainbow Trout *Oncorhynchus mykiss* epidermis was inhibited at 5 d postinfection after the administration of feed additives, including garlic *Allium sativum*, oregano *Origanum onites*, thyme *Thymus vulgaris*, coriander *Coriandrum sativum*, and astaxanthin (a carotenoid derived from the alga *Haematococcus pluvialis*). However, no inhibition was observed at 8 d postinfection. We investigated whether the early inhibition was caused by (1) the feed additives' direct effect on the parasite and/or (2) stimulation of the Rainbow Trout immune responses. Garlic exhibited the highest in vitro killing capacity toward theronts (the infective stage of the parasite), followed by oregano, thyme, and astaxanthin, whereas coriander had no in vitro parasitocidal effect. Immune reactions were measured by plasma lysozyme activity in Rainbow Trout after feeding and by recording immune gene expression in trout leukocytes that were exposed to feed additives. Oregano-fed fish showed a significantly ($P < 0.05$) elevated plasma lysozyme activity. Oregano and thyme—and to a lesser extent, garlic, astaxanthin, and coriander—induced a significant regulation of innate and adaptive immune genes in vitro. The results suggest that the investigated additives inhibit early parasite development directly as well as indirectly (i.e., by elevating the host immune response).

The parasitic ciliate *Ichthyophthirius multifiliis* is the causative agent of white spot disease (also known as ichthyophthiriosis) in freshwater fish worldwide (Buchmann et al. 2001; Matthews 2005; Dickerson 2006; Jørgensen 2016). The infection is characterized by macroscopically visible white spots in the epidermis and may be associated with heavy mortality and morbidity (Hines and Spira 1973; Dickerson 2006; Buchmann 2019). The life cycle of the parasite is direct but comprises several stages. The trophont is the feeding stage, residing in the epidermis of the skin. After a period of growth, it escapes the

epidermis and appears as a freely swimming tomont, which subsequently encysts in a gelatinous capsule (the tomocyst stage) in which tomites (daughter cells) are produced by mitotic divisions. The tomites develop into infective theronts after leaving the cyst; they then seek and penetrate the external surfaces of the fish host (Matthews 2005; Dickerson 2006; Buchmann 2019).

No vaccines are available for prevention of *I. multifiliis* infection, and control is currently based on repeated treatments using chemicals and management (Heinecke and Buchmann 2009). Malachite green and formalin are

*Corresponding author: heidi.mathiessen@sund.ku.dk
Received January 16, 2020; accepted September 9, 2020

effective compounds, but due to their toxicity and carcinogenicity, farmers are replacing these compounds with less environmentally damaging chemicals, such as hydrogen peroxide (Rach et al. 2000; Rintamäki-Kinnunen et al. 2005), sodium percarbonate (Buchmann et al. 2003; Heinecke and Buchmann 2009), and peracetic acid (Rintamäki-Kinnunen et al. 2005; Meinelt et al. 2009; Straus and Meinelt 2009; Bruzio and Buchmann 2010). Replacement of formalin and malachite green by the alternative compounds has challenged fish farm operations. Several of the compounds mentioned can partly control *I. multifiliis* infections but are less successful than malachite green treatment (Lahnsteiner and Weismann 2007). Furthermore, treatments using chemicals such as formalin may damage the fish epidermis, leaving the fish susceptible to other pathogens (Buchmann et al. 2004).

Alternatives to this laborious practice may be found in functional feeding, as various feed additives have been shown to decrease the susceptibility of Rainbow Trout *Oncorhynchus mykiss* to *I. multifiliis* infection (Xueqin et al. 2012). Garlic *Allium sativum* as a feed additive was reported to increase growth, immunity, appetite, and the response to bacterial and fungal pathogens (Shakya and Labh 2014), including *Aeromonas hydrophila* (Mahfouz et al. 2009; Nya and Austin 2009) and monogenean infestations (Inoue et al. 2016). Oregano *Origanum onites* in feed has been shown to confer some resistance toward *Lactococcus garvieae* (Diler et al. 2017) and infections by the parasites *Ichthyobodo salmonis* and *Trichodina truttae* (Mizuno et al. 2018). Thyme *Thymus vulgaris* as a feed additive also conferred some resistance toward *Streptococcus iniae* infection (Yilmaz et al. 2013). Although the mechanisms have not been fully characterized, astaxanthin (a carotenoid derived from the alga *Haematococcus pluvialis*) was suggested to have immune-stimulating effects (Thompson et al. 1995; Amar et al. 2004; Moghaddam et al. 2015; Liu et al. 2016). Astaxanthin feeding was also associated with reduced mortality due to *Edwardsiella tarda* infection (Kim et al. 2012).

The objective of this study was to evaluate the effect of feed additives, including garlic, oregano, thyme, and coriander *Coriandrum sativum* extracts and astaxanthin, on the susceptibility of Rainbow Trout to *I. multifiliis*. Fish susceptibility was assessed by an exposure to *I. multifiliis* infection following feeding, and the mechanisms behind any potential effect were further assessed by conducting in vitro assays. A previous study pointed to a direct effect on the parasite. A garlic extract killed theronts within 900 min at 62.5 mg/L and within 180 min at 312.5 mg/L, and the extract killed tomocysts at 570 mg/L (Buchmann et al. 2003). Compounds like those mentioned above may also have an immune-stimulating effect on the host (Xueqin et al. 2012; Diler et al. 2017; Mizuno et al. 2018). This was tested by the in vitro assays.

We conducted three experiments: (1) an in vivo feeding study that evaluated Rainbow Trout susceptibility to *I. multifiliis* infection, (2) an in vitro immobilization study that investigated the direct effects of feed additives on the parasite, and (3) an in vitro leucocyte stimulation study that assessed the immune-stimulating properties of the feed additives.

METHODS

Fish

Rainbow Trout of the Rakkeby strain (Jutland, Denmark) were brought to the University of Copenhagen after being hatched and reared under pathogen-free conditions at the Bornholm Salmon Hatchery (Xueqin et al. 2012). Smaller fish were used for the in vivo feeding trial and larger fish were used for the in vitro leucocyte trial, as the latter study demanded large amounts of head kidney cells. All fish tanks were kept in the same room with a temperature of 16.5°C during acclimatization (14 d) and during subsequent feeding and infection.

In total, 98 fish (7 treatments \times 2 replicates \times 7 fish = 98 fish) were used for the in vivo study; mean body weight was 31.4 g (SD = 6.6), and mean total body length was 13 cm (SD = 2.5). For the in vitro leucocyte exposure trial, 20 fish with a mean body weight of 48 g (SD = 5.5) and a mean body length of 15.75 cm (SD = 1.25) were used for isolation of leucocytes for measurement of immune gene expression via quantitative PCR (qPCR).

Preparation of Additives

The herbs used in the experiment were dried oregano (Santa Maria, Brøndby, Denmark), thyme (Santa Maria), and coriander (Urtekram, Mariager, Denmark), while the garlic (ØGO) used was fresh. Oregano and thyme batches were ground using a mortar to a maximum particle size of 200 μ m, while garlic was pressed by a garlic presser to release fluid. The astaxanthin used was purchased from Sigma-Aldrich (Catalog Number [CN] SML0982, Darmstadt, Germany).

Parasites for Infection

Trophonts of *I. multifiliis* originating from a Danish trout farm in Jutland were harvested from infected Rainbow Trout that were euthanized by an overdose (300 mg/L) of anaesthetic (tricaine methanesulfonate [MS-222]; CN A5040; Sigma-Aldrich). The fish were left in clean water for approximately 4 h, allowing trophonts to leave the epidermis. The trophonts transformed into tomonts and, via the tomocyst stage, into infective theronts (Heinecke and Buchmann 2009).

In Vivo Experiment

In vivo feeding trial.—The experiment investigating the effect of feed additives was conducted using seven groups in duplicate (Figure 1). The fish were acclimatized in a total of 14 fish tanks, each with seven fish, in a volume of 100 L (aerated municipal water at $16.5 \pm 1.5^\circ\text{C}$) and containing internal Eheim Pickup 160 biofilters (Eldorado, Haderslev, Denmark) for 14 d under a 12-h light : 12-h dark cycle; a daily feeding regime corresponding to 2% of body weight at day 0 (EFICO Enviro 920 Advance; Bio-Mar, Brande, Denmark) was administered for 7 d. Treatment groups were subjected to seven feeding regimes as follows: (1) garlic added at 60 g/kg feed; (2) oregano at 60 g/kg feed; (3) thyme at 60 g/kg feed; (4) coriander at 60 g/kg feed; (5) astaxanthin at 100 mg/kg feed; (6) astaxanthin at 200 mg/kg feed; and (7) control, with no feed additive. The concentration (60 g/kg) used for the herbal feed additives was determined based on a previous experiment regarding the fish's willingness to eat; however, the two concentrations of astaxanthin were chosen based on existing literature. To differentiate the treatment groups, fish were tagged by fin clipping according to the feed additive. On day 7, blood samples (from 7 treatments \times 2 replicates \times 3 fish-replicate⁻¹ \cdot treatment⁻¹ = 42 fish) were collected by caudal vein puncture using heparinized syringes. The blood samples were centrifuged at $3,000 \times g$ for 10 min at 4°C ; plasma was extracted and stored at -80°C (Alishahi and Buchmann 2006) until lysozyme analysis.

The remaining 56 fish were exposed to infection with *I. multifiliis* at approximately 2,100 parasites/fish (58,800 trophonts/tank) in two 100-L tanks (7 treatments \times 4 fish/treatment = 28 fish/tank). Each of the two tanks contained four fish from each of the seven feeding regimes. At 5 and 8 d postinfection, the fish were anaesthetized and subjected to parasitological examination of the dorsal, caudal, and pelvic fins. The fish were monitored in the tanks until release of the parasite.

Feed preparation for in vivo trials.—One milliliter of rapeseed oil was used to bind the feed additives to the dried feed pellets (EFICO Enviro 920 Advance, 3 mm). The same oil was also applied to the control feed but without applying feed additives. The astaxanthin was mixed with the oil before adding it to the feed pellets for proper distribution. The herbal feed additives were added to the feed before the oil was applied.

Trophont enumeration in exposed fish.—The visible parasites from four anaesthetized (MS-222 at 50 mg/L) fish from each tank were microscopically (Leica L2; Leica Microsystems, Brønshøj, Denmark) counted at 5 and 8 d postinfection using a dissection microscope (4–40 \times magnification; Singh and Buchmann 2001).

Lysozyme activity measurement.—Lysozyme in plasma was measured by a spectrophotometric assay using *Micrococcus lysodeikticus* (CN M3770; Sigma-Aldrich) as substrate (Skov et al. 2018). A 10- μL volume of plasma was

added to each well in a 96-well microplate in duplicate. A 190- μL quantity of a suspension containing *M. lysodeikticus* (0.2 mg/mL) in sodium phosphate buffer was added to the well. The plate was spun at $574 \times g$ for 10 s, and the optical density was measured at 450 nm every 15 s for 30 min (Epoch Spectrophotometer; Holm & Halby, Brøndby, Denmark).

In Vitro Immobilization Experiment

Preparation of solutions for in vitro immobilization.—Stock solutions of the aqueous extract from the herbal feed additives were applied in a concentration of 1 g herb per 7 mL of distilled water. Distilled water was used as a control. Astaxanthin was prepared with a solvent (1 g of astaxanthin dissolved in 1 mL of dimethyl sulfoxide [DMSO]) according to the manufacturer's recommendation. A stock solution was prepared using 250 μL from the preparation mixed with 750 μL of distilled water. The control for astaxanthin was 250 μL of DMSO mixed with 750 μL of distilled water. Dilution series (twofold) based on the stock solutions mentioned above were prepared for in vitro exposure of theronts.

Immobilization assay.—The in vitro antiparasitic capacity of the feed additives was evaluated by an immobilization assay performed on *I. multifiliis* theronts. Dilution series (twofold) based on the aforementioned stock solutions were prepared, and a 50- μL volume of the dilutions was added to 50 μL of water containing a suspension of the parasite in a 24-well glass plate. Each well contained 5–10 theronts, and their motility was recorded every 15 min for a total of 60 min under the dissection microscope with subillumination (Leica). Theronts with a total loss of motility were considered inactivated.

In Vitro Leukocyte Stimulation Experiment

Cell isolation.—The cell isolation was performed according to Chettri et al. (2011). In brief, head kidneys from 20 Rainbow Trout were removed, homogenized, and subsequently centrifuged ($400 \times g$ for 30 min at 4°C) on Percoll (51%; CN GE17-0891-01; Sigma-Aldrich). Leukocytes were harvested, and the concentration of live cells was determined using the trypan blue exclusion method (1:1 ratio) and adjusted to 1.0×10^7 cells/mL using Fast-Read 102 slides (CN 630-1893; VWR, Søborg, Denmark). Subsequently, 100 μL of cell suspension were added to each well in a flat-bottom, 96-well microtiter plate (CN 655180; Fisher Scientific, Waltham, Massachusetts, USA) and incubated for 2 h at 15°C . Incubation was followed by removal of nonattached cells by washing, after which the cells were left to acclimatize overnight at 15°C .

Stimulation of cells.—The Rainbow Trout leukocytes were stimulated with a high (70 $\mu\text{g}/\text{mL}$) or a low (35 $\mu\text{g}/\text{mL}$) feed additive dosage for 1 h; the feed additives were then replaced with 100 μL of lysis buffer containing 2-mercaptoethanol (CN M3148; Sigma-Aldrich; Chettri et al.

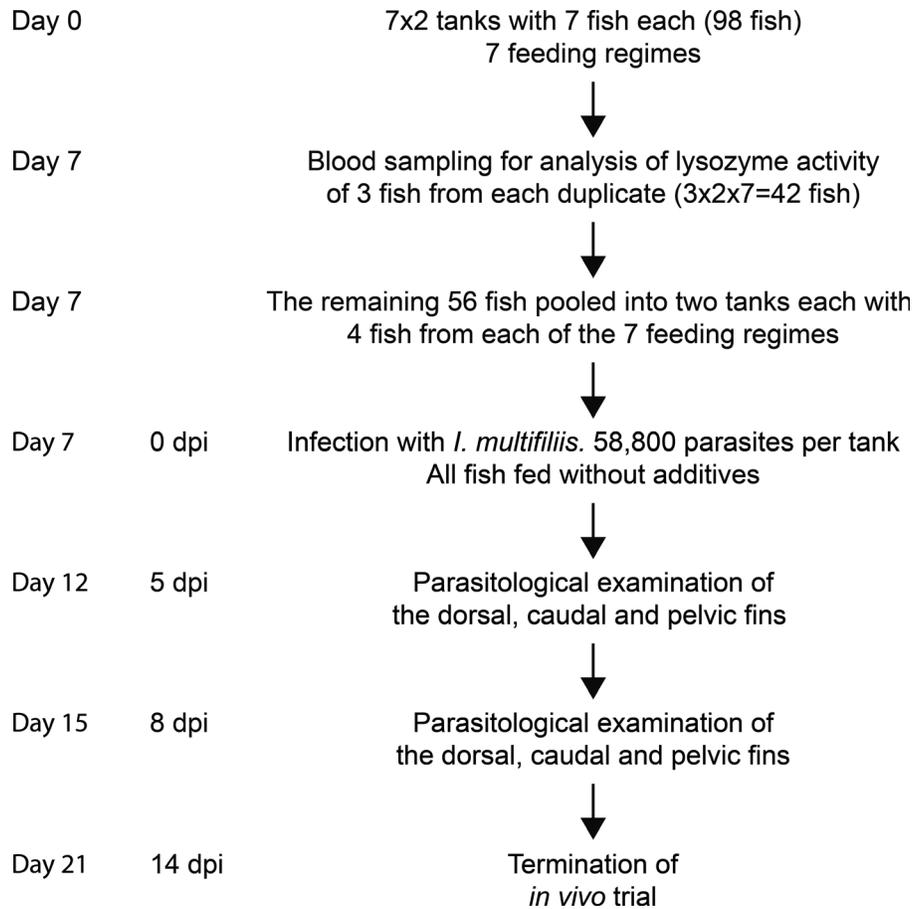


FIGURE 1. Design of the *in vivo* trial with Rainbow Trout. On day 0, 98 fish were divided into seven duplicated groups (seven feeding regimes in two tanks with seven fish each). The seven duplicates were tagged by individual fin clipping. On day 7, three fish from each tank ($3 \times 7 \times 2 = 42$) were used for blood sampling. The remaining fish were pooled into two tanks (each tank infected with 58,800 *Ichthyophthirius multifiliis* trophonts), after which the fish were fed without additives. At 5 and 8 d postinfection (dpi), the fish were subjected to parasitological examination for *I. multifiliis*.

2010, 2011). A lipopolysaccharide preparation from *Escherichia coli* 0111:B4 (100 + 400 $\mu\text{g}/\text{mL}$; CN L2630; Sigma-Aldrich) was applied as a positive control since it has a well-documented ability to induce a response in this *in vitro* leukocyte stimulation assay (Chettri et al. 2011).

Real-time quantitative PCR.—Total RNA from leukocytes was extracted using the GenElute Mammalian Total RNA Miniprep Kit (CN RTN350; Sigma-Aldrich) and treated with DNase (CN AMPD1; Sigma-Aldrich) after extraction to remove potential DNA contamination. The RNA concentration was measured spectrophotometrically using a NanoDrop spectrophotometer (Saveen & Werner ApS, Jyllinge, Denmark), and the samples were kept at -80°C . The RNA was subjected to reverse transcription in a BioRad T100 Thermal Cycler (Thermo Fisher Scientific, Roskilde, Denmark) by means of TaqMan Reverse Transcription Reagents (CN N8080127; Thermo Fisher Scientific) using random hexamers in a 40- μL reaction volume containing a maximum of 1,000 ng of RNA. The reactions occurred under the following conditions: 10 min at 25°C ,

60 min at 37°C , and 5 min at 95°C . Complementary DNA was diluted four times by adding 120 μL of RNase-free water (CN 10977-035 RNase; Thermo Fisher Scientific) and was subsequently stored at 5°C . To perform real-time qPCR assays, an AriaMx Real Time PCR System (AH Diagnostics, Tilst, Denmark) was used. Real-time qPCR was conducted using the Brilliant III Ultra-Fast Master Mix Kit (CN 600880; AH Diagnostics), 2.5 μL of complementary DNA template, 0.8- μM primers, and 0.4- μM probes in a 12.5- μL reaction. The final concentration of MgCl_2 was 5.5 mM. Primers and probes are listed in Table S1 (available in the Supplement in the online version of this article). The PCR conditions were set to one cycle for 10 min at 94°C followed by 40 cycles at 94°C for 10 s and 60°C for 15 s. Each run included wells without template and wells with template but without reverse transcriptase. The genes chosen in this study comprised genes that regulate both innate and adaptive immunity.

Genes encoding an innate immune response were pro-inflammatory cytokines (interleukin [IL] 1 beta [IL-1 β],

tumor necrosis factor alpha [TNF- α], and IL-6), a regulatory cytokine (IL-10), a chemokine (IL-8), acute-phase proteins (serum amyloid A [SAA], hepcidin, and precerebellin), and an antimicrobial enzyme (lysozyme). Genes encoding an adaptive response were immunoglobulins M and T (IgM and IgT), T-cell markers (clusters of differentiation 4 [CD4] and 8 [CD8]) and a Th2 cytokine (IL-4/13A). Finally, complement factors 3 and 5 (C3 and C5) were also included; however, these are involved in both innate and adaptive immunity.

Calculations and Statistical Analysis

Data were analyzed using Microsoft Office Excel (Microsoft, Redmond, Washington, USA) and GraphPad Prism 7 and 8 (GraphPad Software, San Diego, California, USA). Data from the susceptibility trial followed a normal distribution, which allowed application of a one-way ANOVA with Dunnett's multiple comparison test to compare the treatments with the control. Furthermore, a two-way ANOVA was conducted to investigate whether there were differences between the treatments or between the two time points (significance was assessed at $P < 0.05$). Lysozyme activity in plasma was also distributed according to a normal distribution; in this case, the data were analyzed using a Student's *t*-test ($P < 0.05$). The Mantel-Cox test and Gehan-Breslow-Wilcoxon test were performed to evaluate the significant difference between parasite immobilization curves ($P < 0.05$). Relative immune gene expression was analyzed using the simplified $2^{-\Delta\Delta C_q}$ method, where C_q is the cycle quantification value (Livak and Schmittgen 2001), as all of the qPCR assays had efficiencies within $100 \pm 5\%$ (Schmittgen and Livak 2008). Student's *t*-test was applied for evaluating the effect of the feed additives on immune gene expression; only cases having both a P -value < 0.05 and a fold change of at least 2 were considered significant. In some cases, too few C_q values were obtained in one of the groups compared to use the quantitative $2^{-\Delta\Delta C_q}$ method. In these cases, the gene expression regulation was assessed by a qualitative approach using the presence/absence of C_q values and the nonparametric Mann-Whitney test.

Ethics and legislation.—The experiments were performed under Animal Experimental Inspectorate License 2013-15-2934-00794 and in accordance with the general welfare guidelines at the University of Copenhagen.

RESULTS

In Vivo Experiment

The total number of parasites and mean intensity recorded were higher at 8 d postinfection compared to 5 d postinfection (Figure 2). The highest number of parasites

was recorded in the control fish (both 5 and 8 d postinfection), and the lowest number was found in the groups receiving astaxanthin at 100 mg/kg feed. The parasite escaped the epidermis at 14 d postinfection. At 5 d postinfection, all of the feed additives resulted in a significantly lower parasite burden compared to the control fish. No significant difference between the different feed additives was found ($P = 0.5579$). At 8 d postinfection, the infection intensities did not differ significantly between fish that received feed additives and the control group. There was a significant difference ($P < 0.0001$) between the infection levels at 5 and 8 d postinfection.

The plasma lysozyme activity in fish receiving oregano was significantly ($P = 0.0045$, $P = 0.0406$) elevated compared to that in the control fish for both laboratory experiments performed. Plasma lysozyme activity in fish receiving the other feed additives did not differ from that of controls (Table 1).

In Vitro Immobilization Experiment

All herbal feed additives except coriander successfully immobilized theronts. The garlic preparation was the most effective, as we found a significantly ($P < 0.0001$) lower mobility of the parasite in all concentrations until a 1:1,024 dilution ($P = 0.1195$). Oregano resulted in a significantly ($P < 0.0001$ and $P = 0.0012$) higher immobilization until a dilution of 1:256 ($P = 0.7032$). Thyme also affected the parasite, but we merely found a significant ($P < 0.0001$) difference between thyme and the control until a 1:32 dilution ($P = 0.9892$). We noted a higher theront immobilization in astaxanthin compared to the corresponding DMSO control group, but only an astaxanthin dilution of 1:8 ($P = 0.0013$) significantly increased immobilization of the parasite (see Table S2 for exact data).

In Vitro Leukocyte Stimulation Experiment

The additives significantly influenced expression of immune genes in Rainbow Trout leukocytes (Figures 3 and 4). The expression of genes encoding IL-1 β ($P = 0.02$), C3 ($P = 0.003$), IgT ($P = 0.025$), IL-6 ($P = 0.01$), CD4 ($P = 0.046$), CD8 ($P = 0.021$), and IL-4/13A ($P = 0.002$) was significantly upregulated after exposure to the low concentration of garlic. The high dosage of garlic merely resulted in a significant upregulation of IL-6 ($P = 0.04$) and IL-4/13A ($P = 0.001$) genes in Rainbow Trout leukocytes, whereas the SAA gene (low concentration: $P = 0.035$, high concentration: $P = 0.002$) was significantly downregulated at both garlic concentrations.

The low concentration of oregano significantly upregulated the genes encoding CD4 ($P = 0.002$), CD8 ($P = 0.00001$), precerebellin ($P = 0.03$), hepcidin ($P = 0.01$), and IL-4/13A ($P = 0.008$), whereas the high concentration upregulated the genes encoding C5 ($P = 0.0003$), IL-6 ($P = 0.01$), IL-10 ($P = 0.001$), CD4 ($P = 0.003$), CD8 ($P =$

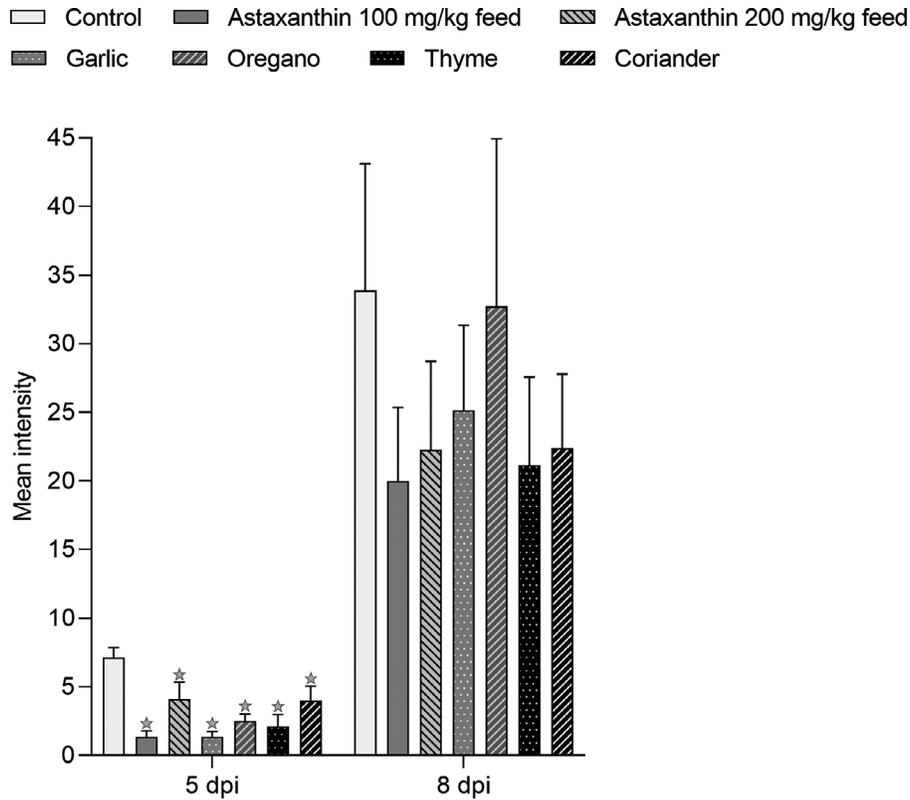


FIGURE 2. Intensity of the *Ichthyophthirius multifiliis* infection in Rainbow Trout that received feed additives (dpi = d postinfection). The intensity was calculated as the mean number of parasites per fish (all fish infected). Error bars indicate the SE. Stars indicate significant differences ($P < 0.05$) in comparison with the control at the given time point (dpi).

TABLE 1. Plasma lysozyme activity (mean and SE; mL) in Rainbow Trout that received different feed additives. An asterisk indicates a significant difference from the control ($P < 0.05$).

Feed additive	Mean	SE
Control	12.47	0.73
Garlic	13.30	1.30
Oregano	16.65*	1.14
Thyme	8.67	1.85
Coriander	11.91	0.61
Astaxanthin (200 mg/kg feed)	12.56	2.04

0.0085), precerebellin ($P = 0.02$), hepcidin ($P = 0.04$), and IL-4/13A ($P = 0.001$). The gene encoding IL-8 (low concentration: $P = 0.000003$, high concentration: $P = 0.00119$) was significantly downregulated at both concentrations of oregano. Furthermore, the low oregano concentration resulted in downregulation of genes encoding IgM ($P = 0.0002$), SAA ($P = 0.012$), TNF- α ($P = 0.002$), and lysozyme ($P = 0.0002$). The high oregano concentration induced a suppression of IgM ($P = 0.0034$) and lysozyme ($P = 0.0005$) gene expression.

Exposure to a low concentration of thyme led to a significant upregulation of the genes encoding C3 ($P = 0.001$), C5 ($P = 0.0158$), IgT ($P = 0.002$), CD8 ($P = 0.0045$), and IL-4/13A ($P = 0.002$). Several of these genes were also upregulated significantly at the high thyme concentration (C5: $P = 0.0347$; IgT: $P = 0.02$; CD4: $P = 0.013$; CD8: $P = 0.0013$; precerebellin: $P = 0.02$; hepcidin: $P = 0.02$; IL-4/13A: $P = 0.001$). However, thyme also elicited a significant downregulation of some genes. At the low thyme concentration, genes encoding IL-8 ($P = 0.00458$), SAA ($P = 0.0002$), and TNF- α ($P = 0.003$) were downregulated. At the high thyme concentration, genes encoding IgM ($P = 0.0173$), IL-8 ($P = 0.0005$), SAA ($P = 0.0008$), TNF- α ($P = 0.019$), and lysozyme ($P = 0.0002$) were also significantly downregulated. Coriander affected gene expression less but resulted in a significant downregulation of the gene encoding SAA ($P = 0.0009$).

When leukocytes were exposed to a low dosage of astaxanthin, it resulted in a significant downregulation of the SAA gene ($P = 0.0039$). Exposure to a high dose of astaxanthin resulted in upregulation of the gene encoding IgT ($P = 0.023$) and a downregulation of the gene encoding precerebellin ($P = 0.04$). Exposure to a

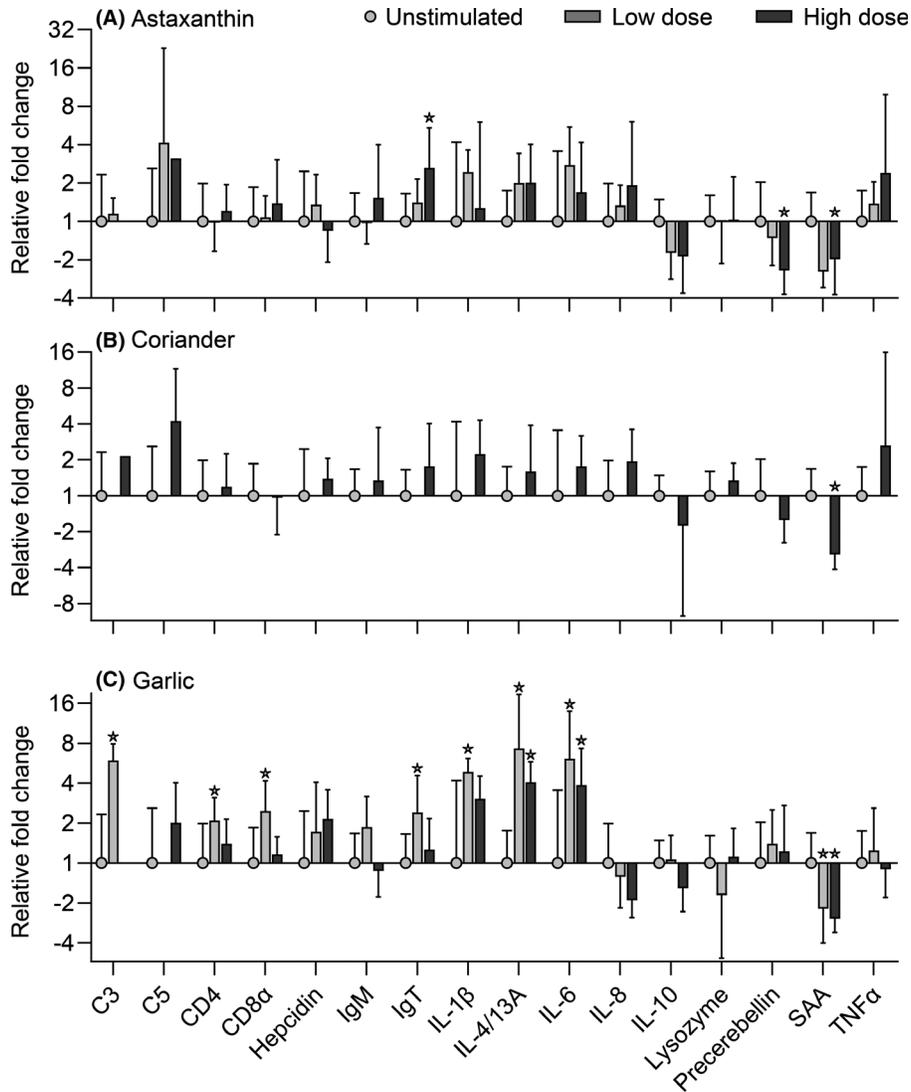


FIGURE 3. Regulation of immune genes in Rainbow Trout leukocytes exposed in vitro to three different feed additives (astaxanthin, coriander, and garlic; high dose = 70 $\mu\text{g}/\text{mL}$; low dose = 35 $\mu\text{g}/\text{mL}$). Stars indicate significant differences ($P < 0.05$) from the control. Abbreviations are defined in Methods.

low concentration of lipopolysaccharide led to a significant upregulation of the genes encoding IL-8 ($P = 0.0125$), TNF- α ($P = 0.004$), and IL-4/13A ($P = 0.045$). The high concentration strongly stimulated the expression of immune genes encoding IL-1 β ($P = 0.01$), IL-6 ($P = 0.04$), IL-8 ($P = 0.000997$), IL-10 ($P = 0.001$), TNF- α ($P = 0.001$), and lysozyme ($P = 0.02$).

DISCUSSION

Control of infectious diseases in fish aquaculture should optimally rely on prophylactic principles, but in many cases the treatment of infected fish is necessary. Availability of effective chemotherapeutants is limited, and their usage

raises toxicity and environmental concerns. Sustainable control agents, including herbal extracts from naturally occurring plants, may be considered relevant in this regard. A series of studies has indicated that these plant products have some effect on both bacterial and parasitic infections (Mahfouz et al. 2009; Nya and Austin 2009; Zheng et al. 2009; Amar et al. 2012; Kim et al. 2012; Yilmaz et al. 2013; Breyer et al. 2015; Inoue et al. 2016; Diler et al. 2017; Jørgensen 2017; Mizuno et al. 2018; Skov et al. 2018).

In Vivo Experiment

In the present study, we conducted an in vivo feeding trial using extracts from garlic, oregano, thyme, and coriander along with astaxanthin. This resulted in a reduced parasite

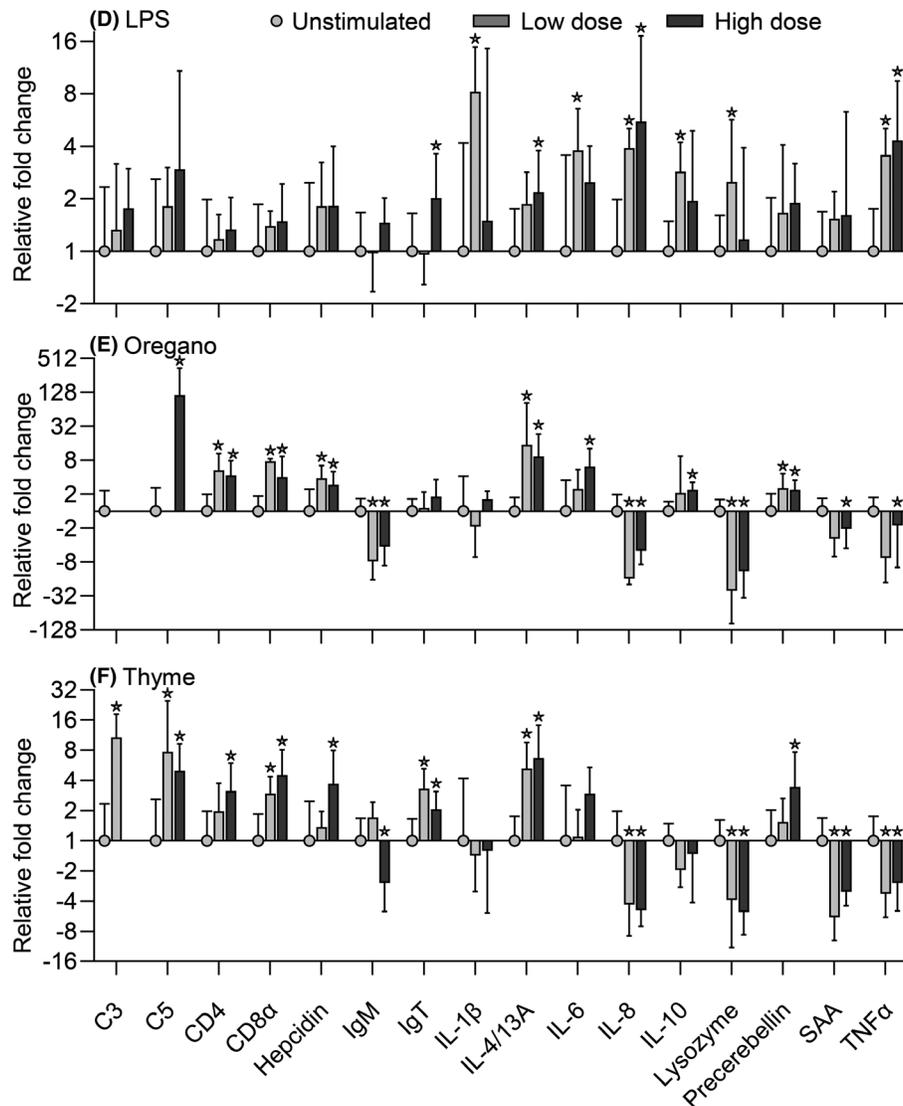


FIGURE 4. Regulation of immune genes in Rainbow Trout leukocytes exposed in vitro to three different feed additives (lipopolysaccharide [LPS], oregano, and thyme; high dose = 70 $\mu\text{g}/\text{mL}$; low dose = 35 $\mu\text{g}/\text{mL}$). Stars indicate significant differences ($P < 0.05$) from the control. Abbreviations are defined in Methods.

burden at 5 d postinfection, suggesting that the garlic, oregano, thyme, and coriander extracts and astaxanthin potentially inhibit early development of *I. multifiliis* trophonts in Rainbow Trout skin, making them harder to detect under the microscope. The effect of the feed additives did not last and was of short duration at best, as no significant difference between treatments was found at 8 d postinfection. Theoretically, the observations could be explained by a direct antiparasitic effect, an immune-stimulating effect, or both. The results indicate an acute and short-lived effect.

Aqueous extracts of garlic, oregano, and thyme along with astaxanthin showed a direct parasitocidal effect on theronts, which could explain our observations, at least partly. In addition, Rainbow Trout that were fed oregano

showed a higher lysozyme activity in plasma, which indicated an effect on the immune system of the fish. In the in vitro leukocyte stimulation experiment, we observed a marked effect of most feed additives, which suggests that activated immune reactions on the fish's body surface may inhibit the early development of trophonts after their invasion. In this context, it is noteworthy that a corresponding delay of *Ichthyobodo* and *Trichodina* infections in salmonids was achieved by a similar administration of oregano (Mizuno et al. 2018). At 5 d postinfection in our study, the parasites were rather small and difficult to discern, which may have led to an underestimation of their numbers. If the size of the parasites was even smaller after hosts were fed the additives, the parasites could go

unnoticed. The suggested slower development was supported by an extended trophont maturation period in the skin of the host, as the parasite did not escape the epidermis until 14 d postinfection. In control fish, the parasites escaped the epidermis after 7–11 d. The fish were fed for a total of 7 d, although other published studies have chosen feeding periods spanning from 14 to 90 d (Mahfouz et al. 2009; Nya and Austin 2009; Zheng et al. 2009; Yilmaz et al. 2013; Breyer et al. 2015; Inoue et al. 2016; Diler et al. 2017; Mizuno et al. 2018). In this way, we could measure the direct effect on the immune system before any adaptation was induced. Components from fish feed accumulate over time (Montero et al. 2005), and a reduced effect of feed additives has been observed over time (Breyer et al. 2015; Mizuno et al. 2018).

In Vitro Immobilization Experiment

Garlic, oregano, thyme, and astaxanthin all showed an in vitro capacity for immobilization of *I. multifiliis*, whereas coriander had no in vitro effect on the parasite. The active compounds in the herbal feed additives have not been clearly defined but are likely to differ from source to source.

In Vitro Leukocyte Stimulation Experiment

Astaxanthin was previously shown to activate immune parameters, such as complement, phagocytic activity (Amar et al. 2004), and neutrophil adherence (Kim et al. 2012), but not lysozyme. Since garlic and thyme components may also stimulate the host immune system (Mahfouz et al. 2009; Nya and Austin 2009; Gulec et al. 2013), we cannot exclude the possibility that the parasite inhibition effect observed at 5 d postinfection could be a result of the immunostimulatory effects of the feed additives.

Our in vitro stimulation of leukocytes showed that different feed additives may affect immune reactions differently. It may be hypothesized that thyme and oregano stimulated the immune genes encoding effector molecules, and depression of genes facilitating an inflammatory response suggests that *I. multifiliis* may profit from inflammation-associated skin processes but may also be adversely affected by effector molecules. Oregano, garlic, and thyme successfully induced upregulation of several genes involved in the adaptive immune response. Garlic at a low dose led to significant upregulation of the C3 gene, supporting previous observations (Breyer et al. 2015). A high garlic dose increased the expression of genes encoding IL-6 and IL-4/13A, whereas other genes were unregulated. Astaxanthin administered in feed has been demonstrated to increase survival in virus-infected fish and to regulate different immune components, such as complement, phagocytic activity (Amar et al. 2004), and neutrophil adherence (Kim et al. 2012). However, during in vitro stimulation of leukocytes, we did not record an

effect on genes encoding C3 and C5—regardless of whether the dosage was low or high. Coriander was associated with an inhibitory effect on trophont development at 5 d postinfection but showed no direct parasitocidal effect or any broad influence on immune gene expression in vitro, suggesting that feed additives may affect other immune mechanisms that were not addressed in the present study.

In conclusion, the use of feed containing extracts of garlic, oregano, thyme, and coriander resulted in a significantly lower detection of parasites at 5 d postinfection after administration of additives was discontinued. No significant effect was seen at 8 d postinfection despite a generally lower infection intensity. This suggests that additives exerted an inhibitory effect on early trophont growth and that the effects of the feed additives are acute or short-lived. Host immune factors may influence the development of the early trophont stage in the epidermis of the fish. It is therefore noteworthy that feed containing oregano was associated with an increased lysozyme activity in Rainbow Trout plasma. Most of the additives had a direct parasitocidal effect when tested in vitro. Garlic showed the highest immobilizing effect on theronts, followed by oregano, thyme, and astaxanthin. Coriander had no direct effect on the parasite. In vitro stimulation of Rainbow Trout leukocytes with the herbal extracts resulted in significant regulation of several immune genes that may have influenced the parasite infection. The inhibition of early trophont development along with stimulation of the immune response may be beneficial for fish farmers and the aquaculture industry. However, further studies should elucidate the antiparasitic mechanisms in the fish and optimize the feed administration before use of feed additives on a wider scale.

ACKNOWLEDGMENTS

The present study was supported by GUDP/ICROFS (Green Development and Demonstration Programme/International Centre for Research in Organic Food Systems) Grant 34009-18-1381 (ShelterFish). This work was associated with the European Union Horizon 2020 project ParaFishControl (Grant Agreement 634429). This output reflects only the authors' view, and the European Union cannot be held responsible for any use that may be made of the information contained herein. There are no conflicts of interest to declare.

ORCID

Heidi Mathiessen  <https://orcid.org/0000-0003-0558-7069>
Rzgar Mohammad Jaafar  <https://orcid.org/0000-0003-2377-3818>

Azmi Al-Jubury  <https://orcid.org/0000-0002-8039-1886>
 Louise von Gersdorff Jørgensen  <https://orcid.org/0000-0002-6005-3249>
 Per Walter Kania  <https://orcid.org/0000-0002-6000-165X>
 Kurt Buchmann  <https://orcid.org/0000-0002-2747-237X>

REFERENCES

- Alishahi, M., and K. Buchmann. 2006. Temperature-dependent protection against *Ichthyophthirius multifiliis* following immunisation of Rainbow Trout using live theronts. *Diseases of Aquatic Organisms* 72:269–273.
- Amar, E. C., V. Kiron, T. Akutsu, S. Satoh, and T. Watanabe. 2012. Resistance of Rainbow Trout *Oncorhynchus mykiss* to infectious hematopoietic necrosis virus (IHNV) experimental infection following ingestion of natural and synthetic carotenoids. *Aquaculture* 330–333:148–155.
- Amar, E. C., V. Kiron, S. Satoh, and T. Watanabe. 2004. Enhancement of innate immunity in Rainbow Trout (*Oncorhynchus mykiss* Walbaum) associated with dietary intake of carotenoids from natural products. *Fish and Shellfish Immunology* 16:527–537.
- Breyer, K. E., R. G. Getchell, E. R. Cornwell, G. A. Wooster, H. G. Ketola, and P. R. Bowser. 2015. Efficacy of an extract from garlic, *Allium sativum*, against infection with the furunculosis bacterium, *Aeromonas salmonicida*, in Rainbow Trout, *Oncorhynchus mykiss*. *Journal of the World Aquaculture Society* 46:273–282.
- Bruzio, R., and K. Buchmann. 2010. The effect of peracetic acid products on parasites causing white spot disease. *Fish Farmer* 6:25–27.
- Buchmann, K. 2019. Immune response to *Ichthyophthirius multifiliis* and role of IgT. *Parasite Immunology* 42(8):e12675.
- Buchmann, K., J. Bresciani, and C. Jappe. 2004. Effects of formalin treatment on epithelial structure and mucous cell densities in Rainbow Trout, *Oncorhynchus mykiss* (Walbaum), skin. *Journal of Fish Diseases* 27:99–104.
- Buchmann, K., P. B. Jensen, and K. D. Kruse. 2003. Effects of sodium percarbonate and garlic extract on *Ichthyophthirius multifiliis* theronts and tomocysts: in vitro experiments. *North American Journal of Aquaculture* 65:21–24.
- Buchmann, K., J. Sigh, C. V. Nielsen, and M. Dalgaard. 2001. Host responses against the fish parasitizing ciliate *Ichthyophthirius multifiliis*. *Veterinary Parasitology* 100:105–116.
- Chettri, J. K., L. Holten-Andersen, and K. Buchmann. 2010. Factors influencing *in vitro* respiratory burst assays with head kidney leucocytes from Rainbow Trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* 33:593–602.
- Chettri, J. K., M. K. Raida, L. Holten-Andersen, P. W. Kania, and K. Buchmann. 2011. PAMP induced expression of immune relevant genes in head kidney leukocytes of Rainbow Trout (*Oncorhynchus mykiss*). *Developmental and Comparative Immunology* 35:476–482.
- Dickerson, H. W. 2006. *Ichthyophthirius multifiliis* and *Cryptocaryon irritans* (phylum Ciliophora). Pages 116–153 in P. T. K. Woo, editor. *Fish diseases and disorders, volume 1: protozoan and metazoan infections*. CAB International, Oxfordshire, UK.
- Diler, O., O. Gormez, I. Diler, and S. Metin. 2017. Effect of oregano (*Origanum onites* L.) essential oil on growth, lysozyme and antioxidant activity and resistance against *Lactococcus garvieae* in Rainbow Trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture Nutrition* 23:844–851.
- Gulec, A. K., D. Danabas, M. Ural, E. Seker, A. Arslan, and O. Serdar. 2013. Effect of mixed use of thyme and fennel oils on biochemical properties and electrolytes in Rainbow Trout as a response to *Yersinia ruckeri* infection. *Acta Veterinaria Brno* 82:297–302.
- Heinecke, R. D., and K. Buchmann. 2009. Control of *Ichthyophthirius multifiliis* using a combination of water filtration and sodium percarbonate: dose–response studies. *Aquaculture* 288:32–35.
- Hines, R. S., and D. T. Spira. 1973. *Ichthyophthirius multifiliis* (Fouquet) in the Mirror Carp, *Cyprinus carpio* L. I. Course of infection. *Journal of Fish Biology* 5:385–392.
- Inoue, L. A. K. A., P. Oliveira Maciel, E. Gusmão Affonso, C. de Lima Bojink, and M. Tavares-Dias. 2016. Growth, parasitic infection and hematology in *Colossoma macropomum* Cuvier, 1818 fed diets containing *Allium sativum*. *Journal of Applied Ichthyology* 32:901–905.
- Jørgensen, L. G. 2016. Infection and immunity against *Ichthyophthirius multifiliis* in Zebrafish (*Danio rerio*). *Fish and Shellfish Immunology* 57:335–339.
- Jørgensen, L. G. 2017. The fish parasite *Ichthyophthirius multifiliis*—host immunology, vaccines and novel treatments. *Fish and Shellfish Immunology* 67:586–595.
- Kim, S., J. Song, and K. Kim. 2012. Effects of dietary astaxanthin on innate immunity and disease resistance against *Edwardsiella tarda* in Olive Flounder *Paralichthys olivaceus*. *Israeli Journal of Aquaculture-Bamidgeh* 64:1–6.
- Lahnsteiner, F., and T. Weismann. 2007. Treatment of ichthyophthiriasis in Rainbow Trout and Common Carp with common and alternative therapeutics. *Journal of Aquatic Animal Health* 19:186–194.
- Liu, F., H. Shi, Q. Guo, Y. Yu, A. Wang, F. Lv, and W. Shen. 2016. Effects of astaxanthin and emodin on the growth, stress resistance and disease resistance of Yellow Catfish (*Pelteobagrus fulvidraco*). *Fish and Shellfish Immunology* 51:125–135.
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. *Methods* 25:402–408.
- Mahfouz, N. B., M. Fathi, M. Y. A. Zead, and S. Mesalhy. 2009. Effect of garlic and yeast in the culture of Nile Tilapia (*Oreochromis niloticus*). *Abbassa International Journal for Aquaculture Special Issue for Global Fisheries and Aquaculture Research Conference* 453–465.
- Matthews, R. A. 2005. *Ichthyophthirius multifiliis* Fouquet and ichthyophthiriasis in freshwater teleosts. Pages 159–241 in J. R. Baker, R. Muller, and D. Rollinson, editors. *Advances in parasitology*. Elsevier, Oxford, UK.
- Meinelt, T., S. Matzke, A. Stüber, M. Pietrock, A. Wienke, A. J. Mitchell, and D. L. Straus. 2009. Toxicity of peracetic acid (PAA) to tomonts of *Ichthyophthirius multifiliis*. *Diseases of Aquatic Organisms* 86:51–56.
- Mizuno, S., S. Urawa, M. Miyamoto, M. Hatakeyama, Y. Sasaki, N. Koide, S. Tada, and H. Ueda. 2018. Effects of dietary supplementation with oregano essential oil on prevention of the ectoparasitic protozoans *Ichthyobodo salmonis* and *Trichodina truttae* in juvenile Chum Salmon *Oncorhynchus keta*. *Journal of Fish Biology* 93:528–539.
- Moghaddam, M. R. M., H. Janmohammadi, and N. Sheikhzade. 2015. Comparison and investigation of the effects of natural carotenoids and dietary astaxanthin on carcass pigmentation, growth performance and serum lysozyme activity of Rainbow Trout (*Oncorhynchus mykiss*). *International Journal of Biosciences* 6:235–242.
- Montero, D., L. Robaina, M. J. Caballero, R. Ginés, and M. S. Izquierdo. 2005. Growth, feed utilization and flesh quality of European Sea Bass (*Dicentrarchus labrax*) fed diets containing vegetable oils: a time-course study on the effect of a re-feeding period with a 100% fish oil diet. *Aquaculture* 248:121–134.
- Nya, E. J., and B. Austin. 2009. Use of garlic, *Allium sativum*, to control *Aeromonas hydrophila* infection in Rainbow Trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* 32:963–970.
- Rach, J. J., M. P. Gaikowski, and R. T. Ramsay. 2000. Efficacy of hydrogen peroxide to control parasitic infestation on hatchery-reared fish. *Journal of Aquatic Animal Health* 12:267–273.

- Rintamäki-Kinnunen, P., M. Rahkonen, A. L. Mannermaa-Keränen, L. R. Suomalainen, H. Mykrä, and E. T. Valtonen. 2005. Treatment of ichthyophthiriasis after malachite green. I. Concrete tanks at salmonid farms. *Diseases of Aquatic Organisms* 64:69–76.
- Schmittgen, T. T., and K. J. Livak. 2008. Analyzing real-time PCR data by the comparative C_T method. *Nature Protocols* 3:1101–1108.
- Shakya, S. R., and S. N. Labh. 2014. Medicinal uses of garlic (*Allium sativum*) improves fish health and acts as an immunostimulant in aquaculture. *European Journal of Biotechnology and Bioscience* 2:44–47.
- Sigh, J., and K. Buchmann. 2001. Comparison of immobilization assays and enzyme-linked immunosorbent assays for detection of Rainbow Trout antibody-titres against *Ichthyophthirius multifiliis* Fouquet, 1876. *Journal of Fish Diseases* 24:49–51.
- Skov, J., J. K. Chettri, R. M. Jaafar, P. W. Kania, I. Dalsgaard, and K. Buchmann. 2018. Effects of soluble immunostimulants on mucosal immune responses in Rainbow Trout immersion-vaccinated against *Yersinia ruckeri*. *Aquaculture* 492:237–246.
- Straus, D. L., and T. Meinelt. 2009. Acute toxicity of peracetic acid (PAA) formulations to *Ichthyophthirius multifiliis* theronts. *Parasitology Research* 104:1237–1241.
- Thompson, I., G. Choubert, D. F. Houlihan, and C. J. Secombes. 1995. The effect of dietary vitamin A and astaxanthin on the immunocompetence of Rainbow Trout. *Aquaculture* 133:91–102.
- Xueqin, J., P. W. Kania, and K. Buchmann. 2012. Comparative effects of four feed types on white spot disease susceptibility and skin immune parameters in Rainbow Trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* 35:127–135.
- Yilmaz, S., S. Ergün, and N. Soytaş. 2013. Herbal supplements are useful for preventing streptococcal disease during first-feeding of tilapia fry, *Oreochromis mossambicus*. *Israeli Journal of Aquaculture-Bamidgeh* 65:1–5.
- Zheng, Z. L., J. Y. W. Tan, H. Y. Liu, X. H. Zhou, X. Xiang, and K. Y. Wang. 2009. Evaluation of oregano essential oil (*Origanum heracleoticum* L.) on growth, antioxidant effect and resistance against *Aeromonas hydrophila* in Channel Catfish (*Ictalurus punctatus*). *Aquaculture* 292:214–218.

SUPPORTING INFORMATION

Additional supplemental material may be found online in the Supporting Information section at the end of the article.