

Cytokine Gene Expression, Immune Responses and Disease Resistance of *Oncorhynchus mykiss* after *Raphanus sativus* By-products Supplementation

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Abstract

In the present study, we examined the effects of aqueous methanolic extract of radish seed (*Raphanus sativus*) by-products (RS) on innate immune responses and growth performance of rainbow trout (*Oncorhynchus mykiss*). The fish was fed diets containing 4 different doses of RS (0 % (Control), 0.1% (RS0.1), 0.5% (RS0.5) and 1% (RS1)) for 14 days. The results showed an increased activity of respiratory burst in fish of treatment groups compared to that of control on 14th day ($P<0.05$). An enhanced bacterial killing activity was observed in R.05 and RS1 treatment groups compared to control ($P<0.05$) on 7th day of the study. Lysozyme activity was elevated in fish of RS1 group on 7th day, and in all treatment groups on 14th day compared to that of the control. Myeloperoxidase activity increased significantly in RS1 and RS0.1 groups compared to the control on 7th day. IL-1b was up-regulated in head kidney of fish in RS0.1 group on 7th day and in RS0.5 group on 14th day of the study. In intestine of fish of experimental groups, IL-1b expression was significantly elevated on 7th day compared to the control. IL-12 was also up-regulated both in kidney and intestine of treatment group fish on 7th day. Similar results were observed on IL-10 expression. IL-8 was up-regulated both in kidney and intestine of treated fish groups. Growth performance was affected positively in the RS1 group compared to the control. However, FCR value did not vary among different groups. Survival also improved against *Aeromonas hydrophila* infection in RS administered fish. All these results suggest that supplementation of RS through diets for 7 days could improve immune responses and growth in rainbow trout.

Introduction

As a rapidly developed industry, aquaculture plays a significant role in the world economy. Thus, aquaculture contributes more than 53 percent to the world fisheries production with its USD 232 billion value (FAO, 2018). In recent years, improved new technologies (Recirculatory aquaculture systems, intensive fish farming units, oxygen supplementation etc.) contribute to this advancement of aquaculture

(Bilen et al., 2015). However, disease outbreaks, climate change risks, and unavailability of feed ingredients also seem new challenges in aquaculture (FAO, 2018).

In the last few decades, fish producers and researchers have focused on improving fish health using different types of immunostimulants (Elbesthi et al., 2020). For this purpose, medicinal herbs are also taken into consideration for their large species diversity and possessing many potential ingredients that have antibacterial, antiviral, and antifungal activities (Dulger

et al., 2005; Dulger et al., 2009). Also, medicinal plants are used as a reproductive promoter in some cases (Sonmez et al., 2019).

Radish (*Raphanus sativus*) belongs to the family Brassicaceae and has various medicinal properties. Chevalier (1996) reported laxative effect of radish in digestive system. It was also used traditionally for treatment of pulmonary complications and asthma (Duke and Ayensu, 1985). Radish has also very efficient antibacterial and anti-fungal activities even against fish pathogen, such as *Hafnia alvei* (Rani et al., 2008).

Rainbow trout is one of the most cultivated fish species in Europe. However, fish farms are often infected by pathogens, such as *Aeromonas* spp., *Flavobacterium* spp. (Capkin et al., 2015) and *Yersinia ruckeri* (Öztürk and Altınok, 2014). Use of medicinal plants as an alternative remedy to improve fish health to fight against pathogens is a new approach (Bilen and Elbeshti, 2019).

Cytokines are important protein mediators in immune system (Ravan and Sakai 2006). Epithelial cells, mast cells, lymphocytes, macrophages and granulocytes are the main sources of the cytokines. Cytokines regulate apoptosis, hematopoiesis, inflammation, anti-inflammation, proliferation and cell migration (Sakai et al., 2020).

Aeromonas hydrophila, is a Gram-negative rod-shaped bacterium, responsible from motile *Aeromonas* septicemia (Jun et al., 2013). The bacteria were previously observed in many different fish species and resulted with high losses (Zhu et al., 2020; Jun et al., 2013; Qin et al., 2016; da Silva et al., 2012).

Cold pressed oil production from medicinal plants and their seed is an important industry and these oils are well known for their beneficial health effects. However, after oil production, dregs are used for farm animal feeding or generally wasted. In the present study, using different extraction techniques we obtained several contents from the by-product of radish seed. Then, possible immunostimulant effects of the radish by-product aqueous methanolic compounds were assessed by examining immune responses in peripheral blood serum, and also in kidney and intestine tissues of rainbow trout. Treated fish was also challenged with the *Aeromonas hydrophila* at the end of the study.

Material and Methods

Experimental Fish and Experimental Design

Rainbow trout (Mean weight: 31.81 ± 0.04 g) juveniles were obtained from a commercial aquaculture farm in Kastamonu. Before the start of the experiment, the fish were acclimatized in a recirculatory tank system maintained at 16°C and fed with a commercial diet for two weeks in the Aquarium Unit at Kastamonu University, Faculty of Fisheries. Each treatment was provided with triplicate (40 fish per aquarium, total 12 aquariums, 110 L each) in recirculation system (8.2–9,3

mg/L dissolved oxygen, 8.1–8.4 pH, 0.02–0.05 mg/L ammonium, 0.03–0.05 mg/L nitrite.). During the study, the water temperature was maintained at 16°C, and 20% of the water was exchanged daily. *Raphanus sativus* aqueous methanolic extract was added to the fish diet at a rate of 0% (Control), 0.1% (RS0.1), 0.5% (RS0.5) and 1% (RS1). The fish were fed with these diets twice daily for 14 days.

Preparation of *Raphanus sativus* Extract

Radish seeds were ground into a fine powder in a mechanical grinder, and 50 g sample was added to 1 L of 40% methanol (Sigma-Aldrich). The mixture was then kept at room temperature for 3 days and stirred every day. After 3 days, *Raphanus sativus* extract was filtered through a filter paper (Whatman filter No. 1), and the filtrate was collected and evaporated in a rotary evaporator at 55–65°C to remove the methanol. The final product was dissolved in distilled water. Extract solution was added to experimental diets at specified concentrations. The methanolic extract of *Raphanus sativus* was diluted with 50 mL of distilled water and then, the *Raphanus sativus* was sprayed on the fish diet at concentrations of 0.1 (RS0.1), 0.5 (RS0.5), and 1 g kg⁻¹ (RS1) and stored in plastic zip pack at –20 °C until use (Bilen et al., 2016).

Sample Collection

On 7th and 14th day of the study, kidney, intestinal tissues and blood samples were collected from the animals of each experimental group and sampled separately. For this, three fish from each aquarium (total nine fish from each experimental group) were randomly selected, and were anesthetized with phenoxyethanol at 0.01 mL/L.

Nonspecific Immune Parameters

Respiratory Burst Activity

Respiratory burst activity of the phagocytes was carried out according to Siwicki et al. (1994). Briefly, heparinized blood sample (0.1 mL) was inserted to the bottom of a glass tube to investigate nitroblue tetrazolium (NBT) reduction activity. Then, 0.2% NBT was added and incubated for another 30 min at 25°C. One mL N, N-dimethylformamide was added to the mixture to stop activity and it was centrifuged at 3000x g for 10 min.

Bacterial Killing Activity

The bacterial killing activity of blood phagocytic cells was measured according to the methodology modified by Siwicki et al. (1994). Initially, 50 µL of blood was added to 50 µL culture medium that includes fetal bovine serum and antibiotic mix in a microtiter plate.

The mixture was incubated at room temperature for 1 h in order to ensure the cells adhered to the plastic surfaces. Then the supernatant and non-adherent cells were removed using a pipette. Fifty μL NBT in PBS solution containing live 1×10^8 *Aeromonas hydrophila* cells was added. This suspension was centrifuged at $1500 \times g$ for 5 min to bring the bacteria into contact with the adherent cells and incubated at room temperature for 30–60 min. Then, the supernatant was pipetted and 200 μL absolute methanol was added to fix cells. After incubation for 2–5 min, the supernatant was thrown out. The cells were rinsed four times with 70% methanol and then the microplate was dried in fume cupboard for 40 min to move away methanol. After that, 100 μL of 2M KOH and 70 μL of dimethyl sulfoxide (DMSO) were added to solubilize the formazan. This mixture was incubated for 5 min, and the plates were placed in the microplate reader set for readings at 620 nm; the KOH-DMSO combination was used as the blank.

Lysozyme Activity Test

Lysozyme activity (LA) was determined according to Ellis (1990) using the turbidimetric assay with minor modifications. In brief, 160 mL suspension of *Micrococcus lysodeikticus* (Sigma-Aldrich) was mixed with 40 mL of fish serum. The rate of lysis was detected against *M. lysodeikticus*. Mixture was measured after 0 and 4 min at 530 nm wavelength by a microplate reader (Thermo Multiskan Go).

Myeloperoxidase Activity Test

Total myeloperoxidase (MPO) content was measured by the method described by Sahoo et al. (2005). In a 96-well plate, 30 μL serum was diluted with 370 μL of Hank's Balanced Salt solution without Ca^{2+} or Mg^{2+} (Sigma Aldrich, Germany). Thousand μL of 0.1 mg mL^{-1} 3, 3', 5, 5'-tetramethylbenzidine dihydrochloride and 0.006% fresh hydrogen peroxide were added to the diluted serum. The reaction was followed kinetically by measuring the increase in absorbance ratios. Reaction velocities were determined as IU, defined as the amount of enzyme required to produce a 0.001 increase in absorbance per minute for 0.5 mL of reaction mixture ($\Delta A 450/\text{min}/\text{mL}$).

Total Protein and Total IgM Content

Total protein was measured by Bradford assay using bovine serum albumin (BSA) as the standard. Total IgM was determined according to Anderson and Siwicki (1995). Briefly, 100 μL of serum was mixed with an equal volume of 12% polyethylene glycol (Sigma P3015). After 2 h incubation, the sample was centrifuged for 10 min at 5000 rpm. Total IgM content was calculated by subtracting the protein content of supernatant from the total protein content in plasma.

Anti-protease Activity

Serum anti-trypsin activity was measured according to Lange et al. (2001). Twenty μL of trypsin (Sigma-Aldrich, 5 mg/ml) was incubated with same amount of serum for 10 min at 22°C. After that, 200 μL of 0.1 M PBS and 250 μL of 2% azocasein solution were mixed. After 1 h at 22 °C, 10% trichloro acetic acid (TCA) was added to stop the reaction. After 20 min of incubation, the mixture was centrifuged at $6000 \times g$ for 5 min. The supernatant was transferred to a 96-well plate containing 100 μL of 1 N NaOH. The absorbance was read at 410 nm. Positive control (100%) was prepared by replacing the serum with buffer. For negative control, buffer replaced both serum and trypsin. The percentage inhibition of trypsin activity was calculated by comparing it with a positive control sample.

Serum Alternative Complement (ACH50) Activity

The hemolytic complement activity was determined according to Ortuno et al. (1998) with a slight modification. Hundred μL of serum sample was serially diluted up to 11th well with an equal volume of HBSS in a 96-well plate. Hundred μL of 3% SRBC in HBSS was added to all the wells. Then, 100 μL distilled water or HBSS was added to SRBC to calculate 100% and 0% hemolysis, respectively and the plate was incubated at 22°C for 1 h. After that, samples were centrifuged at 1500 rpm for 4°C. One hundred fifty μL of supernatant was transferred to a fresh plate and the hemoglobin content of the supernatant was assessed by measuring its OD at 540 nm. The lysis curve was generated by plotting percent hemolysis against the volume of test serum added (ml). The volume yielding 50% hemolysis (ACH50 units/ ml) was determined for each group.

Analysis of Cytokine Gene Expression

RNA Extraction

Approximately 30 mg of kidney and intestine samples were taken from each fish and stored in RNAlater solution for RNA extraction. From the total RNA extracted, cDNA synthesis was achieved via reverse transcription using a BIOLINE kit (ISOLATE II RNA Mini Kit) according to the manufacturer's protocol. The quantity and quality of all RNA samples were checked using a Multiskan GO spectrophotometer (ThermoFischer Scientific, USA).

cDNA Synthesis

The extracted RNAs were subjected to treatment with 1 U DNase I (BIOLINE) in order to completely remove genomic DNA. The extracted total RNA was used for cDNA synthesis via reverse transcription from 1 μg mRNA using a BIOLINE kit (SensiFAST™ cDNA Synthesis

Kit). The cDNA reaction mixture contained 1 µg of template RNA, 15 pmol/µL oligo dT primer, 4 µL 5× TransAmp Buffer, 1 µL of Reverse Transcriptase, oligo dT primer and was made up to 20 µL with nuclease free water (NFW). The reaction mixture was incubated for 10 min at 25°C for primer annealing, 15 min at 42°C for reverse transcription and 5 min at 85°C for inactivation of reaction in a thermal cycler (ThermoFischer Scientific).

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) Analyses of Gene Expression

After cDNA synthesis, qRT-PCR was performed using CFX Real-Time PCR Detection Systems (Bio-Rad, ABD) and SensiFAST SYBR No-ROX PCR Kit (BIOLINE, ABD). Gene specific primer sequences and references are enlisted in Table 1. qRT-PCR mixture included 12.5 µL of 2× SYBR Green Master Mix, 0.1 µg of template DNA, 0.4 µM of each gene specific forward and reverse primer (IL-1β, IL-6, IL-8, IL-10 and IL-12) and NFW to the final volume of 20 µL. qRT-PCR steps involved were: denaturation at 95°C for 20 s, annealing at 60°C for 1 min and extension step at 72°C for 30 s. Fluorescence signals were picked up at 530 nm wavelength from 60°C to 95°C at every 0.5°C per second to implement melting curve analysis. qRT-PCR was achieved with 3 different samples from experimental and control groups, and 3 technical replicates were evaluated for each sample. The ΔCT and $\Delta\Delta CT$ were estimated by $\Delta CT = CT_{\text{target}} - CT_{\text{reference}}$ and $\Delta\Delta CT = \Delta CT_{\text{treated sample}} - \Delta CT_{\text{control sample}}$. The results were measured using $2^{-\Delta\Delta CT}$ method to calculate relative gene expression. The standard error of means between replicates was computed simultaneously.

Gas Chromatography–mass Spectrometry (GC-MS) Analysis of Radish Seed By-Product (*Raphanus sativus*)

The GC-MS analysis of the samples extorted after aqueous methanolic extracts was determined using a Shimadzu Mass Spectrometer- QP 2010 Ultra equipped with Rtx-5MS capillary column (30 m·0.25 mm; coating thickness 0.25 µm). The compounds were identified by comparing with the standards, or the mass spectra were matched with the Wiley Data Library (Table 2).

Challenge Test

At the end of feeding trial, fish were challenged with *Aeromonas hydrophila* (SBAh1) (Bilen et al., 2019b). The strain was previously isolated from diseased fish and identified by biochemical and PCR methods. Challenge test was performed as described in our previous study (Bilen et al., 2016). Briefly, *A. hydrophila* with 1×10^7 CFUs mL⁻¹ mixed in 100 mL PBS was injected to all fish intra-peritoneally at the end of the dietary feeding trial (after 14 days). Dead *O. mykiss* was removed from the tank and mortality was recorded daily for 10 days. *A. hydrophila* was re-isolated from the dead fish to confirm the mortality caused by the bacterial infection. After 10 days post injection, survival rate of groups was calculated using this formulae: SR (%) = (number of fish survived/number of fish injected) × 100.

Statistical Analysis

One-way analysis of variance (ANOVA) and Duncan's Multiple Range Test were used to determine any difference in non-specific immune parameters, cytokine gene expressions and survival rate at P<0.05.

Table 1. Primers used in qRT-PCR for analysis of immune gene expressions in the study.

Gene	Primer Sequence	Reference
B-actin	F5'ATGGAAGGTGAAATCGCC 3' R5' TGCCAGATCTTCTCCATG 3'	Sigh at al. 2004
IL-1β	F5'ACCGAGTTCAAGGACAAGGA 3' R5' CATTTCATCAGGACCCAGCAC 3'	Awad at al. 2011
IL-8	F5'CACAGACAGAGAAGGAAGGAAAG 3' R5'TGTCATCTTGGGGTTACAGA 3'	Awad at al. 2011
IL-6	F5'CAAGGATGCTTGCTACGAAGG 3' R5'GCTCTTTGATCAGGATGTTGCT 3'	Awad at al. 2011
IL-10	F5' CGACTTTAAATCTCCCATCGAC 3' R5'GCATTGGACGATCTCTTTCTTC 3'	Raida and Buchmann 2008
IL-12	F5' GAACCCAGACGACGATGATT 3' R5' GTTCAAACCTCCAACCCTCCA 3'	Komatsu et. al. 2009
IFN-1	F5'CAAGAAGTGGGCATGTGATCTGT 3' R5' AGAATGCCCCAGTCTTTTCC3'	Ooi at al 2008
IFN-2	F5'GTTGAGGGCCATGGATGTG 3' R5' TCCAGCCCATCAAGCAGAA3'	Ooi at al 2008
TGF-β	F5' AGATAAATCGGAGAGTTGCTGTG 3' R5'CCTGCTCCACCTTGTGTTGT 3'	Awad at al 2011
TNF-α	F5' CAAGAGTTTGAACCTTGTTCAA 3' R5' GCTGCTGCCGCACATAGAC 3'	Panigrahi at al 2007

Homogeneity of variances was determined using Kolmogorov-Smirnov test and Levene's test.

Results

In the present study, at the end of 14-day trial adaptive immune responses were presented in Table 3.

The results showed no differences in NBT reduction at 7th day of the study ($P > 0.05$). However, on 14th day of the study, in all treatment groups, respiratory burst was significantly increased compared to that of control. Bacterial killing activity was elevated in RS0.5 and RS1 groups compared to RS0.1 and control at 7th day of the study. The highest activity was observed in RS1 on 14th day compared to other treatments ($P < 0.05$). LA was enhanced in RS0.5 and RS1 ($P < 0.05$) and no differences were observed between RS0.1 and control ($P > 0.05$) on 7th day of the study. In all treatment groups, LA was significantly increased at 14th day of the study. MPO was elevated in RS0.5 and RS1 groups compared to RS0.1 and control ($P < 0.05$) at 7th day of the study. At the end

of the study, interestingly MPO activity in all treatments as well as in control was not changed. Total protein was significantly increased in RS0.1 group compared to other on 7th day and in all experimental groups compared to control on 14th day of the study. IgM levels were significantly increased on both the sampling times in all treatment groups compared to that of control. ACH50 was also significantly increased in all treated fish groups compared to control and the highest level was observed in RS1 fish group ($P < 0.05$). An elevated anti-protease activity in treatment groups RS0.1 and RS1 was determined at both sampling times compared to control ($P < 0.05$). No significant difference was observed between control and RS0.5 group.

The results of innate immune gene expressions were summarized in Figures. 1, 2, 3, 4, 5, 6, 7, 8, and 9.

IL-1 β expression was up-regulated in kidney and intestine in all experimental groups on 7th and 14th day of the study ($P < 0.05$).

IL-12 expression result was provided in Figure 2.

Table 2. Composition of aqueous methanolic extract of radish seed (*Raphanus sativus*) by-products determined using GC-MS

Peak#	Area%	Name
1	0.39	Ethanedioic Acid
2	2.38	Acetic acid (CAS)
3	0.96	2-Propanone, 1-hydroxy-
4	0.16	1-Penten-3-one (CAS)
5	0.25	2-Propenoic acid, methyl ester (CAS)
6	0.56	Propanoic acid, 2-oxo-, methyl ester (CAS)
7	2.73	1,1-Diethoxypropanal
8	0.12	Ethyl orthoformate
9	0.38	Formamide, N-methoxy-
10	0.74	Formamide, N-methoxy-
11	1.00	Formamide, N-methoxy-
12	0.22	2-Furanmethanol (CAS)
13	0.39	1,2-Cyclopentanedione
14	0.35	Dimethyl trisulfide
15	0.58	2(5H)-Thiophenone, 5-methyl-
16	0.16	Benzyl alcohol
17	0.65	Cyclopentane, (methylthio)-
18	0.22	Epithiovaleronitrile
19	0.31	1,2,3-Propanetriol (CAS)
20	0.14	Benzaldehyde, 3,4-dimethyl-
21	0.29	4-Methoxymethylphenol
22	0.25	Guaiacol <4-vinyl->
23	0.27	Vanillin
24	0.79	3',5'-Dimethoxyacetophenone
25	0.14	Diethyl Phthalate
26	13.32	Benzaldehyde, 4-hydroxy-3,5-dimethoxy-
27	13.48	.alpha.-D-Glucopyranoside, methyl
28	3.41	N-F ormyl-dl-valine
29	11.85	Tetradecanoic acid
30	30.97	3H-1,2,4-Triazole-3-thione, 1,2-dihydro-4-methyl- (CAS)
31	0.12	5-ISOPROPYLTETRAHYDROTHIOPHEN-3-ONE
32	0.37	Palmitic acid
33	1.83	n-Hexadecanoic acid
34	8.62	Oleic Acid
35	1.59	Octadecanoic acid
	100.00	

Highlighted ones are the most abundant contents in the extract (%).

The result demonstrated that IL-12 gene was up-regulated almost 30 folds in kidney of fish in all treatment groups compared to control on 7th day of the study ($P<0.05$). Similar to that, an increased level of expression was also observed in intestine (almost 30 folds) on 14th day of the study.

Similar to kidney results, in intestine, an increased IL-12 expression was observed in fish of all treatment groups ($P>0.05$) on both sampling time. IL-10 gene expression pattern was similar to that of IL-12 (Figure 3).

Results of IL-6 gene expression were depicted in Figure 4. Results were showed significant increase on both experimental time and both in the kidney and the intestine. IL-8 gene expression level increased in kidney and intestine of fish of treatment groups ($P<0.05$) on 7th and 14th day. In intestine, IL-8 gene expression was up-regulated on both 7th and 14th day of the study in all experimental groups.

Results of IFN-1 and IFN-2 gene expressions were summarized in Figure 6. and 7.

Expression of both of the genes was significantly increased on 7th and 14th day of the study in all treated fish groups compared to control. The highest level of IFN-1 gene expression was observed both in kidney and the intestine on 7th day of the study in RS1 fish group ($P<0.05$).

Results of TGF- β gene expression were provided in Figure 8. In kidney, there was no significant difference in fish of the treatment groups compared to that of the control. However, in intestine, TGF- β gene expression was significantly elevated in fish of all treated groups compared to the control on 7th and 14th day of the study.

In Figure 9, TNF- α gene expression was presented. Similar to IL-10 results, an increased level of expression was observed in head kidney and intestine at both

sampling times ($P<0.05$) in all experimental groups compared to that of control.

Growth performance results were presented in Table 4.

At the end of the study, final weight of fish in RS1 group was significantly higher. Similar to this, the highest SGR was also determined in RS1 treatment. In all treatment groups, FCR values were decreased but were not significantly lower compared to that of control ($P>0.05$).

Survival of rainbow trout after challenged with *A. hydrophila* was presented in Figure 10. The result demonstrated that survival rate was significantly higher in all experimental groups compared to control ($P<0.05$). Interestingly, the highest survival rate was observed in RS0.1 treatment group ($P<0.05$).

Discussion

Over the last few decades, potential effects of medicinal herbs on immune responses, protection to pathogens and growth promotion have been demonstrated in fish (Bilen et al., 2016; Bilen and Elbeshti, 2019; Bilen et al., 2019d). Besides, antimicrobial resistance especially against fish pathogens forced aquaculturists to find alternative and natural sources. In the present study, positive effects of radish have been observed on fish immune system and growth promotion, even though it was used for a short span. Protection against one of the most important fish pathogens, *A. hydrophila* was also significantly elevated.

Respiratory burst activity increased during last sampling time in the radish extract treated fish groups but it was not significantly higher. Similarly, in earlier studies, an enhanced respiratory burst activity was observed after *Melissa officinalis* (Bilen et al., 2019a)

Table 3. Non-specific immune responses of rainbow trout fed with different doses of radish seed (*Raphanus sativus*) by-products aqueous methanolic extract.

		Control	RS0.1	RS0.5	RS1
Respiratory Burst (mg/ml)	7 th Day	0.68±0.01	0.76±0.02	0.82±0.01	0.87±0.19
	14 th Day	0.70±0.03 ^b	1.19±0.24 ^a	1.09±0.06 ^a	1.19±0.04 ^a
Bacterial Killing Activity	7 th Day	0.66±0.01 ^b	0.63±0.03 ^b	1.25±0.01 ^a	1.57±0.05 ^a
	14 th Day	1.08±0.06 ^{ab}	1.07±0.06 ^{ab}	0.91±0.04 ^b	1.37±0.15 ^a
Lysozyme Activity (U/ml)	7 th Day	1.14±0.11 ^c	1.06±0.04 ^c	2.40±0.63 ^{ab}	3.75±0.76 ^a
	14 th Day	0.66±0.13 ^b	2.10±0.09 ^a	2.01±0.29 ^a	2.40±0.29 ^a
Myeloperoxidase Activity (540 nm)	7 th Day	40.37±7.06 ^c	80.78±15.11 ^c	310.31±58.86 ^b	831.77±69.32 ^a
	14 th Day	194.98±10.51	297.16±52.83	274.85±70.50	333.33±23.37
Total Protein (g/dl)	7 th Day	5.71±0.85 ^b	6.05±0.41 ^a	5.81±0.14 ^b	5.83±0.35 ^b
	14 th Day	5.75±1.02 ^b	5.98±0.21 ^a	5.83±0.51 ^a	5.91±0.27 ^a
Total IgM (g/dl)	7 th Day	2.08±0.02 ^b	2.75±0.1 ^a	2.85±0.024 ^a	2.97±0.09 ^a
	14 th Day	2.01±0.012 ^b	2.70±0.012 ^a	2.81±0.014 ^a	2.86±0.024 ^a
ACH50 Activity (U/ml)	7 th Day	110±1.26 ^c	126±2.65 ^b	124±2.14 ^b	141±1.47 ^a
	14 th Day	115±2.36 ^c	125±3.84 ^b	121±1.95 ^b	138±2.05 ^a
Antiprotease Activity (%)	7 th Day	72.35±2.65 ^{bc}	81.25±1.92 ^a	76.35±2.01 ^b	79.35±1.47 ^a
	14 th Day	74.25±3.02 ^b	82.45±3.54 ^a	76.38±1.74 ^b	81.25±1.45 ^a

RS0.1, RS0.5 and RS1, radish seed (*Raphanus sativus*) by-products aqueous methanolic extract at 0.1, 0.5 and 1% diet, respectively. Values are expressed as mean \pm SE. Different superscript letters on values indicate significant differences between groups in a row ($P<0.05$).

and *Centella asiatica* (Srichaiyo et al., 2020) administration. This study exhibited an increase in bacterial killing activity in RS1 group. Similar to our study, seabass and seabream had higher protection against bacteria after common mallow and tetra extract administration (Bilen et al., 2019c).

Myeloperoxidase is also an important enzyme released from neutrophils (Siwicki et al 1994). In the present study, increasing MPO activity was observed in fish of RS1 and RS0.1 groups. Similarly, an increased MPO activity was noticed in rainbow trout fed with *Malva sylvestris* (Bilen et al., 2020), goldfish fed with *Urtica dioica* (Bilen et al., 2014) and Nile tilapia fed with *Camellia sinensis* (Van Doan et al., 2019). However, no effect on MPO activity was observed in carp fed with different doses of *Tilia tomentosa* (Almabrok et al., 2018).

Lysozyme is a lytic enzyme (Magnadóttir, 2006) that prevents colonization of the bacterial pathogens by lysing bacterial cell walls (Alexander and Ingram, 1992). Lysozyme activity was increased in RS0.1 and RS1 fish groups during both the sampling times. Different components of the RS could be responsible for increasing lysozyme activity. Similarity to our result, *Apium graveolens* fed carp displayed increasing lysozyme activity (Mohamed et al., 2018). In zebrafish, an elevated lysozyme activity was obtained after administration of apple cider vinegar (Ahmadifar et al., 2019). On the contrary, no effect was observed on carp lysozyme activity after dietary administration of *Chenopodium album* (Amhamed et al., 2018).

Total protein and Total IgM were significantly increased in the present study in RS fish groups. Elevated total protein and IgM results directly indicate an enhanced immune response in rainbow trout. Similar to that, elevated total protein was determined on rainbow trout fed with *Aloe vera* (Mehrabi et al., 2019). ACH50 is an important innate immune component and many different proteins assigned on immune responses in complement system (Pearce et al., 2003). In the present study, an increased ACH50 activity was determined in all treated fish groups as it has been observed on total protein and IgM levels. Similarly, an elevated activity was also observed in rainbow trout fed with barberry root (Ramezanzadeh et al., 2020). Anti-protease also increased in RS0.1 and RS1 fish groups. Probably, the increased anti-protease activity also

regulated the immune response against *A. hydrophila* infection. In line with our study, an elevated anti-protease activity was also reported by several authors (Awad et al., 2013; Awad et al., 2020; Maldonado-Garcia et al., 2019).

IL-1 β is one of the key pro-inflammatory cytokines (Zou and Secombes 2016). It activates immune cells against pathogens in fish. The present study demonstrated its up-regulation in both kidney and intestine of treated fish at different sampling times. An increased IL-1 β gene expression in rainbow trout leucocytes was detected after exposure to cyclopamine (Sönmez et al., 2018). IL-1 β gene expression was also elevated in *Cyprinus carpio* fed with raffinose (Karimi et al., 2020) and ferula (Safari et al., 2016), and in rainbow trout treated with *Usnea barbata* (Bilen et al., 2019d) and tetra (Bilen and Elbeshti, 2019).

IL-12 is a heterodimeric cytokine that regulates IFN- γ production in natural killer cells, B and T cells (Wang et al., 2014). Also, IL-12 activates Th1 and Th2 based immune response (Wangkahart et al., 2019). An elevated transcription of this gene was observed in intestine and kidney of fish in RS treatments. This result may suggest that neutrophils and T cells were stimulated after RS treatment. The enhanced IL-12 transcription could cause high survival in RS treated rainbow trout against *A. hydrophila* infection. Similarly, Juniperus extract has caused IL-12 gene up-regulation in rainbow trout (Bilen et al.).

IL-8 chemokine is also involved in an early inflammatory reaction since it has a chemo-attractive effect on neutrophils in trout (Zhang et al., 2002). During infection, it is released by different immune cells (Tran et al., 2019). It was clearly demonstrated that IL-8 initiates inflammatory reaction in fish against bacterial infection (Wiens et al., 2006). In the present study, IL-8 gene expression was up-regulated in intestine and kidney during both sampling times in RS fish groups. Likewise, Bilen et al. (2019d) detected an increase on IL-8 transcription in rainbow trout treated with *Usnea barbata*. Contrary to our study, Altunoglu et al. (2017) demonstrated a down-regulation in IL-8 gene expression in rainbow trout administered with black cumin aqueous methanolic extract.

IL-10 is an anti-inflammatory cytokine that may cause down-regulation of inflammation (Raida and Buchmann, 2008; Tran et al., 2019). At the all sampling

Table 4. Growth performance of rainbow trout fed with different doses of radish seed (*Raphanus sativus*) by-products aqueous methanolic extract.

	Initial Weight (g)	Final Weight (g)	WG (%)	SGR	FCR
Control	31.88±0.08	42±0.19 ^b	31.74±0.65 ^b	1.97±0.04 ^b	1.16±0.05
RS0.1	31.78±0.04	42.7±0.69 ^{ab}	34.35±1.00 ^{ab}	2.11±0.05 ^{ab}	1.05±0.01
RS0.5	31.80±0.04	43.61±1.32 ^{ab}	37.15±2.40 ^{ab}	2.25±0.12 ^{ab}	1.12±0.03
RS1	31.78±0.03	44.73±0.24 ^a	40.66±1.38 ^a	2.44±0.07 ^a	1.09±0.01

RS0.1, RS0.5 and RS1, radish seed (*Raphanus sativus*) by-products aqueous methanolic extract at 0.1, 0.5 and 1 % diet, respectively. Values are expressed as mean \pm SE. Different superscript letters on values indicate significant differences between groups in a column (P<0.05).

time, IL-10 gene expression was up-regulated in RS fish groups both of the intestine and kidney. Depending on the IL-1 β up-regulation, up-regulated IL-10 could normalize anti-inflammatory immune response. Similar to this result, Gharaei et al. (2020) reported an increase in IL-10 gene expression in rainbow trout fed with sumac. IL-6 is a pro-inflammatory cytokine that activates

inflammatory cells and induces acute phase protein synthesis (Dong, 2008). IL-6 gene expression increased clearly in kidney and intestine of RS fish groups. The increased IL-6 transcription caused a pro-inflammatory response. Increased IL-6 gene expression was also observed in rainbow trout fed with *Aloe vera* extract (Mehrabi et al., 2019).

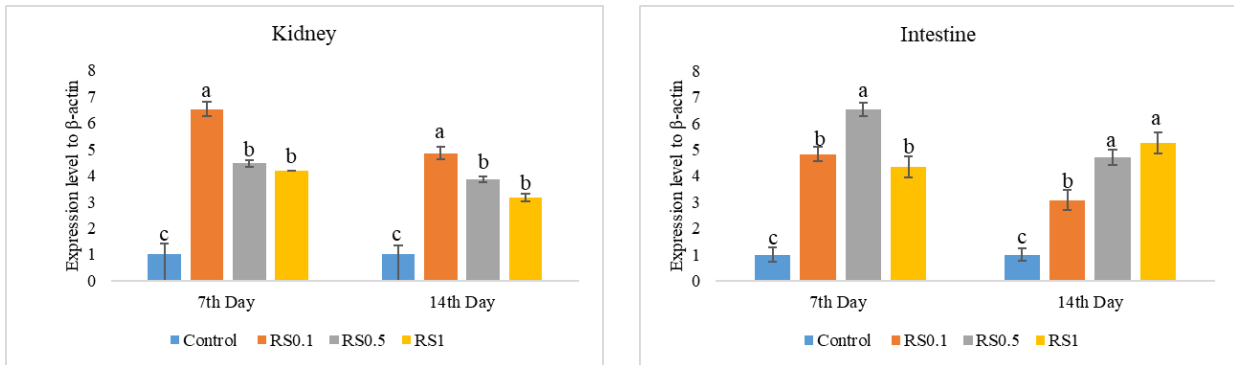


Figure 1. Relative gene expression of IL-1 β in the head kidney and intestine cells of rainbow trout treated with different doses of radish seed (*Raphanus sativus*) by-products aqueous methanolic extract. RS0.1, RS0.5 and RS1, radish seed (*Raphanus sativus*) by-products aqueous methanolic extract at 0.1 0.5 and 1% diet, respectively. Values are expressed as mean \pm SE. Different letters above bars indicate significant differences between groups at each sampling day (P<0.05).

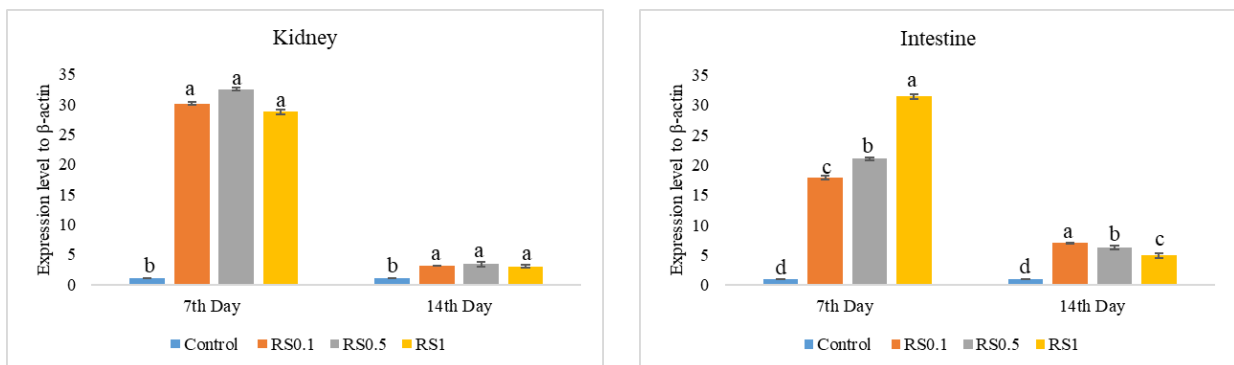


Figure 2. Relative gene expression of IL-12 in the head kidney and intestine cells of rainbow trout treated with different doses of radish seed (*Raphanus sativus*) by-products aqueous methanolic extract. RS0.1, RS0.5 and RS1, radish seed (*Raphanus sativus*) by-products aqueous methanolic extract at 0.1 0.5 and 1% diet, respectively. Values are expressed as mean \pm SE. Different letters above bars indicate significant differences between groups at each sampling day (P<0.05).

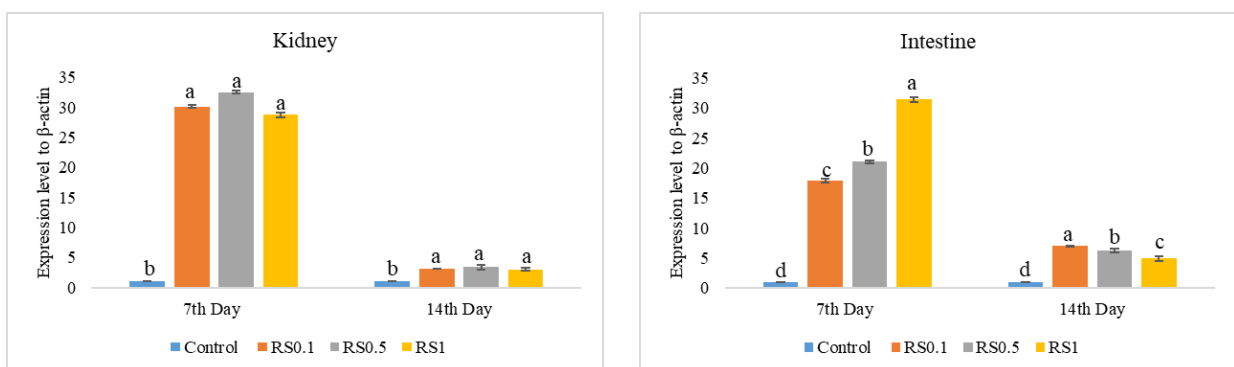


Figure 3. Relative gene expression of IL-10 in the head kidney and intestine cells of rainbow trout treated with different doses of radish seed (*Raphanus sativus*) by-products aqueous methanolic extract. RS0.1, RS0.5 and RS1, radish seed (*Raphanus sativus*) by-products aqueous methanolic extract at 0.1 0.5 and 1% diet, respectively. Values are expressed as mean \pm SE. Different letters above bars indicate significant differences between groups at each sampling day (P<0.05).

Interferons are generally responsible for defending viruses (Ooi et al., 2008). Both IFN-1 and IFN-2 genes were up-regulated in kidney and intestine of the treated fish. Kucukgul and Gulsafak (2019) also observed an up-regulation of IFN gene in kidney of rainbow trout after carvacrol supplementation. TGF- β was identified from several fish species, and it possesses an immunosuppressive activity (Zou and Secombes, 2016).

TGF- β could prevent the nitric oxide response of TNF- α activated macrophages, and also inhibited the LPS induced elevation of TNF- α , IL-1 β and IL-8 in monocytes/macrophages (Haddad et al., 2008). TGF- β gene expression did not show any difference in kidney at both sampling times. However, in intestine, an elevated expression level was observed (up to 14 folds). It is clear from the result that TGF regulation is stronger

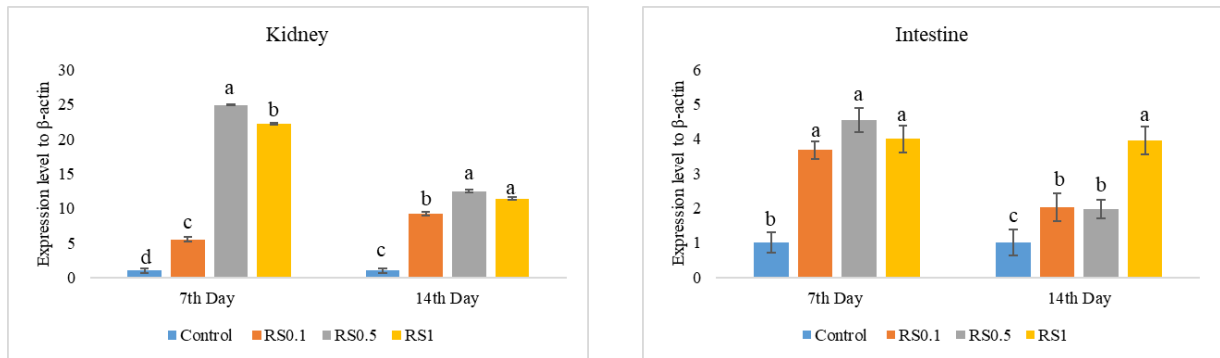


Figure 4. Relative gene expression of IL-6 in the head kidney and intestine cells of rainbow trout treated with different doses of radish seed (*Raphanus sativus*) by-products aqueous methanolic extract. RS0.1, RS0.5 and RS1, radish seed (*Raphanus sativus*) by-products aqueous methanolic extract at 0.1 0.5 and 1% diet, respectively. Values are expressed as mean \pm SE. Different letters above bars indicate significant differences between groups at each sampling day (P<0.05).

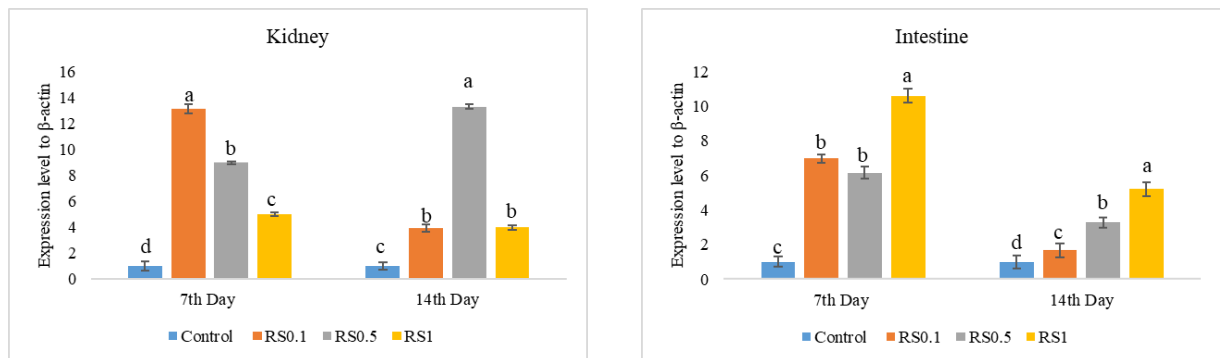


Figure 5. Relative gene expression of IL-8 in the head kidney and intestine cells of rainbow trout treated with different doses of radish seed (*Raphanus sativus*) by-products aqueous methanolic extract. RS0.1, RS0.5 and RS1, radish seed (*Raphanus sativus*) by-products aqueous methanolic extract at 0.1 0.5 and 1% diet, respectively. Values are expressed as mean \pm SE. Different letters above bars indicate significant differences between groups at each sampling day (P<0.05).

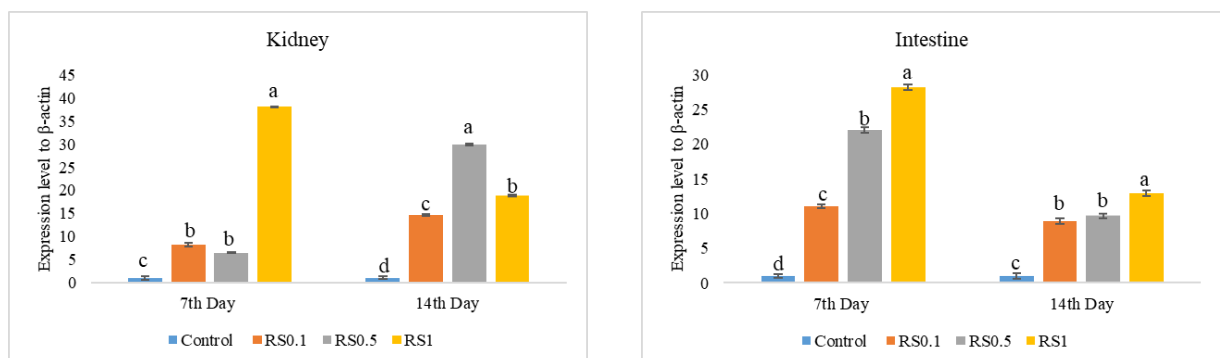


Figure 6. Relative gene expression of IFN-1 in the head kidney and intestine cells of rainbow trout treated with different doses of radish seed (*Raphanus sativus*) by-products aqueous methanolic extract. RS0.1, RS0.5 and RS1, radish seed (*Raphanus sativus*) by-products aqueous methanolic extract at 0.1 0.5 and 1% diet, respectively. Values are expressed as mean \pm SE. Different letters above bars indicate significant differences between groups at each sampling day (P<0.05).

in the intestine. RS could decrease or regulate the inflammation in intestine. TNF- α is a pro-inflammatory cytokine and has important role on inflammation, defense against bacteria, cell apoptosis and differentiation (Ware, 2003). TNF is secreted in the kidney mainly. The results showed an elevation in both kidney and intestine of the RS fish groups. Similar to that, an increased TNF- α gene expression was observed

in rainbow trout after *Mentha longifolia* application (Heydari et al., 2020).

To determine growth performance, both SGR and FCR are important variables. It is not important to obtain or elevate any positive growth performance in fish fed with a medicinal herb which is being tested for immunostimulant properties. However, in this type of study, we generally focus on assessing any side effects

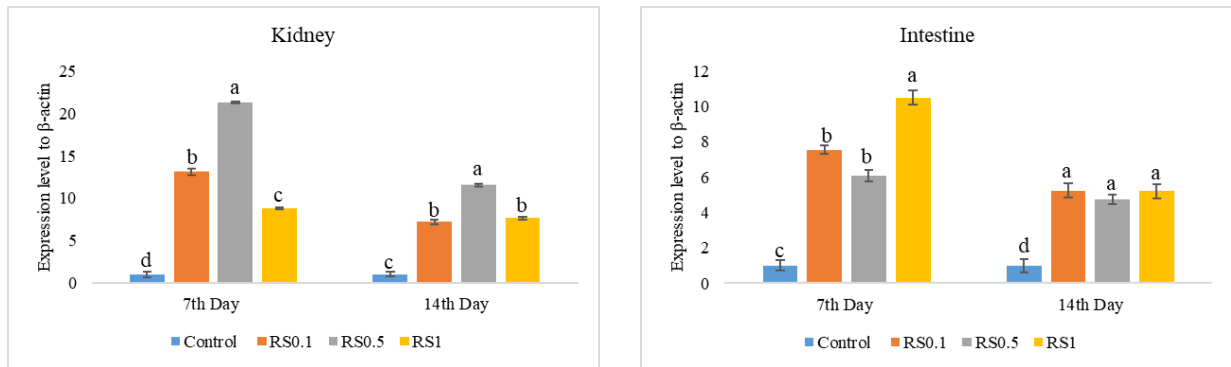


Figure 7. Relative gene expression of IFN-2 in the head kidney and intestine cells of rainbow trout treated with different doses of radish seed (*Raphanus sativus*) by-products aqueous methanolic extract. RS0.1, RS0.5 and RS1, radish seed (*Raphanus sativus*) by-products aqueous methanolic extract at 0.1 0.5 and 1% diet, respectively. Values are expressed as mean \pm SE. Different letters above bars indicate significant differences between groups at each sampling day ($P < 0.05$).

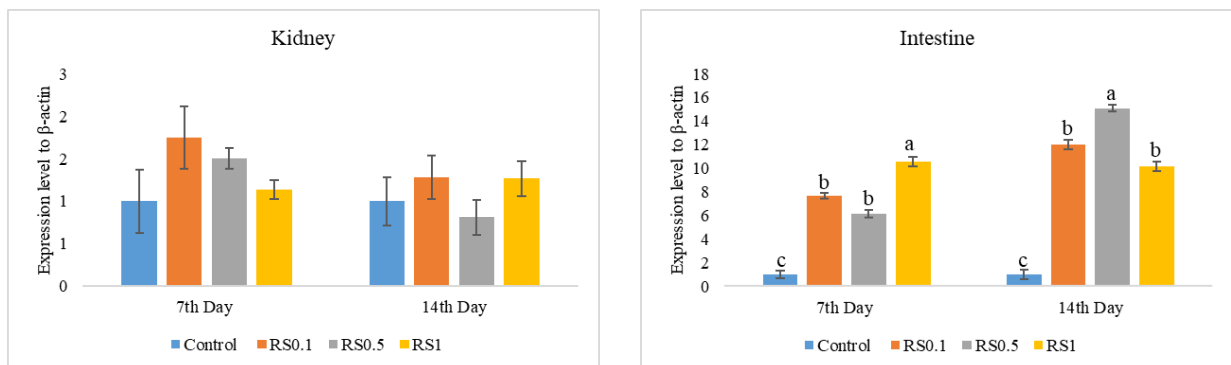


Figure 8. Relative gene expression of TGF- β in the head kidney and intestine cells of rainbow trout treated with different doses of radish seed (*Raphanus sativus*) by-products aqueous methanolic extract. RS0.1, RS0.5 and RS1, radish seed (*Raphanus sativus*) by-products aqueous methanolic extract at 0.1 0.5 and 1% diet, respectively. Values are expressed as mean \pm SE. Different letters above bars indicate significant differences between groups at each sampling day ($P < 0.05$).

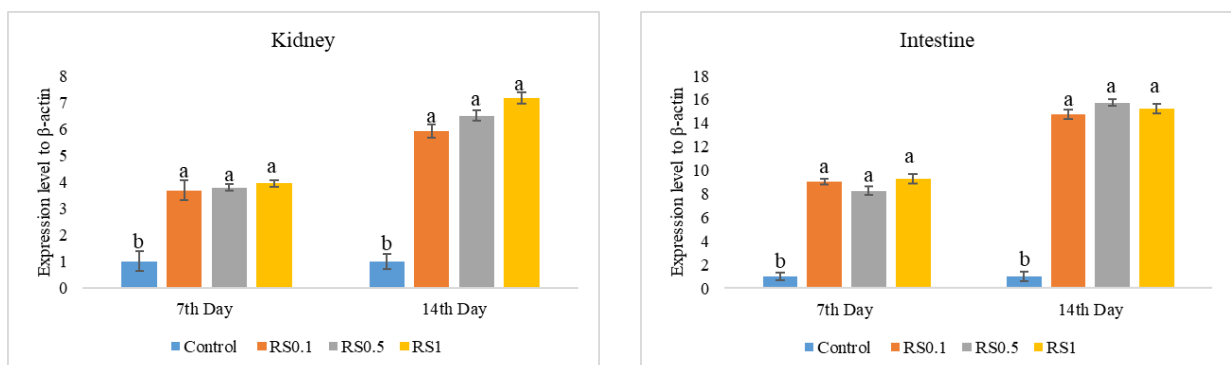


Figure 9. Relative gene expression of TNF- α in the head kidney and intestine cells of rainbow trout treated with different doses of radish seed (*Raphanus sativus*) by-products aqueous methanolic extract. RS0.1, RS0.5 and RS1, radish seed (*Raphanus sativus*) by-products aqueous methanolic extract at 0.1 0.5 and 1% diet, respectively. Values are expressed as mean \pm SE. Different letters above bars indicate significant differences between groups at each sampling day ($P < 0.05$).

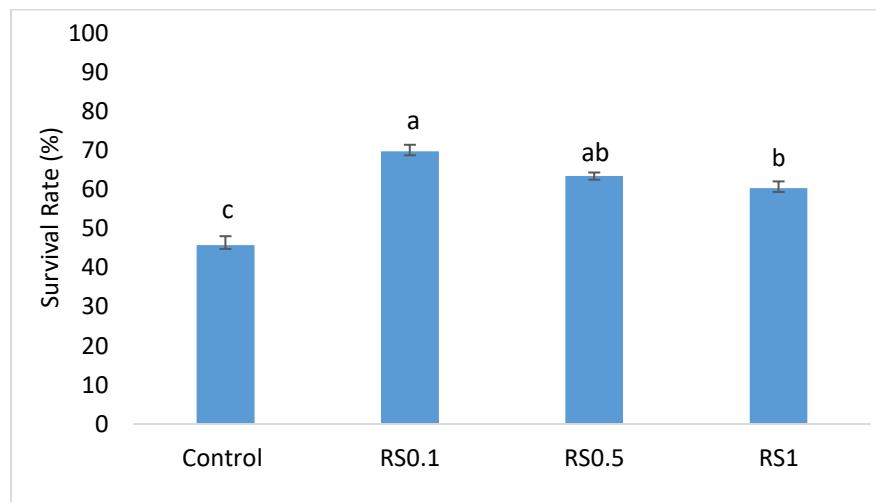


Figure 10. Survival rate (%) in rainbow trout treated with radish seed (*Raphanus sativus*) by-products aqueous methanolic extract after challenge with *Aeromonas hydrophila*. RS0.1, RS0.5 and RS1, radish seed (*Raphanus sativus*) by-products aqueous methanolic extract at 0.1 0.5 and 1% diet, respectively. Values are expressed as mean \pm SE. Different letters above bars indicate significant differences between groups ($P < 0.05$).

of the herb on growth performance. In the present study, final weight in all treatment groups was enhanced compared to that of control but the highest final weight and SGR were recorded in RS1 fish group. Besides, FCR levels in all treated fish groups did not change compared to that of the control. Similarly, *Malva sylvestris* administration in carp (Bilen et al., 2019b), seabream (Bilen et al., 2019c) and rainbow trout (Rashidian et al., 2020) caused a growth promoting effect. On the contrary, in few previous experiments, no growth promotion effect was observed in goldfish fed with nettle (Bilen et al., 2014), and in rainbow trout fed with tetra and laurel (Bilen and Bilen, 2012).

Survival in RS treated fish improved against *A. hydrophila* infection in the present study. Radish contains many components in its extract. When we screened the radish seed extract, an antimicrobial, antifungal and antitumor (Ullah et al., 2015) chemical, benzaldehyde (13.32%) was obtained in the extract. Triazole is also an important component of the radish extract (30.98%). Triazole is a well-known antifungal agent (Verweij et al., 2016). However, we speculate that with its antimicrobial effects benzaldehyde probably caused an enhanced survival in treated fish in the study and it was supported by the increased bacterial killing activity in the fish blood. Also, an elevated lysozyme level with its opsonin property could induce high survival rate against *A. hydrophila*.

Conclusion

Radish seed by-product aqueous methanolic extract was tested as a growth promoter and immunostimulant even with a short span administration. Also, an elevated survival in treated fish against *A. hydrophila* infection is a positive sign. All these results suggest that application radish extract through fish diets for 14 days can improve innate immune responses and growth in rainbow trout.

Ethical Statement

The present study was performed as per the guidelines and permission according to Directive 2010/63/EU obtained from the local Ethics Committee for Animal Research Studies at the Kastamonu University (2019.27).

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The study was not supported by any other project.

Author Contribution

All was done by Gökhan Arslan

Conflict of Interest

The author declares that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper."

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The names, degrees, and affiliations who have contributed substantially to a study but do not fulfill the criteria for authorship can be listed in the Acknowledgments section.

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