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Original Article

The use of *Zingiber officinale* extract against *Yersinia ruckeri* and its effects on the antioxidant status and immune response in *Oncorhynchus mykiss*

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Abstract: The present work aimed to explore the effects of nutritional ginger on rainbow trout. The fish were fed various concentrations of ginger extract i.e. 1, 3, 6, and 10 g/kg; the control was fed no ginger. Having fed for 56 days, the fish were exposed to *Yersinia ruckeri* and any case of death was documented for two weeks. The diet with the extract of ginger could check the experimental infection in rainbow trout. The highest survival rate, i.e. 91.4%, was attained in the group nourished with ginger at 10 g/kg while the survival rate for the control was 41.6%. Ginger diet of 6 and 10 g/kg affected the biochemical parameters and the immune response. Total serum protein and total serum immunoglobulin, together with lysozyme, phagocytic, bactericidal and antioxidant enzymes activities, significantly improved in some groups, other than the control, in a dose-reliant mode ($P<0.05$). No variations were detected in the levels of alanine aminotransferase, lactate dehydrogenase, and aspartate aminotransferase activities in the ginger-fed groups, indicating no liver toxicity. The findings of the current research revealed that some doses of nutritional ginger (6 and 10 g/kg) could reinforce the non-specific immunity and diminish the vulnerability of rainbow trout to *Y. ruckeri*.

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Introduction

As one of the most extensively introduced fish species in the world, rainbow trout has been introduced into at least 99 countries (Stanković et al., 2015). The production of rainbow trout in freshwater amounted to 576.902 metric tons (MT) globally in 2014 (FAO, 2016). Iran, Turkey, and Italy stood as the top three producers and produced 126.515, 107.533, and 34.400 MT, respectively, comprising almost 47% of the worldwide culture of rainbow trout (FAO, 2016).

Diseases are still the main limit for the development of aquaculture. Enteric red mouth disease (ERMD), also known as yersiniosis, caused by *Yersinia ruckeri*, is an important pathogen in salmonid fish (Gregory et al., 2010). As a Gram-negative enterobacterium, this pathogen is isolated from different fish species (Haig et al., 2011; Chettri et al., 2013; Soltani et al., 2014).

Chemotherapies are extensively used to control and prevent bacterial diseases in intensive aquaculture (Austin and Austin, 2007); however, the broad usage of antibiotics may cause environmental risks, lead to the development of resistant pathogens, and threaten human health (Esiobu et al., 2002; Jones et al., 2004; Rao et al., 2006). Immune enhancers augment resistance to stress and disease by enhancing the immune responses in aquatic animals (Harikrishnan et al., 2011). Herbal immunostimulants consist of numerous terpenoid, polyphenolic, polypeptide, phenolic, quinone, lectine, and alkaloid complexes, most of which are very adequate replacements to chemical drugs (Talpur et al., 2013). They also support growth, are anti-stress and antimicrobial in aquaculture (Citarasu et al., 2001; Maqsood et al., 2011). Currently, much attention has been paid to herbal immunostimulants in aquaculture because they are inexpensive and environmentally friendly (Abdel-Tawwab et al., 2018; Hoseini and Yousefi, 2018; Adeshina et al., 2018; Yousefi et al., 2019).

Zingiber officinale Roscoe, commonly known as ginger, comprises flavonoids, alkaloids, steroids, gingerols, shogaols, polyphenols, carotenoids,

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Table 1. Formulation and element constitution of basal diet.

Ingredients	(g/kg)	Approximate constitution of additives (g/kg)				
		Crude protein	Crude fat	Fiber	Ash	Moisture
Sardine Fish meal	350	650	80	10	190	70
Anchovy Fish meal	250	700	90	10	140	60
Soybean meal	75	400	20	60	70	120
Wheat flour	184	100	10	25	10	180
Soybean oil	60	0	990	0	0	10
Fish oil	30	0	990	0	0	10
Wheat Gluten	25	750	20	20	15	75
Gelatin	10	750	0	0	130	110
Vitamin and mineral mix	16					

Mineral and vitamin mixture (mg/ kg or IU/kg): Vitamin: retinol acetate (A), 1200000 IU; thiamin mononitrate (B1), 200 mg; riboflavin (B2), 3600 mg; calcium D-pantothenate (B3), 7200 mg; niacinamide (B5), 9000 mg; pyridoxine hydrochloride (B6), 2400 mg; folic acid (B9) 600 mg; cyanocobalamin (B12), 4 mg; L-ascorbic acid (C), 5400 mg;Cholecalciferol (D3), 400000; D-biotin (H2), 200 mg; DL-a-tocopheryl acetate (E), 30 IU;menadione sodium bisulfite (K3), 1200 mg;antioxidant500 mg: Mineral: Cu, 500 mg; Se, 50 mg; Co, 50 mg; Zn, 6000 mg; Fe, 4500 mg; I, 150 mg; choline chloride, 150 000 mg; Mn,5000 mg; Carrier up to 1 kg.

zingeron, vitamins, and minerals (Iheanacho et al., 2017). Ginger is a significant immunostimulant and also has antibacterial, antiviral, antifungal, antioxidant, and anti-inflammatory effects on various living organisms (Nya and Austin, 2009a; Otunola et al., 2010). To the best of our knowledge, no data is available regarding the anti-infectious potential of ginger against *Y. ruckeri* in fish species. Therefore, the objective of the current study was to determine the impact of nutritional ginger on immunity response and disease resistance against *Y. ruckeri* in rainbow trout.

Materials and Methods

Preparation of ginger extracts and diets: Ginger fresh rhizomes were bought from a local shop in Karaj, Iran. Clean water was used to wash the ginger. Then, it was skinned, chopped, and shade-dried at room temperature. Then, it was dried in an oven at 60°C and powdered using an electrical blender. Samples were then homogenized in ethanol (75%) for three days. Then, the mixture was filtered through Whatman filter paper (No. 1) and became concentrated at 40°C by a rotary evaporator. The dried extract was put in closed bags and kept at 4°C for further use. The pellet feed (Faradaneh Company, Shahrekord, Iran) was used as the basal diet. Table 1 presents how the basal diet was formed. The extract was homogenized in 75% ethanol and sprayed gently over the feed pellets in five dissimilar levels (0, 1, 3, 6, and 10 g/kg feed). For drying out the pellets, they were put in an oven at 35°C for 24 h.

The feed pellets were covered with 4% aqueous gelatin dissolved in distilled water at a ratio of 5:40 (v/w) for keeping the plant extract intact (Wu et al., 2013). The pellets were kept at room temperature (25°C) for 6 h to be air-dried and were then conserved in airtight bags at 4°C for further use. The approximate ingredients (crude lipid, fiber, crude protein, dry matter, and ash) of the feeds used in the study were analyzed in triplicate based on the association of official analytical chemists (AOAC, 2005). The diets were examined for the presence of nitrogen-free extract (NFE) and the amount of this extract was estimated based on the following formula (Shaluei et al., 2016):

NFE = dry matter – (crude lipid + crude protein + fibre +ash).

Table 2 shows the approximate constitutions of test diets.

Experimental design: The research tests were performed at a private trout farm at the Hashtgerd, Karaj, Iran. A total of 750 juvenile rainbow trout (45.8±3.6 g), were arbitrarily distributed into five different groups with three repeats for each. The fish (50 per replicate) were held in 15 circular tanks (3000 L), supplied with well water (constant temperature 15±0.5°C; pH=7.6; oxygen>7.5 mg/ L; NH_3<0.01 mg/L; NO_2<0.1 mg/L and total hardness 278 mg/L),

Table 2. Approximate constitution of diets containing different concentrations of ginger extract.

Approximate analysis (g/kg dry weight)	Nutritional ginger extract inclusion levels (g/kg)				
	0	1	3	6	10
Dry matter	918.24±8.48[a]	918.62±8.38[a]	920.26±6.14[a]	934.63±9.45[a]	944.64±10.26[a]
Crude protein	492.36±8.26[a]	503.34±7.16[a]	496.34±10.12[a]	502.23±9.14[a]	494.26±10.14[a]
Crude lipid	164.42±8.66[a]	165.36±6.82[a]	172.26±7.45[a]	170.56±8.45[a]	167.26±7.86[a]
Fiber	19.24±4.28[a]	20.12±3.68[a]	21.14±4.22[a]	22.12±3.86[a]	24.18±4.48[a]
Ash	108.36±6.42[a]	110.42±5.57[a]	117.34±6.23[a]	120.14±8.24[a]	122.35±6.89[a]
NFE	135.36±18.3[a]	116.34±20.46[a]	118.54±15.42[a]	121.26±17.32[a]	137.42±12.8[a]
Gross energy (MJ/kg)	22.26±0.12[a]	22.58±0.16[a]	23.14±0.17[a]	20.18±0.08[a]	19.76±0.13[a]

Data in rows assigned with different letters are significantly different at 5% level of significance.

with a 10:14 h light: dark natural photoperiod. Water parameters were measured every two weeks. Oxygen and total hardness were measured based on standard methods (Clesceri et al., 1999), and pH using pH meter (model: Hanna Instrument model No. H1 8915 ATC). Ammonia, nitrite and nitrate were monitored using a commercial testing kit (Fish Farming Test Kit, Model FF- 1A; HACH Co., Loveland, CO, USA) and maintained in standard ranges. The basal diet was used to nourish the control while the experimental groups were supplied with the basal diet supplemented with four concentrations of ginger extract (1, 3, 6, and 10 g/kg). As proposed elsewhere, all the groups were fed to proximate the estimated levels (2% biomass per day) at a frequency of thrice a day for 8 weeks (56 days) (Merrifield et al., 2010). Following every 30 min of feeding interval, siphoning was used to remove uneaten feed and feces for all the groups (Li et al., 2015).

Blood and serum collection: After every 2 weeks, the fish were first fasted for 24 h and then anesthetized with eugenol (25 mg/ml) before blood sampling was done. In each group, 15 fish were randomly selected for blood collection (five fish per group replicate), and a 2-ml syringe was used to collect blood from their caudal vein. For each blood sample, one part of it was transferred into pre-heparinized plastic Eppendorf tubes, whereas the other part was transferred into Eppendorf tubes without anticoagulant and left to clot there for 2 h at room temperature. The tubes were kept at 4ºC during the night and then centrifuged at 2500g for 15 min. Next, the collection of the supernatant serum was done and kept at -20ºC in screw cap glass vials to be used later.

Immunoglobulin assay: The total serum immunoglobulin (Ig) was measured based on the method suggested by Siwicki and Anderson (1993). Immunoglobulins were separated from serum by precipitation with 10000 kDa polyethylene glycol (PEG, Sigma). Serum (100 µl) and an equal volume of 12% PEG solution were mixed together and the mixture was shaken for 2 h at room temperature. At first, the supernatant was centrifuged at 5000 g for 15 min; then, it was removed and the proteins concentration was detected based on Biuret method (Goldenfarb et al., 1971). To determine the total immunoglobulin content, this value was subtracted from the total serum concentration of the protein.

Lysozyme assay: Jian and Wu's (2003) method was used to evaluate serum lysozyme activity. Concisely, a suspension of *Micrococcus luteus*, which had been grown during the night, was produced by dissolving 20 mg of *M. luteus* into 100 ml of 0.067 mol/L sodium phosphate buffer, pH 6.4. One hundred µl of fish serum was added to a 3 ml suspension of *M. luteus* at 22°C and absorbance at 540 nm was read after 0.5 and 4.5 min. One unit of lysozyme activity was considered as the quantity of lysozyme, inducing a reduction in absorbance of 0.001 per minute.

Phagocytic activity *Ex vivo*: Phagocytic activity was examined according to Zhou et al. (2002), and the commercial baker's yeast, *Saccharomyces cerevisiae*, was used as an indicator. Dried live yeast was mixed in 2% sucrose solution (pH 3-4) for 2 h at 30°C and boiled for 30 min. Then, the yeast was centrifuged (800 g for 10 min); the pellet was washed two times

and it was re-suspended in 0.85% saline (at 2×10^8 cell/ml). After this, an aliquot [20 µl] of suspension, together with 40 µl heparinized whole blood, was added to a 0.1 ml Eppendorf tube. The mixture was incubated at 22°C for 30 min and shaken mildly. Afterward, the preparation of glass smears took place and Wright-Giemsa was used to stain the slides which had been air-dried earlier. Phagocytic activity (PA) was estimated by assessing 100 phagocytes per slide under a light microscope. The evaluation was done on three slides/fish. The mean PA was determined as 100 × Number of phagocytic cells and the engulfed yeast cells/Number of phagocytes were enumerated.

Complement activity: The assessment of the components of the complement (C3 and C4) was done using laboratory kits (Pars Azmoon Co., Tehran, Iran) based on immunoturbidimetry (Sun et al., 2010). Total serum protein was determined as described in the Biuret method (Goldenfarb et al., 1971).

Serum enzymes activities: Lactate dehydrogenase (LDH), Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (AKP) activities were detected in serum colorimetrically using the kits offered by Ziest Chem diagnostics, Tehran, Iran (Fazlolahzadeh et al., 2011).

Serum bactericidal activity: Bactericidal activity of serum was detected according to Rao et al. (2006). The centrifugation of the fresh nutrient broth culture of *Y. ruckeri* was done for 3000 g for 15 min; also, the washing and suspension of the pellet was done in PBS. The suspension optical density was accustomed to 0.5 at 546 nm. The dilution of the bacterial suspension was done serially (1:10) with PBS and it was repeated for five times. Two ml of this diluted *Y. ruckeri* suspension was incubated with 20 µl of serum in an Eppendorf tube for 1 h at 22ºC to evaluate the bactericidal activity of serum. PBS substituted the serum in the bacterial control group. Once the incubation finished, the counting of the viable bacteria was started by enumerating the colonies grown on a nutrient agar plate for 24 h at 22ºC.

Antioxidant-related parameters assay: Total antioxidant capacity (TAC) and the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) in serum were measured using a spectrophotometer (UV-2100, Shanghai Jinhua Technology Instrument Co., Ltd., Shanghai, China) at 520, 550, 412, 405, and 520 nm, respectively (Deng et al., 2013). The antioxidant-related parameter detection kits (TAC, CAT, GSH-Px, and SOD) were ordered from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). One unit of TAC was considered as a 0.01 rise of the absorbance of the reaction induced by serum per mL reacting at 37˚C for 1 min. One unit of CAT activity was interpreted as the quantity of the enzyme that accelerated the breakdown of 1 mmol of H_2O_2 per min. One unit of GSH-Px activity was defined as the quantity of the enzyme that decreased the GSH level in the reaction system at 1 mmol/ L per minute. One unit of SOD activity was considered as the quantity of the enzyme needed to induce a 50% inhibition of the nitroblue tetrazolium reduction rate determined at 550 nm.

Challenge experiment: A virulent isolate of *Y. ruckeri* (FJ870985) was cultured in tryptic soy broth (TSB, Germany) for 48 h at 22°C. The feeding continued for 8 weeks. At the end of the 8 week feeding trial, the intraperitoneal injection of all the groups (6 fish from each tank in triplicate, n=18) was done with 0.1 ml of *Y. ruckeri* suspension, grown in one day, in PBS containing 10^6 cells/ml. Formerly, the challenge dose was established to cause 50% mortality (LD50) in non-supplemented fish. To form a control, some fish were injected with 0.1 mL PBS. After the bacterial challenge, all the fish (ginger supplemented and non-supplemented) were monitored for two weeks to document any strange behavior, clinical signs, and daily fish death. Eventually, the following equation was used to measure the values of the relative percent survival (RPS) (Amend, 1981):

$$RPS = 1 - [(\text{treatment mortality}/\text{control mortality}) \times 100].$$

Statistical analysis: The data are presented as Mean±SD of the number of fish per treatment. One way analysis of variance (ANOVA) and Tukey's multiple comparison test were performed for the analysis of the data. Statistical analysis was performed

Table 3. Average serum biochemical parameters for rainbow trout juveniles fed with 0, 1, 3, 6 and10 g ginger per 1000 g of feed for 8 weeks.

Parameters	G ginger per 1000g of feed	Experimental period			
		2th week	4th week	6th week	8th week
ALT (U/L)	0	9.38±0.18	9.56±0.18	9.23±0.19	9.40±0.25
	1	9.49±0.23	9.61±0.16	9.31±0.17	9.36±0.18
	3	9.54±0.24	9.46±0.18	9.29±0.22	9.70±0.05
	6	9.56±0.21	9.65±0.24	9.33±0.18	9.36±0.14
	10	9.41±0.16	9.28±0.22	9.52±0.19	9.40±0.05
AST (U/L)	0	224.3±9.8	236.6±10.2	241.5±9.7	234.8±9.8
	1	238.4±8.6	242.8±7.9	224.9±8.4	239.1±10.3
	3	246.8±9.3	238.6±8.5	247.3±8.3	241.3±9.4
	6	233.6±7.4	227.8±8.4	251.4±10.3	245.9±8.8
	10	246.3±8.6	248.3±9.2	250.4±9.8	252.6±7.2
LDH (U/L)	0	729.6±33.4	732.4±28.7	724.8±32.6	744.6±27.2
	1	736.5±34.2	728.3±26.9	738.8±34.1	755.9±34.2
	3	764.8±27.2	783.7±34.8	792.6±31.9	773.7±28.4
	6	775.8±28.3	796.7±35.8	8·4.8±28.6	809.6±29.2
	10	786.4±33.6	813.8±26.7	812.8±29.1	810.7±32.8
AKP (U/ L)	0	22.36±0.42	23.64±0.53	22.17±0.64	23.38± 0.67
	1	21.14±0.51	24.18±0.49	22.46±0.43	19.95±0.53
	3	22.43±0.62	23.91±0.57	24.17±0.68	25.74±0.86
	6	23.19±0.48	25.16±0.64	26.11±0.72*	28.45±0.97*
	10	25.47±0.73	27.34±0.86*	29.27±1.08*	31.54±2.79*
Total protein (g/dL)	0	3.68±0.25	3.49±0.18	3.56±0.24	3.75±0.19
	1	3.52±0.27	3.59±0.26	3.73±0.17	3.89±0.26
	3	3.65±0.18	3.82±0.23	3.93±0.18	4.12±0.21
	6	3.89±0.22	4.17±0.26	4.59±0.26	5.14±0.24*
	10	4.08±0.27	4.48±0.19	5.17±0.24*	5.86±0.23*

Data in a column assigned with asterisk indicates significant difference from the control (P<0.05) (n=15).

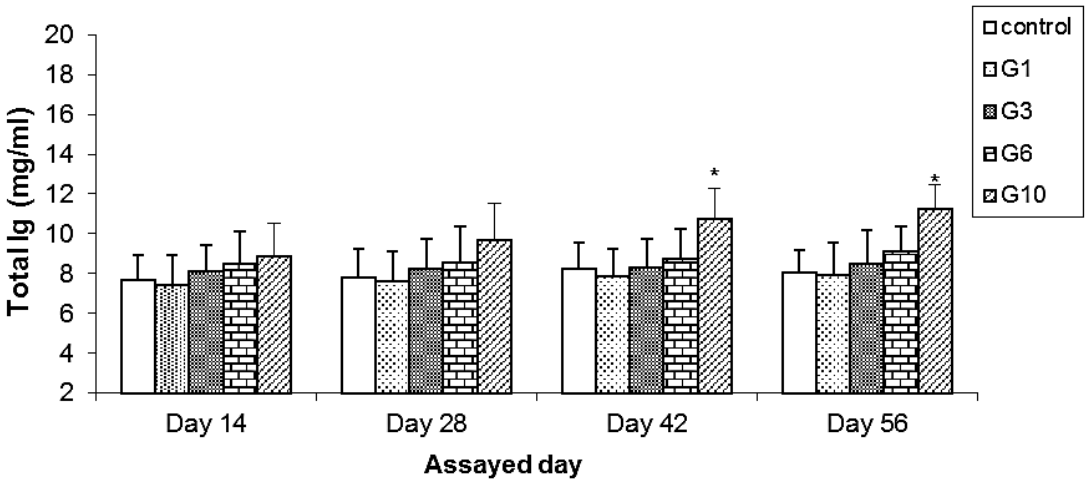

Figure 1. Alterations in serum total Ig of rainbow trout juveniles fed with 0, 1, 3, 6 and10 g ginger per 1000 g of feed for 2, 4, 6 and 8 weeks. Bars assigned with asterisk indicate significant difference from the control (P<0.05) (n=15).

using SPSS software (version 16.0, Chicago, IL) and the probability of P<0.05 was considered statistically significant.

Results

No abnormal behavior or mortality was noticed throughout the experimental period. The effects of *Z. officinale* as feed additive on total protein and total Ig can be observed in Figure 1 and Table 3. After 4 and 8 weeks of feeding, the total protein and total Ig increased significantly in the 10 g ginger-fed group compared to those of the other groups (P<0.05).

Table 4. Effects of dietary ginger supplementation on antioxidant capacity parameters in serum of rainbow trout.

Parameters	G ginger per 1000g of feed	Experimental period			
		2th week	4th week	6th week	8th week
SOD	0	5.63±0.46	7.11±0.83	6.44±0.58	7.18 ±0.72
(U/ mg protein)	1	8.24±0.56	7.79±0.61	9.33±0.68	10.25±0.73
	3	8.86±0.67	9.27±0.84	11.66±0.96	13.81±1.27
	6	12.29±1.08	14.48±1.37	18.26±1.93*	22.36±2.17*
	10	15.43±1.59*	18.49±2.07*	23.18±2.56*	29.62±3.24*
CAT	0	6.12±0.42	5.86±0.54	6.43±0.59	6.94±0.46
(U/ mg protein)	1	4.28±0.46	5.88±0.51	6.83±0.43	8.85±0.47
	3	7.52±0.43	8.26±0.53	9.12±0.47	10.24±0.51*
	6	9.47±0.59	11.27±0.64	14.52±0.82*	18.21±1.13*
	10	12.67±0.73	16.48±0.94*	23.4±1.26*	29.36±2.47*
GSH-Px	0	0.08±0.01	0.09±0.01	0.11±0.01	0.08±0.01
(U/ mg protein)	1	0.11±0.01	0.09±0.01	0.12±0.01	0.11± 0.01
	3	0.09±0.01	0.10±0.01	0.11±0.01	0.12±0.00
	6	0.11±0.01	0.12±0.01	0.11±0.01	0.13±0.01*
	10	0.11±0.01	0.13±0.01	0.12±0.01	0.14±0.01*
TAC	0	3.61±0.24	3.76±0.28	3.54±0.22	3.78± 0.20a
(U/ mg protein)	1	3.74±0.29	3.88±0.23	4.31±0.27	5.22±0.21ab
	3	4.42±0.23	4.84±0.27	5.11±0.36	5.81±0.31ab
	6	4.75±0.28	5.07±0.23	5.87±0.36	6.12±0.28b*
	10	5.37±0.31	6.24±0.26*	6.88±0.38*	7.52±0.42b*

Data in a column assigned with asterisk indicates significant difference from the control (P<0.05) (n =15).

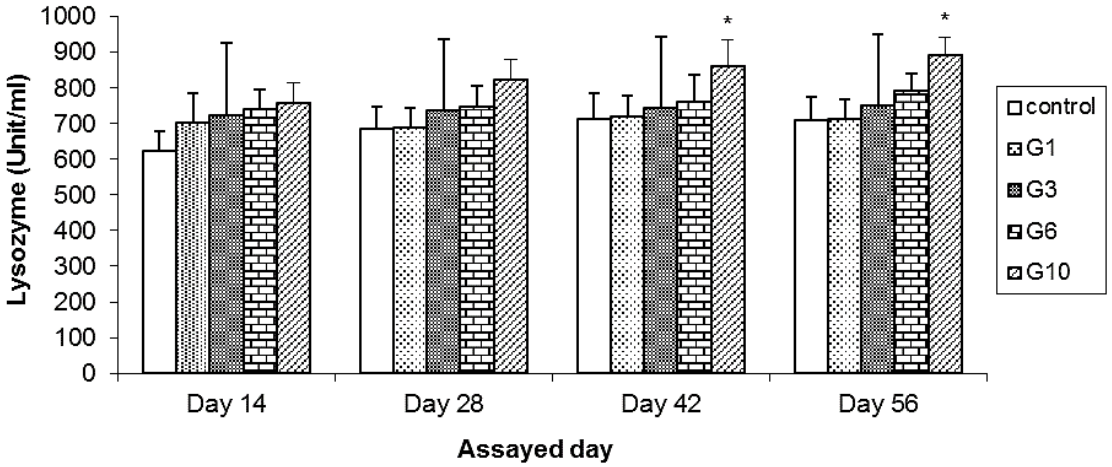

Figure 2. Alterations in serum lysozyme activity of rainbow trout juveniles fed with 0, 1, 3,6 and10 g ginger per 1000 g of feed for 2, 4, 6 and 8 weeks. Bars assigned with asterisk indicate significant difference from the control (P<0.05) (n=15).

Lysozyme activity enhanced significantly at the end of weeks 6 and 8 in the fish fed with 10 g extract compared to that of the other groups (P<0.05) (Fig. 2). Furthermore, a significant enhancement, starting when the second week of the feeding was about to finish, was observed in the phagocytic activity of the fish supplemented with 10 g plant additive compared to other groups (Fig. 3). This value was maximized when the feeding trial reached the end of the 8th week. A significant increase was observed in the complement components (C3 and C4) in the 10 g ginger-fed group, compared to the control groups, only by the end of the 8-week feeding trial (Figs. 4, 5). In the present research, the activities of AST, ALT, and LDH did not show any significant change when the fish diet was supplemented with ginger (P>0.05) (Table 3). As regards the serum bactericidal activity, the number of viable bacterial colonies showed a dose-reliant reducing trend in fish supplemented with ginger. A significant increase was observed in the serum bactericidal activity of the fish which were fed with both 6 and 10 g plant additive, compared to their

Table 5. Effects of dietary ginger supplementation on relative percentage survival (RPS) against LD50 concentration of *Yersinia ruckeri* in rainbow trout.

G ginger per 1000g of feed	Survival (%)	Mortality (%)	RPS (%)
0 (Control)	41.6[d]	58.4	-
1	48.4[d]	51.6	11.6
3	55.4[c]	44.6	23.6
6	65.2[b]	34.8	40.5
10	91.4[a]	8.6	85.3

Values with the same superscript in a column do not differ significantly (P<0.05) (n =15).

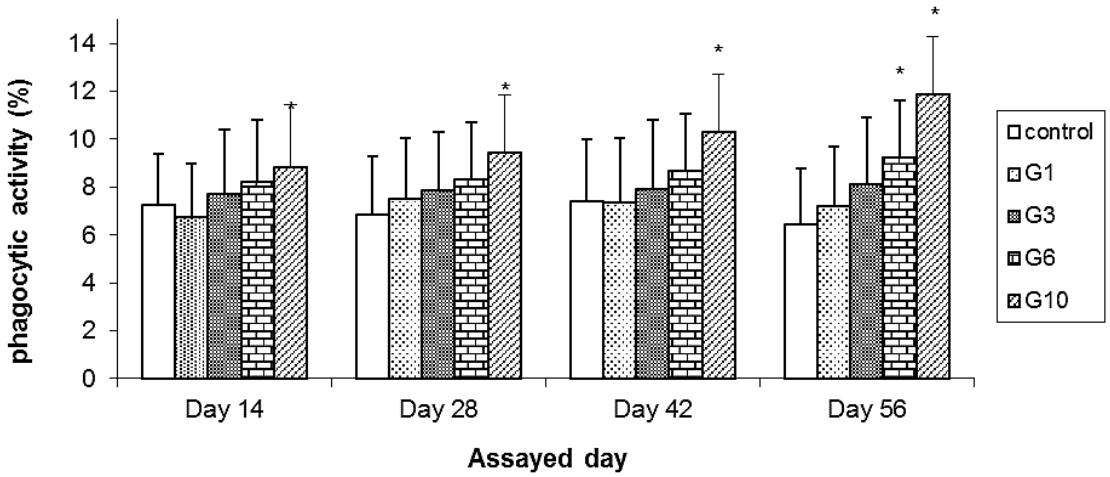

Figure 3. Alterations in phagocytic activity of peripheral blood leukocytes of rainbow trout juveniles fed with 0, 1, 3, 6 and10 g ginger per 1000 g of feed for 2, 4, 6 and 8 weeks. Bars assigned with asterisk indicate significant difference from the control (P<0.05) (n=15).

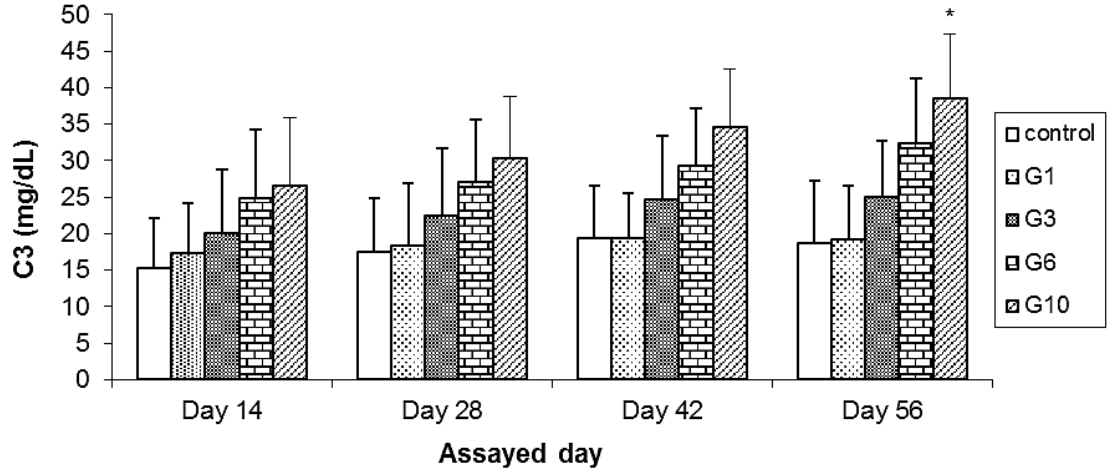

Figure 4. Alterations in serumC3 component of complement of rainbow trout juveniles fed with 0, 1, 3,6 and10 g ginger per 1000 g of feed for 2, 4, 6 and 8 weeks. Bars assigned with asterisk indicate significant difference from the control (P<0.05) (n=15).

respective control groups, at the end of weeks 2, 4, 6, and 8 (Fig. 6).

Dietary ginger supplementation largely increased the activities of serum antioxidant enzymes in a dose-dependent manner (Table 4). The serum SOD activity elevated significantly in the fish fed ginger at 10 g level compared to the control group; this elevation started at the end of week 2 and peaked by the end of the 8-week feeding trial. Besides, a significant increase in TAC, CAT, AKP and GSH-Px activities was first noticed at the end of week 4 in fish fed ginger at 1% level which continued by both 6 and 10 g plant additive and peaked by the end of feeding trial.

The fish mortality trends during the two weeks after the presentation of *Y. ruckeri* challenge are tabulated in Table 5. The incorporation of ginger extract in the

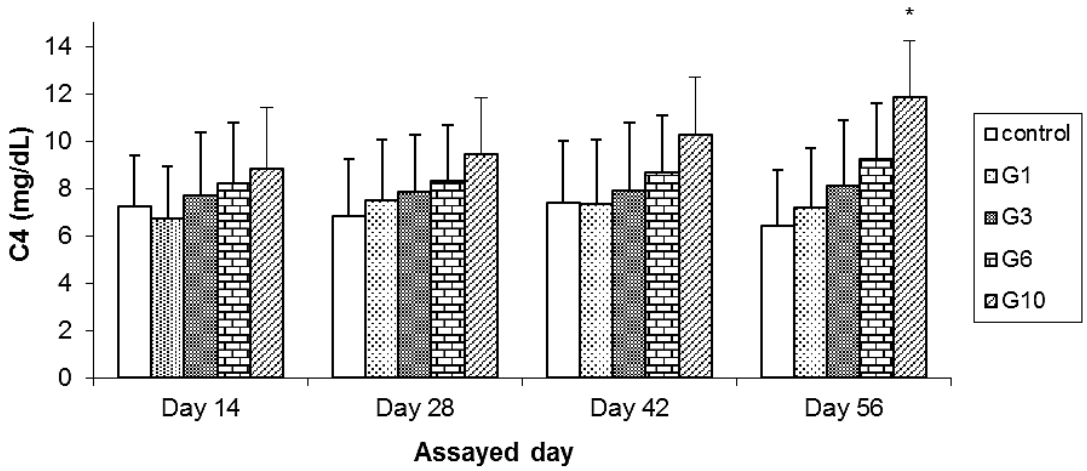

Figure 5. Alterations in serum C4 component of complement of rainbow trout juveniles fed with 0, 1, 3, 6 and10 g ginger per 1000 g of feed for 2, 4, 6 and 8 weeks. Bars assigned with asterisk indicate significant difference from the control (P<0.05) (n=15).

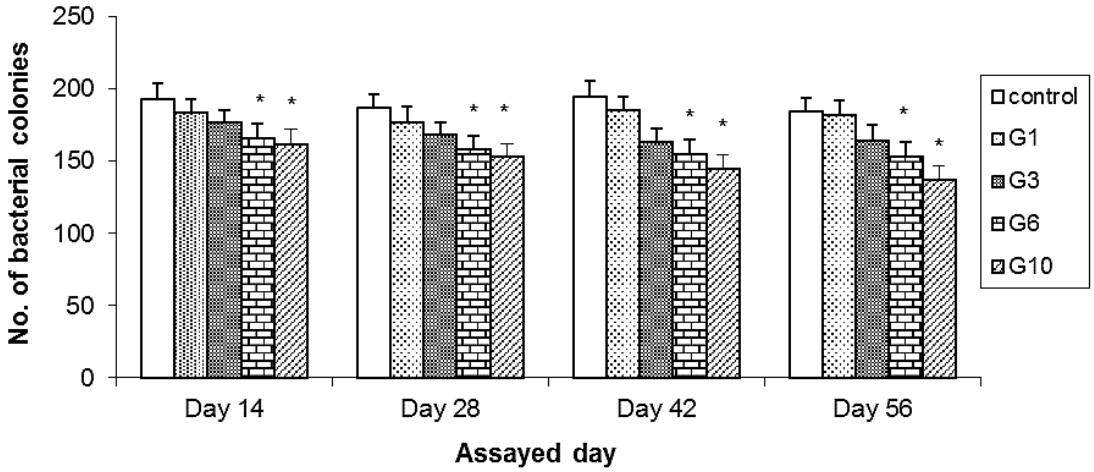

Figure 6. Alterations in serum bactericidal activity of rainbow trout juveniles fed with 0, 1, 3,6and10 g ginger per 1000 g of feed for 2, 4, 6 and 8 weeks. Bars assigned with asterisk indicate significant difference from the control (P<0.05) (n=15).

diet of rainbow trout led to a significant decrease in the cases of fish death after the bacterial challenge was presented. The control group showed 58.4% mortality whereas the groups supplemented with different concentrations of the ginger extract had mortalities ranging 8.6–51.6% in a dose-reliant fashion with the lowermost mortality rate (i.e. highest resistance) noticed in fish fed with 10 g plant extract. Furthermore, no clinical signs of the disease were observed in the survived fish belonging to the group fed with ginger at the end of the bacterial challenge.

Discussions

Over the past two decades, significant attention has been paid to the use of herbal immune enhancers in aqua farming. The present research aimed to evaluate the biochemical and immunological responses of young rainbow trout fed with experimental diets supplemented with various levels (1, 3, 6, and 10 g per kg feed) of *Z. officinale*.

Some researchers believe that the serum total protein is the most important representative of the nutritional, biochemical, and health condition in fish species (Patriche et al., 2009). The highest serum protein level was found in the group fed with 10 g ginger per 1000 g of feed, a finding which was in parallel with what Nya and Austin (2009a) reported in their study. Similarly, ginger powder as a feed additive could increase the total protein in *L. calcarifer* (Talpur et al., 2013) and juvenile *H. huso* (Gholipour Kanani et al., 2014). Likewise, increased values of total serum protein in beluga (*H. huso*) and rainbow trout after

dietary supplementation with stinging nettle (*Urtica dioica*) have been reported by Binaii et al. (2014) and Bohlouli and Sadeghi (2014). The increment in total serum protein value in fish treated with ginger supplemented diet might be accompanied by the increased levels of immune parameters which have a protein structure, such as total complements and immunoglobulins.

Fish innate immunity is the first barrier against microbial invaders and more critical for fish than mammals (Uribe et al., 2011). Immunoglobulin (IgM) is a main element of the teleost humoral immunity and contributes in the recognition and nullification of exogenic agents such as viruses and bacteria which are harmful to fish health (Magnadóttir, 2006).

In the present work, the total immunoglobulins values elevated following nutritional inclusion of *Z. officinale*. Similar results were found in beluga (*H. huso*) and *Lates calcarifer* following dietary supplementation of ginger (Vahedi et al., 2017; Talpur et al., 2013). Several fish species also presented elevated levels in serum immunoglobulins upon dietary supplementation of the herbal ingredients (Nya and Austin, 2009b; Binaii et al., 2014; Bohlouli and Sadeghi, 2014; Akrami et al., 2015; Ngugi et al., 2015; Bohlouli and Sadeghi, 2016). This might be due to an augmentation effect of herbal extract on lymphocyte activity and their proliferation resulting in provoking immunoglobulin production by B lymphocytes.

Phagocytosis by macrophages and neutrophils is a critical defense activity against pathogenic bacteria (Rao et al., 2006). The increase observed in the phagocytic activity of the fish belonging to the ginger-supplemented groups in this study is believed to have been caused by the bioactive elements of ginger (Tan and Vanitha, 2004), elements which allowed the fish to be less affected by the infection. The findings of the present study are comparable and similar to those of Dügenci et al. (2003), Nya and Austin (2009a), Haghighi and Rohani (2013), and Talpur et al. (2013 a, b), who all reported an enhanced phagocytic activity in rainbow trout and Asia sea bass after they had been fed with ginger.

The serum lysozyme activity is considered as an effective antibacterial parameter against bacterial pathogens (Misra et al., 2006; Zhou et al., 2013). In the current work, the lysozyme activity presented significant increment in the group fed with 10 g ginger. These findings are similar to those of Haghighi and Sharif Rohani (2013) and Talpur et al. (2013a, b), who reported that the dietary administration of ginger could significantly enhance the serum lysozyme activity in *O. mykiss* and *L. calcarifer*, respectively. Ginger supplementation at 2% concentration in *Epinephelus fuscoguttatus*, resulted in an increased seric lysozymeactivity (Apines-Amar et al., 2013). Nevertheless, and despite the findings of the present research, some studies have reported no change in the lysozyme activity of young belugas which were fed with ginger (Gholipour Kanani et al., 2013). Such conflicting results might be due to differences in fish species, fish size, and environmental fluctuation.

The complement system can quickly recognize and opsonize bacteria for phagocytosis or break them up directly by inducing cell membrane disorder (Ahmadi et al., 2012). Complement components (C3 and C4) determined in the present study were significantly improved with 10 g of ginger extract indicating that ginger could influence the activity of the complement system in rainbow trout. This is in agreement with Saeidi-Asl et al. (2017) who recorded elevated levels of complement components C3 and C4 in rainbow trout following nutritional addition of stinging nettle extract. Moreover, similar results were found in rainbow trout supplemented with *Ferulago angulata* extract and Persian oak (*Quercus brantii*) fruit extract, respectively (Bohlouli and Sadeghi, 2016; Bohlouli et al., 2016).

Various studies stated that ginger is an effective antioxidant which has cellular protecting and reformative properties induced by destroying free radicals (Mallikarjuna et al., 2008; Lebda et al., 2012). Live organisms encountered to pathogens and various stressors are exhibited to free radicals generated by oxidative stress (Tovar-Ramırez et al., 2010). To protect themselves against free radicals, the living organisms rely on their enzymatic defense mechanism, and the first line of this mechanism is

formed by antioxidant enzymes, such as SOD, CAT, and GSH-Px (Abdel-Tawwab and Hamed Heba, 2018). Moreover, total antioxidant capacity, which is considered a reliable disease biomarker, could be used to estimate efficiently the antioxidant capability of antioxidants (Kusano and Ferrari, 2008).

Our findings reveal that antioxidant enzymes (SOD, CAT, and GSH-Px) activities and total antioxidant capacity increased in the experimental groups with increased ginger concentration. It was stressed that this may be related with several bioactive ingredients (shagol, gingerols, zingeron, etc.) which exist in the composition of ginger and are responsible for anti-oxidative property of ginger (Şahan et al., 2018). In a similar study conducted in tilapia, a significant increment in the activity of CAT and SOD following intensive feeding with garlic has been reported (Metwally, 2009). Likewise, dietary supplementation of cholesterol considerably improved the serum and hepatic GSH-Px, SOD, CAT and TAC activities in rainbow trout (Denget al., 2013). Noteworthy, Sukumaran et al. (2016) reported that nutritional incorporation of ginger at 0.8 and 1% level remarkably upregulated the expression of antioxidant genes (zinc/copper superoxide dismutase [SOD1] and glutathione peroxidase [Gpx]) in the intestine, kidney, and hepatopancreas of *Labeo rohita* fingerlings.

Alanine aminotransferase, AST, and LDH enzymes are functional indicators of liver cells impairment. Increased values may point to cell necrosis, degeneration, and damage to the liver caused by cellular destruction (Bhardwaj et al., 2010). In the current study, no changes were detected in AST, ALT and LDH levels in the experimental treatments, representing no liver toxicity in fish following dietary supplementation of ginger. These findings are consistent with those of Gholipour et al. (2014) and Binaii et al. (2014), who reported no notable changes in the level of ALT and AST in beluga supplemented with dietary ginger and nettle. Moreover, AST and ALT levels indicated even a considerable reduction in Sobaity sea bream (*Sparidentex hasta*) fry following dietary supplementation of ginger (Jahanjoo et al., 2018).

The serum bactericidal activity has an important task in the killing of the pathogens in fish (Ellis, 2001). Our results showed that serum bactericidal activity increased in the experimental groups with increased ginger concentration. These results are in concord with the findings of Nya and Austin (2009a, b) and Talpur and Ikhwanuddin (2012, 2013), who reported an elevated serum bactericidal activity in rainbow trout and *L. calcarifer* upon diet supplementation with ginger, garlic, and neem leaf (*Azadirachta indica*), respectively.

The microbial challenge is an essential stage to assess the influence of immune enhancers on the immune response of the host, as some immune-relevant indicators do not definitely represent the real health condition of the fish (Adel et al., 2016a). The enhanced disease resistance observed in the current work is due to improvement in immunological responses. The increase in the immune indices tested here well-connected with the enhanced resistance to an infectious pathogen (*Y. ruckeri*). The findings of the current research obviously indicated that when the rainbow trout's diet was supplemented with ginger, the Rainbow trout resistances to *Y. ruckeri* increased to some extent. The maximum protection was detected in the group fed with 10 g of the extract. Likewise, earlier studies found that nutritional addition of various herbal ingredients decreased the mortality against bacterial pathogens (Adel et al., 2016b; Awad and Austin, 2010; Talpur, 2014; Ngugi et al., 2015).

Conclusion

In conclusion, the current research brings some pieces of evidence that nutritional supplementation of ginger at 10 g for 56 days has improved the antioxidant status and stimulated non-specific immune responses in rainbow trout. Evidently, dietary application of ginger has a considerable effect in the control of *Y. ruckeri* infection. Moreover, the incorporation of ginger in the fish diet as a substitute to chemotherapies has a great value in disease control in aquaculture. These findings propose that nutritional doses of ginger should be noticed when durable field trials are carried out.

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