



AMELIORATING EFFECTS OF DIETARY PARSLEY (*PETROSELINUM CRISPUM*) ON AMMONIA TOXICITY IN THE RAINBOW TROUT, *ONCORHYNCHUS MYKISS*: GROWTH, DIGESTIVE ENZYMES, IMMUNITY, AND STRESS RESISTANCE

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Abstract

Use of phytobiotics as immunostimulant is increasing in aquaculture. In this study, the immune and growth promoting effects of parsley extract (PUE) were examined in the rainbow trout. In this regard, the various dietary levels of parsley extract (PUE), *Petroselinum crispum* (0, 0.5, 1.5, 3, and 4.5%) was added to the diet of rainbow trout to assay its potentials on immunity, growth, digestive enzymes, and mitigating ammonia-induced stress. A total of 450 fish (15.43 ± 0.06 g; mean $\pm$ SE) were distributed into fifteen 300-L fiberglass tanks (30 fish per tank) and fed PUE for 60 days and then challenged with unionized ammonia (0.3 mg/l). According to results, PUE mainly at 3–4.5% levels increased the growth, lipase and protease activities, humoral and mucosal immune components and liver antioxidant enzymes activities. These effects were kept even after ammonia stress, where the immune components were higher in PUE-treated fish than the control group. Furthermore, the dietary PUE reduced ammonia-related oxidative stress, as malondialdehyde (MDA) values (as the indicator of lipid peroxidation) decreased in fish fed PUE. The concentrations of liver metabolic enzymes in blood, both before and after ammonia stress declined in the fish fed PUE. Our findings demonstrated the growth and immune boosting and ammonia-stress mitigating properties of PUE in rainbow trout.

Key words: phytobiotics, parsley extract, immunity, antioxidant status, ammonia stress, fish

Rainbow trout (*Oncorhynchus mykiss*) is one of the most important farmed species of Salmonidae in the world. The global production of rainbow trout accounts for about 25% of salmon production (Adeli and Baghaei, 2013). Today, due to the human needs for aquatic protein resources, trout farms are well developed and farmers have turned to intensive aquaculture systems (Schumann and Brinker, 2020). However, fish farming in high density conditions has increased the risk of diseases and the accumulation of toxic organic and inorganic substances (Datta, 2012; Simbeye and Yang, 2014; Dauda et al., 2019; Parvathy et al., 2023; Zhao et al., 2023). In this regard, one of the most important toxic compounds is unionized ammonia, produced in culture medium, as a result of protein metabolism and microbial decomposition of organic matters (mostly wastes and uneaten food) (Wicks and Randall, 2002; Ip and Chew, 2010; Levit, 2010; Shiwanand and Tripathi, 2013; Zhang et al., 2019; Yan et al., 2021).

Under intensive aquaculture, the production of ambient ammonia is intensified, causing toxicity in fish, at high concentrations (Reddy-Lopata et al., 2006; Liu et al., 2008; Ching et al., 2009; Cheng et al., 2015; Shin et

al., 2016; Li et al., 2018; Hoseini et al., 2019 b; Shi et al. 2019; Li et al., 2020; Sallam et al., 2020). The toxic impacts of unionized ammonia are widely studied in fishes (Levit, 2010; Yousefi et al., 2021 a). The sub-lethal concentrations of ambient ammonia can reduce fish feeding, growth, immunity, alter fish behavior and blood biochemistry, disrupt osmoregulation and trigger oxidative stress reactions (Baldisserotto et al., 2014; Sergeant, 2014; Zhang et al., 2018). In addition, ambient ammonia at high concentrations may cause high mortality in fish farms, if its concentrations are not managed (Ip and Chew, 2010; Shiwanand and Tripathi, 2013). Therefore, managing ambient ammonia in fish ponds or increasing the resistance of fish to ammonia can help to enhance the efficiency of aquaculture. Nutritional management has been known as an effective way to improve the fish health against the ammonia stress. In this regard, the protecting impacts of dietary supplements against ammonia stress are indicated in fish (Pan et al., 2011; Yilmaz, 2019). For example, in the common carp (*Cyprinus carpio*), dietary supplements such as olive leaf extract (Rajabiesterabadi et al., 2020), a combination of sodium butyrate, *Bacillus*

amyloliquefaciens, zinc methionine, and digestive enzymes (Abdel-Tawwab et al., 2020), caratenoids (Rama and Manjabhat, 2014), cineole, *Artemisia* leaf extract, phytol (Hoseini et al., 2021), garlic (Yousefi et al., 2020) and arginine (Hoseini et al., 2019 a) enhanced fish immunity and mitigated the ammonia-induced stress. In Nile tilapia, *Oreochromis niloticus*, use of 0.22% to 0.30% menthol essential oil improved immune system and reduced the fish mortality after exposure to ambient ammonia (Magouz et al., 2021).

The parsley, *Petroselinum crispum* belongs to Umbelliferae family that is currently cultivated in many areas of the world (Farag et al., 2021). This plant is originated from Mediterranean region and used in traditional medicine due to its therapeutic properties (Abdellatif et al., 2017). Bioactive compounds of parsley, such as oleic acid, D-limonene, α -pinene and myristicin have antioxidant properties and thus are able to scavenge free radicals (Farag et al., 2021). Although the immune boosting impacts of parsley have been indicated in some vertebrates (Karimi et al., 2012; Aljanaby, 2013; Abou Seif, 2014; Maodaa et al., 2016; Ashry et al., 2021), this function has been rarely studied in fish. This research aimed to determine the potentials of dietary parsley on growth, activity of digestive enzymes, immunity, stress responses and resistance to ammonia-induced stress in the most important farmed fish, rainbow trout.

Material and methods

Preparation and analysis of antioxidant power of the parsley extract

Fresh parsley was harvested from a local farm, washed, and dried in 40°C. 200 g of dried parsley was powdered and added to 80% ethanol with stirring for 24 h. After that, the suspended particles were removed by Büchner funnel, concentrated by rotary evaporation at 40°C and finally pulverized by freeze drying and stored at -18°C until use (Adel et al., 2016).

Extract antioxidant power

The analysis of the antioxidant power of the extract was conducted by four methods as follows and the results are presented in Table 1.

Table 1. Evaluation of the antioxidant power of parsley extract using different methods

Methods	Antioxidant potentials
Total phenolics, mg GAE/g	2.14±0.34
Total flavonoids, mg QE/g	10.25±0.51
DPPH inhibition percentage	59.77±4.05
β -carotene/linoleic acid % oxidative inhibition	49.11±3.4

Free radical scavenging method (DPPH)

The extract (100 μ l) was mixed with 0.2 mL of 2,2 M-2,2-diphenyl-1-picrylhydrazyl (DPPH) in 150 μ M

methanol, incubated at 24°C for 20 min and then absorbance read at 520 nm (Brand-Williams et al., 1995).

Evaluation of total phenol

Total phenolic evaluation was performed using Folin-Ciocalteu method. Briefly, the extract (120 μ l) was mixed with 10% Folin-Ciocalteu reagent (50 μ l) and 20% saturated sodium carbonate solution (30 μ l) and incubated for at 36°C for 60 min. The sample absorbance was read at 735 nm (Da Silva et al., 2019).

Evaluation of total flavonoid content

Evaluation of total flavonoid content was measured at 510 nm using aluminum chloride (Scapin et al., 2016). A solution of diluted aluminum chloride (250 μ l) and 5% sodium nitrate (75 μ l) was prepared. 10% aluminum chloride solution (150 μ l) was added after 5 min and kept at 25°C for 5 min. Eventually, the mixture was diluted by sodium hydroxide (500 μ l).

Evaluation of beta-carotene/ linoleic acid

Methanolic beta-carotene solution (20 mg beta-carotene in chloroform) was made and homogenized in linoleic acid and 200 mg of Tween 40 (28 μ l) (Miller, 1971). After evaporation of chloroform using rotary evaporator at 40°C, an emulsion was prepared by adding 50 ml distilled water and the process was repeated using butylate hydroxy anisole as the antioxidant standard. The absorbance was read at 470 nm every 15 min at same wavelength.

Fish and diets

Rainbow trout (n= 450, mean $\pm$ SE: 15.43 $\pm$ 0.06 g) were transported under continuous aeration to the lab and kept in 300-L fiberglass tanks (15 tanks, 30 fish/tank) with a constant flow of water. After 10 days of adaptation period, fish were fed 3 times a day with tested diets containing various values of parsley extract (PUE) in 5 groups for 60 days. Groups were in three replicates as follows: T1 (control): non-PUE fed fish, T2: fish supplemented with 0.5% PUE, T3: fish supplemented with 1.5% PUE, T4: fish supplemented with 3% PUE and T5: fish supplemented with 4.5% PUE. PUE levels in diets were selected according to those suggested by previous researches (Talpur, 2014; Farsani et al., 2019; Oroji et al., 2021; Yousefi et al., 2021 c). Test feeds were made by mixing PUE with a rainbow trout basal diet. The basal diet composition is presented in Table 2. To make experimental feeds, 300 ml distilled water was added to each 1 kg of basal diet and mixed to form dough. Then, the levels of PUE (0.5, 1.5, 3 and 4.5%) were added to the dough and mixed well for 20 min. The dough was finally mechanically converted into pellets by grinder, dried at 34°C and kept at 4°C. Water quality parameters during the experiment were checked daily for pH: 7.2–7.5; dissolved oxygen: 7.5–8 mg/l; temperature: 14–15°C; unionized ammonia: 0.006–0.01 mg/l.

Table 2. Feedstuffs and composition of the basal diet

Ingredients	(g/kg on dry basis)	Proximate composition	(g/kg)
Fishmeal ¹	320	Crude protein	425
Soybean meal (defatted) ²	260	Crude lipid	164
Wheat flour	168	Crude fiber	32
Meat meal ³	100	Crude ash	96
Fish oil	60		
Soybean oil	50		
Mineral mix ⁴	16		
Vitamin mix ⁴	10		
Cellulose	10		
Phytase ⁵	3		
DL-methionine ⁶	3		
Total	1000		

¹67% protein; 10% lipid, and % ash (Khazar Company, Mazandaeen Iran).

²Gorgan Soya Co., Gorgan, Iran (46% protein).

³60% protein and 18% lipid.

⁴The premixes were provided following Hosseini Shekarabi et al. (2021).

⁵Golbid Co., Tehran, Iran (10000 IU).

⁶Mad Tiour Co., Sanandaj, Iran.

Growth parameters

The biometry of fish was conducted weekly by sampling 5 fish per tank. After 60 days of feeding, the clove powder (140 mg/l)-anesthetized fish were starved for 24 h and growth performance was assayed, as follows:

$$\begin{aligned}
 \text{Weight gain (WG) (g)} &= FWe - IWe \\
 \text{SGR (\%)} &= 100 \times [(\ln FWe - \ln IWe) / \text{day}] \\
 \text{FCR} &= FI / (FWe - IWe) \\
 \text{PER} &= WG / [\text{total protein consumed (g)}] \\
 \text{Condition factor (CF) (g/cm}^3\text{)} &= (\text{whole body wet weight} / [\text{total length}]^3) \times 100 \\
 \text{SR (\%)} &= (Nf / Ni) \times 100
 \end{aligned}$$

Where, *FW*: final weight, *IW*: initial weight, *SGR*: specific growth rate, *FCR*: feed conversion ratio, *FI*: food consumed (g) during feeding period, *PER*: protein efficiency ratio, *SR*: survival rate, *Nf*: number of alive fish after the experiment period, *Ni*: number of alive individuals at the beginning of the experiment.

Ammonia stress experiment

After the experiment period, fish (n=20/tank) were randomly captured and considered for ammonia challenge. Fish were exposed to 0.3 mg/l ammonia (Darmstadt, Germany) for 24 h (Yousefi et al., 2020). During the challenge period, feeding and water flow in the tanks were stopped, but aeration was kept continuously using an air pump.

Collection of mucus and serum

The blood and mucus samples were collected in two times including 1) after 60 days feeding experiment and 2) after ammonia challenge. To assay biochemicals in serum, blood samples of three fish were taken after sedation with clove powder (140 mg/l) by a 1.5 ml syringe.

heparinized blood samples were left at room temperature for 2 h and then, serum was isolated by centrifugation (3000 × g) and kept at −70°C. Mucus was collected and prepared according to Ross et al. (2000).

Serum and mucus immune components

Lysozyme activity was measured at 550 nm upon lysis of *Micrococcus luteus* cell wall by serum and mucus (Ellis, 1990).

ACH₅₀ was estimated by Yano (1992) method using sheep red blood cells. Briefly, 500 µl of serum was serially diluted by veronal buffer in test tubes. 200 µl of red blood cell suspension was added to each tube and incubated for 90 min at 15°C. Hemolysis was stopped by adding 10 mmol EDTA gelatin veronal buffer. After centrifugation (1000 × g, 5 min), the rate of hemolysis in supernatant was measured at 414 nm.

Total immunoglobulins (total Ig) were calculated based on Siwicki (1993) method using 12% polyethylene glycol.

Bactericidal activity of mucus and serum against the bacterium *Yersinia ruckeri* (KC291153) was measured according to Rao et al. (2006). In this method, the bacterium was cultured on Nutrient Broth medium for 24 h and then bacterial cells were collected by centrifugation (3000 rpm for 15 min) and poured into sterile sodium phosphate buffer. The suspension was diluted using phosphate saline buffer and then 200 µl of serum or mucus sample was added to 2 ml of the suspension and kept at 24°C for 60 min. Finally, the suspensions were grown on tryptic soy agar media (TSA). The media was left for 24 h at 25°C and then bacterial colonies counted.

The total protein in serum and mucus was determined using the Bradford (1976) method. Albumin levels were also measured by the Nicholson method at 620 nm. The activity of mucus alkaline phosphatase (ALP) activity in mucus was measured by commercial kit (Pars Azmun CO., Iran) at 405.

Digestive enzymes

For digestive enzyme determinations, the intestine was removed from anesthetized fish (3 fish/tank) and homogenized in Tris buffer (Gisbert et al., 2016). The supernatant was separated by centrifugation (6000 × g) and kept at −80°C.

Amylase was assayed at 600 nm upon reaction of the enzyme on 2% starch solution as substrate (Robyt and Whelan, 1968). Lipase activity was colorimetrically assayed at 405 nm using polyphenol myristate (as target) at 25°C. Protease was measured at 440 nm upon reaction of the enzyme on azo-casein as substrate (García-Carreño, 1992).

Liver antioxidant enzymes

To assay antioxidant enzymes (CAT, GPx, and SOD), fish (n=3) were sampled from each tank randomly and after anesthetizing with clove powder, fish were dissected and liver tissue taken and homogenized in potassium phosphate buffer. The homogenized tissue was centri-

fuged (11,500 g for 16 min at 4°C) and the supernatant kept at -80°C till enzyme assays (Rafieepour et al., 2019). The enzymes were estimated using commercial kits according to manufacturer's protocol (Pars Azmun Co., Tehran, Iran).

Lipid peroxidation index

Oxidative stress was assayed by determining malondialdehyde (MDA) based on its reaction with thiobarbituric acid (TBARS) according to Rajabiesterabadi et al. (2020).

Liver metabolic enzymes

All liver enzymes (ALP, AST, and ALT) were assayed with commercial assay kits (Pars Azmun Co., Tehran, Iran) according to the manufacturer's instructions.

Glucose and cortisol

ELISA method was used to assay cortisol using an assay kit (IBL Co., Germany) and glucose determined colorimetrically by glucose oxidase-peroxidase (GOD-POD) method using commercial kit (Pars Azmun Co., Tehran, Iran).

Data evaluations

Data evaluations were performed by SPSS (Version 16, IBM, USA). Kolmogorov-Smirnov test, one-way ANOVA and Tukey test were used to evaluate data nor-

mal, identify differences and compare the means, respectively ($P < 0.05$).

Results

Growth

Growth results are presented in Table 3. The final weight (FW) showed increases ($P < 0.05$) after feeding experiment in T4 (3% PUE) and T5 (4.5% PUE) in comparison with other groups. WG was enhanced in fish of 3–4.5% PUE compared to non-PUE fish. FCR values were lower in T4 and T5 than in non-PUE and fish of T2. The values of CF were higher ($P < 0.05$) in T4 than in non-PUE fish, T2 and T3. The PER values were higher ($P < 0.05$) in T4 and T5 than in control and fish of T2 after feeding period. The values of SGR increased in T4 and T5 in comparison with non-PUE fish. The SR had no differences ($P > 0.05$) in all groups after the experiment.

Digestive enzymes

No changes in amylase activity ($P > 0.05$) were observed between PUE-treated and non-treated fish (Table 4). In fish of T2, T4 and T5, protease activity was higher ($P < 0.05$) compared to non-PUE fish, with highest activity in T4 (Table 4). The lipase activity in fish of 3–4.5% PUE was elevated ($P < 0.05$) compared to other diets (Table 4).

Table 3. The growth parameters and survival rate in rainbow trout, *Oncorhynchus mykiss* after 60 days supplementation with various dietary levels of *Petroselinum crispum* extract (PUE). T1 (control): non-supplemented fish, T2: fish supplemented with 0.5% PUE, T3: fish supplemented with 1.5% PUE, T4: fish supplemented with 3% PUE and T5: fish supplemented with 4.5% PUE. Data represent as mean $\pm$ SE. Different letters in the same row indicate significant differences ($P < 0.05$)

Parameters	T1 (control)	T2	T3	T4	T5
IW (g)	15.27 $\pm$ 0.13 a	15.33 $\pm$ 0.23 a	15.63 $\pm$ 0.12 a	15.50 $\pm$ 0.15 a	15.42 $\pm$ 0.08 a
FW (g)	60.00 $\pm$ 1.60 c	63.16 $\pm$ 1.58 c	65.50 $\pm$ 1.44 bc	73.00 $\pm$ 1.15 a	70.16 $\pm$ 1.16 ab
WG (g)	44.72 $\pm$ 1.58 c	47.83 $\pm$ 1.81 bc	49.86 $\pm$ 1.53 bc	57.50 $\pm$ 1.27 a	54.73 $\pm$ 1.12 ab
FCR	1.65 $\pm$ 0.06 a	1.57 $\pm$ 0.06 a	1.42 $\pm$ 0.07 ab	1.17 $\pm$ 0.04 b	1.20 $\pm$ 0.04 b
CF (g/cm ³)	0.87 $\pm$ 0.05 c	0.91 $\pm$ 0.02 c	1.01 $\pm$ 0.05 bc	1.41 $\pm$ 0.06 a	1.23 $\pm$ 0.01 ab
PER	1.28 $\pm$ 0.05 b	1.34 $\pm$ 0.04 b	1.49 $\pm$ 0.07 ab	1.80 $\pm$ 0.06 a	1.77 $\pm$ 0.11 a
SGR (%/d)	3.19 $\pm$ 0.04 c	3.32 $\pm$ 0.06 bc	3.39 $\pm$ 0.05 abc	3.61 $\pm$ 0.02 a	3.53 $\pm$ 0.02 ab
SR (%)	94.33 $\pm$ 2.96 a	96.33 $\pm$ 2.02 a	98.66 $\pm$ 1.33 a	95.33 $\pm$ 2.90 a	94.33 $\pm$ 2.96 a

IW: initial weight; FW: final weight; WG: weight gain; FCR: feed conversion ratio; CF: condition factor; PER: protein efficiency ratio; SGR: specific growth rate; SR: survival rate.

Table 4. The digestive enzymes in rainbow trout, *Oncorhynchus mykiss* after 60 days supplementation with various dietary levels of *Petroselinum crispum* extract (PUE). T1 (control): non-supplemented fish, T2: fish supplemented with 0.5% PUE, T3: fish supplemented with 1.5% PUE, T4: fish supplemented with 3% PUE and T5: fish supplemented with 4.5% PUE. Data represent as mean $\pm$ SE. Different letters in the same row indicate significant differences ($P < 0.05$)

Parameters	T1	T2	T3	T4	T5
Amylase (U/mg protein)	0.67 $\pm$ 0.12 a	0.95 $\pm$ 0.08 a	0.95 $\pm$ 0.07 a	0.99 $\pm$ 0.07 a	0.95 $\pm$ 0.08 a
Protease (U/mg protein)	2.85 $\pm$ 0.19 c	3.25 $\pm$ 0.19 bc	3.98 $\pm$ 0.10 ab	4.22 $\pm$ 0.21 a	3.61 $\pm$ 0.15 ab
Lipase (U/mg protein)	0.98 $\pm$ 0.04 b	1.15 $\pm$ 0.03 b	1.30 $\pm$ 0.09 b	1.88 $\pm$ 0.09 a	1.73 $\pm$ 0.11 a

Serum immunological components*Before ammonia challenge*

The immune components showed changes ($P<0.05$) in response to dietary parsley (Table 5). Lysozyme exhibited more activity ($P<0.05$) in T4 than in non-PUE fish in comparison with control. The ACH_{50} activity in T4 and T5 was elevated in comparison with non-PUE fish ($P<0.05$). The Ig value was higher ($P<0.05$) in parsley-supplemented fish in comparison with control after feeding experiment. In addition, Ig levels were higher ($P>0.05$) in fish of 1.5–4.5% PUE than those in T2. The bactericidal activity increased ($P<0.05$) in parsley-supplemented fish compared to non-treated fish, with the highest activity observed in fish of 3% PUE. The albumin concentrations were raised in T4 and T5. The total protein was elevated ($P<0.05$) in fish of 1.5–4.5% PUE in comparison with non-PUE fish. Higher protein content was obtained ($P<0.05$) in T4 and T5 than in T2.

After ammonia challenge

The immune components were altered in ammonia challenge (Table 5). Albumin concentration and lysozyme and ACH_{50} activities were elevated ($P<0.05$) in fish of 3–4.5% PUE compared to other diets. Furthermore, the ACH_{50} activity in T3 was higher ($P<0.05$) than in non-PUE fish. Higher levels of Ig were obtained ($P<0.05$) in T3, T4 and T5 in comparison with other diets. In addition,

the Ig content in T4 and T5 was higher ($P<0.05$) than T3. Bactericidal activity in fish of 1.5–4.5% PUE was elevated ($P<0.05$) compared to non-PUE fish, with highest activity found in T4. The protein concentrations in fish of 1.5–4.5% PUE increased ($P<0.05$) in comparison with non-PUE fish.

Mucus immunological components*Before ammonia challenge*

The lysozyme activity of T4 fish increased ($P<0.05$) in comparison with non-PUE fish and T2 (Figure 1 A). The ALP in T3, T4 and T5 was elevated ($P<0.05$) compared to non-PUE fish (Figure 1 B). The bactericidal activity increased in PUE-fed fish (Figure 1 C). Total protein in fish of 3–4.5% PUE increased ($P<0.05$) in comparison with other diets (Figure 1 D).

After ammonia challenge

The lysozyme activity increased ($P<0.05$) in 1.5–4.5% PUE treatments after ammonia exposure (Figure 1 A). The ALP activity in T4 and T5 was elevated ($P<0.05$) compared to non-PUE fish and T2 (Figure 1 B). With highest activity in fish of 4.5% PUE, bactericidal activity in T3, T4 and T5 increased ($P<0.05$) compared to non-PUE fish, as the number of CFUs were lower in these treatments (Figure 1 C). The total protein concentrations in T4 and T5 increased ($P<0.05$) compared to other diets (Figure 1 D).

Table 5. The serum immune components in rainbow trout, *Oncorhynchus mykiss* after 60 days supplementation with various dietary levels of *Petroselinum crispum* extract (PUE) and after ammonia challenge. T1 (control): non-supplemented fish, T2: fish supplemented with 0.5% PUE, T3: fish supplemented with 1.5% PUE, T4: fish supplemented with 3% PUE and T5: fish supplemented with 4.5% PUE. Data represent as mean $\pm$ SE. Different letters in the same row indicate significant differences ($P<0.05$)

Status	Parameters	T1 (control)	T2	T3	T4	T5
Before challenge	Lysozyme (U/ml)	19.90 $\pm$ 0.46 b	20.81 $\pm$ 0.43 ab	21.31 $\pm$ 0.53 ab	22.30 $\pm$ 0.54 a	21.58 $\pm$ 0.44 ab
	ACH_{50} (U/ml)	269.16 $\pm$ 1.88 c	272.06 $\pm$ 1.21 bc	273.74 $\pm$ 1.27 bc	277.66 $\pm$ 1.72 b	286.91 $\pm$ 1.72 a
	Total Ig (mg/dL)	23.29 $\pm$ 0.70 c	27.30 $\pm$ 0.97 b	30.33 $\pm$ 1.12 ab	33.33 $\pm$ 0.85 a	31.71 $\pm$ 1.09 a
	Bactericidal (log cfu/ml)	4.37 $\pm$ 0.17 a	3.70 $\pm$ 0.13 b	3.32 $\pm$ 0.11 bc	2.85 $\pm$ 0.16 c	3.00 $\pm$ 0.07 c
	Albumin (mg/dL)	1.43 $\pm$ 0.09 c	1.64 $\pm$ 0.10 bc	1.63 $\pm$ 0.06 bc	2.20 $\pm$ 0.10 a	2.01 $\pm$ 0.11 ab
	Total Protein (mg/dL)	3.16 $\pm$ 0.05 c	3.24 $\pm$ 0.06 bc	3.56 $\pm$ 0.11 ab	3.93 $\pm$ 0.11 a	3.86 $\pm$ 0.10 a
After challenge	Lysozyme (U/ml)	13.70 $\pm$ 0.77 b	15.42 $\pm$ 0.58 b	16.17 $\pm$ 0.53 b	23.01 $\pm$ 0.55 a	21.69 $\pm$ 0.86 a
	ACH_{50} (U/ml)	243.16 $\pm$ 1.95 c	251.13 $\pm$ 2.78 bc	258.71 $\pm$ 2.05 b	276.19 $\pm$ 1.80 a	267.60 $\pm$ 1.85 a
	Total Ig (mg/dL)	14.91 $\pm$ 0.83 c	16.43 $\pm$ 0.90 c	20.52 $\pm$ 0.98 b	27.36 $\pm$ 0.76 a	24.44 $\pm$ 0.92 a
	Bactericidal (log cfu/ml)	7.69 $\pm$ 0.43 a	6.62 $\pm$ 0.36 ab	4.98 $\pm$ 0.30 c	4.37 $\pm$ 0.27 c	5.51 $\pm$ 0.22 bc
	Albumin (mg/dL)	1.31 $\pm$ 0.06 b	1.53 $\pm$ 0.08 b	1.52 $\pm$ 0.07 b	2.17 $\pm$ 0.12 a	1.95 $\pm$ 0.11 a
	Total Protein (mg/dL)	3.04 $\pm$ 0.04 c	3.10 $\pm$ 0.05 c	3.38 $\pm$ 0.09 bc	3.88 $\pm$ 0.11 a	3.65 $\pm$ 0.12 ab

Total Ig: total immunoglobulins.

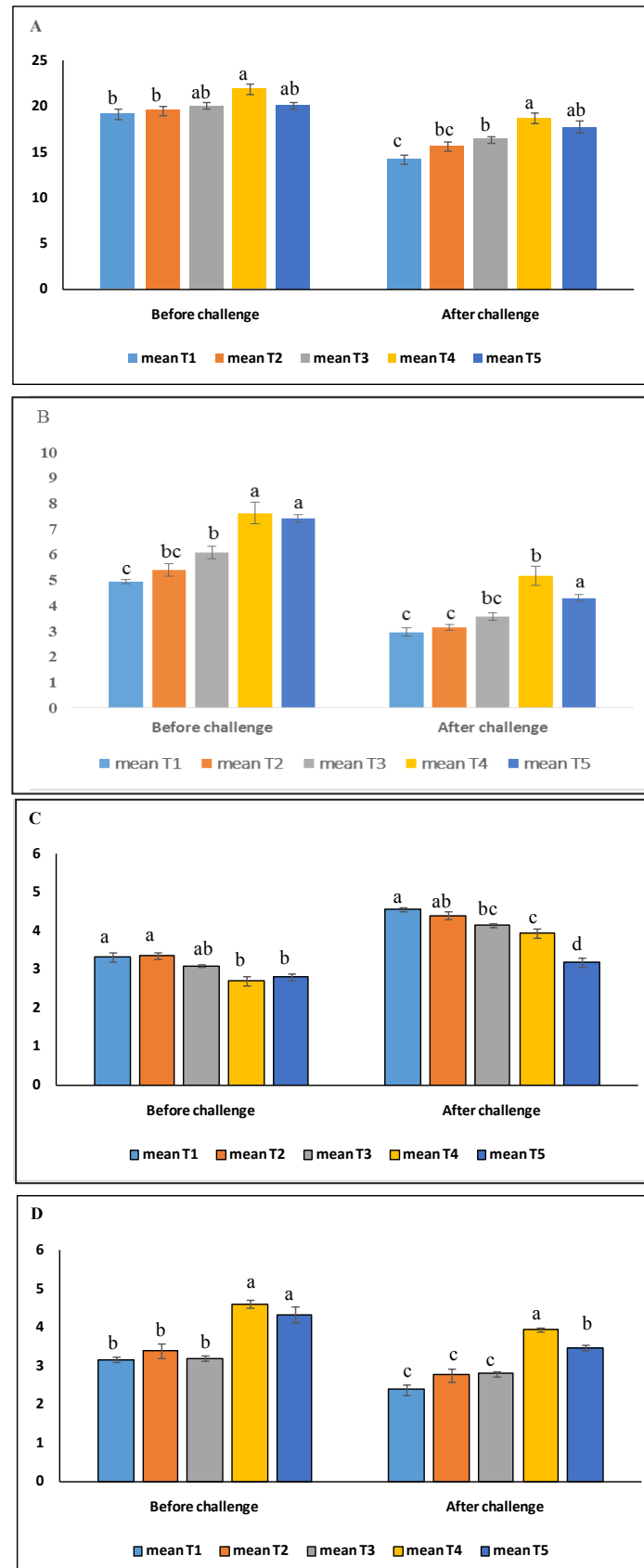


Figure 1. The mucosal levels of immune components (**A**: Lysozyme (U/mg protein); **B**: ALP (alkaline phosphatase) activity (U/l); **C**: Bactericidal activity (log cfu/ml); **D**: Total protein (mg/dL)) in rainbow trout, *Oncorhynchus mykiss* after 60 days supplementation with various dietary levels of *Petroselinum crispum* extract (PUE) and after ammonia challenge. T1 (control): non-supplemented fish, T2: fish supplemented with 0.5% PUE, T3: fish supplemented with 1.5% PUE, T4: fish supplemented with 3% PUE and T5: fish supplemented with 4.5% PUE. Data represent as mean $\pm$ SE. Different letters on bars indicate significant differences ($P < 0.05$)

Table 6. The liver antioxidant enzymes and malondialdehyde (MDA) levels in rainbow trout, *Oncorhynchus mykiss* after 60 days supplementation with various dietary levels of *Petroselinum crispum* extract (PUE) and after ammonia challenge. T1 (control): non-supplemented fish, T2: fish supplemented with 0.5% PUE, T3: fish supplemented with 1.5% PUE, T4: fish supplemented with 3% PUE and T5: fish supplemented with 4.5% PUE. Data represent as mean $\pm$ SE. Different letters in the same row indicate significant differences ($P<0.05$)

Status	Parameters	T1 (control)	T2	T3	T4	T5
Before challenge	MDA (nM/ml)	6.30 $\pm$ 0.09 a	5.93 $\pm$ 0.19 a	4.73 $\pm$ 0.20 b	3.62 $\pm$ 0.15 c	3.81 $\pm$ 0.15 c
	SOD (U/ml)	58.09 $\pm$ 0.75 c	61.75 $\pm$ 1.87 bc	64.84 $\pm$ 0.99 b	75.35 $\pm$ 1.41 a	74.97 $\pm$ 1.05 a
	CAT (U/ml)	16.91 $\pm$ 0.38 d	17.69 $\pm$ 0.27 cd	19.27 $\pm$ 0.28 bc	22.23 $\pm$ 0.71 a	20.15 $\pm$ 0.71 ab
	GPx (U/ml)	0.43 $\pm$ 0.02 c	0.62 $\pm$ 0.03 b	0.69 $\pm$ 0.03 b	1.02 $\pm$ 0.04 a	1.01 $\pm$ 0.04 a
After challenge	MDA (nM/ml)	7.67 $\pm$ 0.31 a	7.22 $\pm$ 0.21 ab	6.60 $\pm$ 0.23 b	4.36 $\pm$ 0.21 c	4.79 $\pm$ 0.28 c
	SOD (U/ml)	56.85 $\pm$ 0.73 c	62.83 $\pm$ 1.76 b	65.71 $\pm$ 0.91 b	76.44 $\pm$ 1.06 a	76.09 $\pm$ 1.32 a
	CAT (U/ml)	11.81 $\pm$ 0.52 d	15.52 $\pm$ 0.37 b	16.32 $\pm$ 0.45 b	21.56 $\pm$ 0.62 b	18.90 $\pm$ 0.78 a
	GPx (U/ml)	0.39 $\pm$ 0.02 c	0.55 $\pm$ 0.02 b	0.66 $\pm$ 0.03 b	1.02 $\pm$ 0.05 a	0.97 $\pm$ 0.04 a

MDA: malondialdehyde; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase.

Table 7. The serum levels of liver metabolic enzymes in rainbow trout, *Oncorhynchus mykiss* after 60 days supplementation with various dietary levels of *Petroselinum crispum* extract (PUE) and after ammonia challenge. T1 (control): non-supplemented fish, T2: fish supplemented with 0.5% PUE, T3: fish supplemented with 1.5% PUE, T4: fish supplemented with 3% PUE and T5: fish supplemented with 4.5% PUE. Data represent as mean $\pm$ SE. Different letters in the same row indicate significant differences ($P<0.05$)

Status	Parameters	T1	T2	T3	T4	T5
Before challenge	ALT (U/L)	26.91 $\pm$ 0.76 a	25.11 $\pm$ 0.38 ab	25.03 $\pm$ 0.87 ab	22.48 $\pm$ 0.62 b	24.29 $\pm$ 0.77 ab
	AST (U/L)	332.57 $\pm$ 0.85 a	328.92 $\pm$ 1.61 ab	325.34 $\pm$ 1.33 bc	319.23 $\pm$ 1.14 d	320.21 $\pm$ 1.57 cd
	ALP (U/L)	503.66 $\pm$ 2.24 a	492.16 $\pm$ 0.83 b	491.33 $\pm$ 1.28 b	479.50 $\pm$ 1.83 c	487.20 $\pm$ 1.01 b
	LDH (U/L)	753.00 $\pm$ 4.08 a	746.16 $\pm$ 5.43 ab	736.00 $\pm$ 4.44 ab	736.16 $\pm$ 3.71 ab	735.16 $\pm$ 2.18 b
After challenge	ALT (U/L)	38.24 $\pm$ 0.95 a	35.77 $\pm$ 0.83 a	32.03 $\pm$ 0.73 b	23.96 $\pm$ 0.45 c	25.66 $\pm$ 0.61 c
	AST (U/L)	375.90 $\pm$ 2.05 a	368.92 $\pm$ 1.80 ab	362.00 $\pm$ 2.68 b	339.23 $\pm$ 3.04 c	344.56 $\pm$ 2.40 c
	ALP (U/L)	547.16 $\pm$ 2.18 a	526.16 $\pm$ 2.24 b	512.16 $\pm$ 1.35 c	482.66 $\pm$ 2.84 e	492.66 $\pm$ 1.14 d
	LDH (U/L)	800.83 $\pm$ 2.52 a	784.50 $\pm$ 2.52 b	767.66 $\pm$ 2.91 c	741.00 $\pm$ 1.65 d	747.66 $\pm$ 1.87 d

ALT: alanine aminotransferase; AST: aspartate transaminase; ALP: alkaline phosphatase; LDH: lactate dehydrogenase.

Antioxidant enzymes and MDA in liver

Before ammonia challenge

The activity of SOD increased in T4 and T5 compared to others (Table 6, $P<0.05$). SOD activity was elevated in 1.5% PUE fish compared to non-PUE fish (Table 6). GPx activity increased ($P<0.05$) in parsley-supplemented fish compared to non-PUE fish (Table 6). The highest levels ($P<0.05$) of GPx were recognized in T4 and T5, respectively (Table 6). The CAT activity increased ($P<0.05$) in 1.5–4.5% PUE treatments (Table 6). MDA decreased ($P<0.05$) in fish of 1.5–4.5% PUE compared to non-PUE fish (Table 6). The lowest MDA value was found in 3% PUE fish (Table 6, $P<0.05$).

After ammonia challenge

The activity of SOD, CAT and GPx with highest levels in fish of 3–4.5% PUE increased ($P<0.05$) in PUE-fed fish (Table 6). MDA showed decreases ($P<0.05$) in fish of 3–4.5% PUE in comparison with control (Table 6).

Liver metabolic enzymes

Before ammonia challenge

The activity of ALT in T4 was lower ($P<0.05$) than those in non-PUE fish after feeding period (Table 7). AST activity in fish of 3–4.5% PUE considerably decreased ($P<0.05$) compared to non-PUE fish (Table 7). ALP activity was reduced in PUE-fed fish compared to non-PUE individuals (Table 7, $P<0.05$). The lowest ALP activity ($P<0.05$) was found in T4 and T5, respectively (Table 7). In T5, LDH activity decreased ($P<0.05$) compared to non-PUE fish (Table 7).

After ammonia challenge

The activity of ALT in fish of 1.5–4.5% PUE declined ($P<0.05$) compared to others. The lowest ($P<0.05$) ALT activity was found respectively (Table 7). AST activity in T4 and T5 declined ($P<0.05$) compared to other groups (Table 7). With lowest activity in T4 and T5 respectively, ALP and LDH activities were reduced (with lowest activity in T4 and T5 respectively) in all parsley-supplemented fish (Table 7, $P<0.05$).

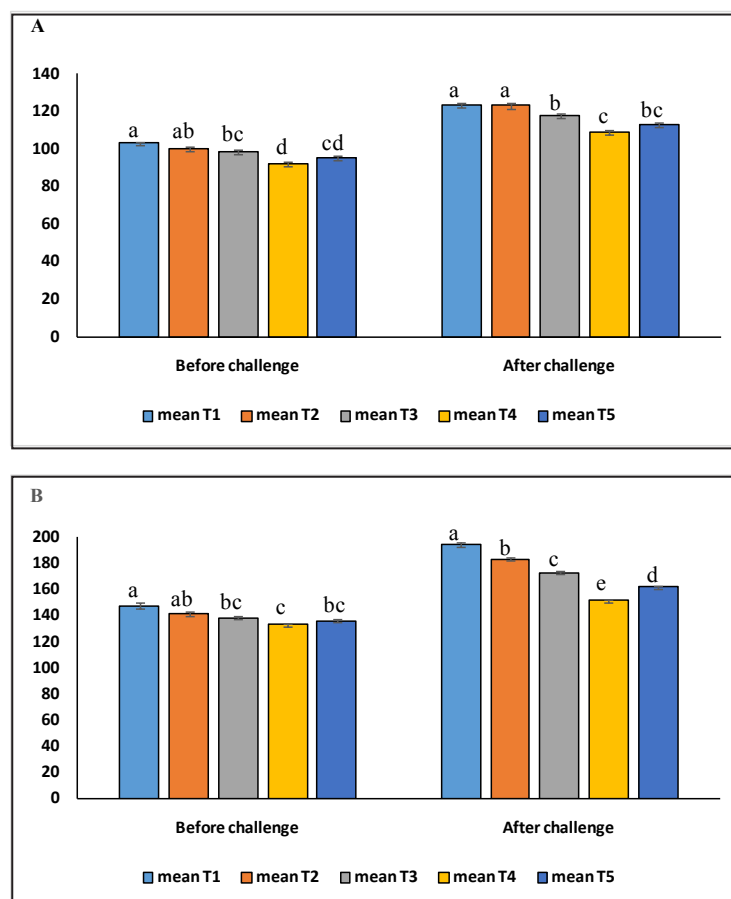


Figure 2. The serum levels of (A): glucose (mg/dL) and (B): cortisol (ng/ml) in rainbow trout, *Oncorhynchus mykiss* after 60 days supplementation with various dietary levels of *Petroselinum crispum* extract (PUE) and after ammonia challenge. T1 (control): non-supplemented fish, T2: fish supplemented with 0.5% PUE, T3: fish supplemented with 1.5% PUE, T4: fish supplemented with 3% PUE and T5: fish supplemented with 4.5% PUE. Data represent as mean $\pm$ SE. Different letters on bars indicate significant differences ($P < 0.05$)

Glucose and cortisol

Before ammonia challenge

Glucose (Figure 2 A) and cortisol (Figure 2 B) concentrations were lower ($P < 0.05$) in T4 and T5 compared to the control and T2 after feeding period.

After ammonia challenge

Glucose (Figure 2 A) had higher levels ($P < 0.05$) in 1.5–4.5% PUE fish than in non-PUE fish ($P < 0.05$). Cortisol concentrations declined ($P < 0.05$) in all fish fed parsley (Figure 2 B). Lowest levels ($P < 0.05$) of cortisol were observed in T4 and T5, respectively (Figure 2 B).

Discussion

Plant-based probiotics are widely used in aquaculture as natural growth and immune boosting components (Awad and Awaad, 2017; Mohammadi et al., 2020; Al-Shawi et al., 2021; Mansour et al., 2022; Yousefi et al., 2022; Almarri et al., 2023; Mansour et al., 2023). Poten-

tials of dietary parsley extract (PUE) were assayed on growth, immunity and resistance of the fish to ammonia stress. PUE significantly improved growth indices, almost all in fish supplemented with dietary levels of 3–4.5%. These results are similar to those that reported the improving effect of plant extracts on fish growth (Yousefi et al., 2021 b, c; Rudiansyah et al., 2022). In koi fish (*Cyprinus carpio*), supplementation of fishes with 0.5% parsley in diet significantly improved growth, FCR and survival rate (Mooraki et al., 2014). However, supplementation of rainbow trout with lyophilized Chinese parsley, *Coriandrum sativum* had no considerable effects on the fish growth (Ren et al., 2006). In this study, the improving effects of PUE on the fish growth may be associated with increased activity of digestive enzymes and digestion efficiency, as the PER value and the activity of protease and lipase increased over 60 days feeding experiment in the treatments T4 and T5. Various studies have also shown the promoting impacts of plant extracts on digestive enzymes of fishes (Zemheri-Navruz et al., 2020; Zhang et al., 2020; Oroji et al., 2021; Raissy et al., 2022). Although the mechanism of action of parsley on

digestive enzymes is still unknown in fish, other studies have shown that some herbal supplements or their derivatives may act as appetizers and stimulated the secretion of digestive enzymes (Akbari et al., 2018; Rashidian et al., 2022). In addition, the role of parsley as appetizer is reported (Amin et al., 2007; Gholami et al., 2014). Therefore, the increasing effect of parsley on digestive enzymes may be related to the role of this plant as an appetizer.

In this study, dietary PUE enhanced the immune system of the fish by increasing Ig levels, lysozyme, ACH₅₀, bactericidal activities, total protein and albumin in serum and also by increasing ALP, lysozyme, bactericidal activities and total protein concentrations in skin mucus. In this regard, the immunostimulating impacts of PUE were more pronounced in the treatments T4 and T5. Lysozyme as an important component of innate immunity destructs bacterial cell wall (Ellis, 1999). Although the immunogenic mechanism of parsley has not been discovered in fish, few studies have shown that the immunogenic effects of parsley may be due to the presence of some immunogenic compounds in its chemical composition such as α -pinene, D-limonene, myristicin, and oleic acid (Farag et al., 2022).

Antibodies and albumin compose total protein and thus total protein can indicate the immune status in fish (Corbel, 1975). Ig is involved in fish immunity through pathogen opsonization and toxin neutralization (Mashoof and Criscitiello, 2016). The complement components include a range of plasma-soluble proteins that are involved in inflammatory reactions and also opsonization of pathogens (Boshra et al., 2006).

Antioxidant system is the first and strong obstacle against toxin-induced oxidative stress in fish (Lushchak, 2016; Hoseinifar et al., 2020). Dietary PUE improved antioxidant system, as SOD, GPx and CAT activities in liver increased in the supplemented fish, especially in those fed 3–4.5% PUE.

In addition, the antioxidant properties of parsley may be a result of some antioxidant compounds, such as kaempferol and quercetin, apigenin and luteolin in its biochemical composition (Duthie, 1999; Fukumoto and Mazza, 2000; Haidari et al., 2011; Stan et al., 2012).

Although the promoting effects of plant-based supplements on the immune and antioxidants system of fish have been reported a lot, there is no data on the antioxidant and immune boosting effects of parsley in fish. Therefore, our study presented new information about the immunogenic effects of parsley in fish.

Dietary PUE also prompted the immune and antioxidant system against ambient ammonia stress, as the serum and mucus immune components and antioxidant enzymes increased in the treated fish after ammonia challenge. These results are in line with studies that have previously reported the ameliorating effects of plant-based supplements on the toxicity induced by ammonia stress.

In this study, 1.5–4.5% PUE reduced MDA levels both before and after ammonia stress compared to con-

trols. The MDA reflects the oxidative stress status in vertebrates including fish (Mendes et al., 2009; Slaninova et al., 2009; Khoubnasabjafari et al., 2016; Ghafarifar-sani et al., 2021 a, b). Therefore, a decline in MDA of treated fish may confirm the role of PUE in ameliorating ammonia-induced oxidative stress. This result supported the researches reporting the mitigating impacts of plant-based compounds in fish on ammonia-induced oxidative stress (Mirghaed et al., 2019, 2020).

Dietary PUE reduced stress in fish both before and after ammonia challenge, as cortisol and glucose concentrations declined in the fish fed PUE. Cortisol, as the most important stress hormone acts on liver to break down glycogen stores and release glucose to blood to meet the energy demands for stressful conditions (Vijayan et al., 2010). Increases in cortisol and glucose following ammonia stress can be mitigated by herbal supplements (Yousefi et al., 2020; Hoseini et al., 2021). Therefore, the lower levels of cortisol and glucose in PUE supplemented fish than in control may suggest an ameliorating effect for PUE against ammonia stress.

Liver metabolic enzymes (ALP, AST, ALT, and LDH) play an important role in detoxifying xenobiotics. However, increased levels of these enzymes in the blood may indicate liver damage and dysfunction (Ghelichpour et al., 2020). Serum levels of liver metabolic enzymes both before and after ammonia stress showed considerable decreases in fish of 1.5–4.5% PUE in comparison with control, which may indicate the mitigating effects of PUE on liver dysfunction and hepatocyte lesions. In consistence with our results, other plant-based supplements also reduced the liver metabolic enzymes in blood in fish exposed to ammonia (Mirghaed et al., 2019) and other stressors (Hegazi et al., 2010; Banaei et al., 2015; Al-Shawi et al., 2021).

Conclusion

In conclusion, PUE improved growth and immunity in the rainbow trout and also mitigated the stress induced by the ambient ammonia. The growth promoting effects of PUE may be due to its stimulating impacts on digestive enzyme activities. PUE also had protective impacts on liver and enhanced antioxidant defense by enhancing antioxidant enzyme levels. The immune enhancing impacts of PUE may be attributed to some immunogenic bioactive compounds in its biochemical composition. Therefore, use of PUE in optimized dietary levels (3–4.5%) may be useful for trout aquaculture enhancement.

Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Funding statement

There is no government or organizational fund for this work.

Conflict of interest

Authors have no conflict of interest to declare for the publication of the present work.

Data availability

The data that support the findings of this study are available upon reasonable request to the corresponding authors.

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Received: 18 VII 2023

Accepted: 27 XI 2023