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Dietary chestnut bee pollen as an immunostimulant for rainbow trout (*Oncorhynchus mykiss*): Effects on the growth, haematological values, immune response, oxidant/antioxidant status, and survival against *Aeromonas salmonicida* subsp. *achromogenes*

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ABSTRACT

The present study was designed to assess the influence of dietary supplementation with chestnut bee pollen at various levels in rainbow trout, *Oncorhynchus mykiss*. For two weeks feeding period, a total of 300 fish were allocated into 12 fiberglass tanks and divided into four equal groups, three replicates each, with chestnut bee pollen (BP) dietary inclusion as follows; the fish group was given a basal diet (C); fish group fed a diet supplemented with BP 1% (BP-1); fish group fed a diet supplemented with BP 2% (BP-2); and fish group fed a diet supplemented with BP 4% (BP-3). At the end of the experiment, growth, haematological values, immune status, antioxidant status, and survival rate against *Aeromonas salmonicida* subsp. *achromogenes* were evaluated. Dietary supplementation with chestnut bee pollen significantly improves growth performance. Fish fed the diets containing chestnut bee pollen had higher the haematological values than those fed the control diet. The results showed that all the immunological parameters in the groups fed with chestnut bee pollen were significantly higher when compared to the control group. Moreover, dietary chestnut bee pollen increased disease resistance against *Aeromonas salmonicida* subsp. *achromogenes* compared to the control group. The tissue SOD, CAT and GSH-Px activities of groups fed with chestnut bee pollen significantly enhanced when compared with the control groups. In contrast, the tissue MDA levels in all groups fed with chestnut bee pollen were significantly decreased. The best values for the antioxidant parameters were determined in the groups fed with 2 and 4% of chestnut bee pollen. Overall, these findings suggest that dietary chestnut bee pollen enhances the growth, the haematological values, the immune and antioxidant response and increases disease resistance against rainbow trout.

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Introduction

Fish farmers use different veterinary medicines and chemotherapeutic agents to treat or prevent fish diseases, which results in enormous economic losses in aquaculture on a global scale. The intensive use of these agents in aquaculture has led to an increase in antibiotic-resistant bacterial genes that are transferred to animals and humans which damages bacterial flora (1,2). Also, they cause immunosuppression, oxidative stress, nephrotoxicity, growth retardation, environmental pollution and accumulation of chemicals in aquatic animal tissues, which can be possibly dangerous to public health (3-5). Therefore, natural immunostimulants such as probiotics, prebiotics, symbiotics, complex carbohydrates, nutritional factors, herbs, hormones, and cytokines are an alternative to chemical agents (4,6,7).

Because natural immunostimulants have anti-stress, antioxidant, immunostimulant, and growth-promoting effects, they are suitable feed additives for aquaculture (8). Bee pollen, a natural product, is a floral type of pollen collected by honeybees. The bee pollen is essential for the reproduction and survival of these creatures (9). The bee pollen is the result of flower pollens agglutination produced by worker honeybees with nectar and salivary components. It is usually used as a prime nutrient resource for the adult and larval development of bees in daily life (10,11). On the other hand, it has bioactive molecules such as protein, amino acids, carbohydrates, fats, polysaccharides, vitamins, minerals, flavonoids, and phenolic components)9,12). These compounds process a wide range of biological functions which include antiinflammatory, anticarcinogenic, antitumor, antioxidant, antibacterial, antiviral, hepatoprotective and hyperglycemic activities, especially immunomodulatory activity (10,11).

Limited data exist regarding the effect of bee pollen on fish. For example, El-Asely et al. (13) have documented that the administration of bee pollen can stimulate immunological, haematological, biochemical and growth parameters in Nile tilapia, *Oreochromis niloticus*. However, there is no detailed study on the effect of bee pollen on the growth, haematology, immunity, antioxidant activity, and disease resistance in rainbow trout, *Oncorhynchus mykiss*, and other important aquaculture species. The study herein,

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therefore, examines the effects of bee pollen on growth performance, haematological values, cellular and humoral immunity, oxidant/antioxidant status, and disease resistance against *A. salmonicida* subsp. *salmonicida* infection in rainbow trout.

Materials and Methods

Collection and chemical analysis of bee pollen

The bee pollen was collected by means of special traps, aimed at foraging bees, placed at the entrance of honeybee hives within the vicinity of Zonguldak province in Turkey. The palynological identification of bee pollen was done by Prof. Dr. Sibel Silici in Erciyes University, Turkey. Nine families of pollen pellets were found in the sample; however, *Castanea sativa* was the dominant species in the pollen mixture.

Firstly, the chestnut bee pollen was ground and then dried at approximately 30 °C without exposure to light. The ground bee pollen was stored at 2–8 °C in desiccators, until extraction. The chemical analysis of chestnut bee pollen was determined as described by the Association of Official Agricultural Chemists (AOAC) standard methods (14-16). pH value was monitored directly from the pH meter (WTW-Inolab, Weilheim, Germany). Color values of the samples were analyzed by using an automatic colorimeter (Lovibond RT Series Reflectance Tintometer). Lovibond tintometer gives three values, *L*, *a**, and *b**. *L* (lightness) values measure the level of black to white (0 to 100), *a** red to green (+=red and −=green), and *b** yellow to blue (+=yellow and −=blue). Main sugar compositions in the chestnut bee pollen samples were determined by high-performance liquid chromatography [(High-Performance Liquid Chromatography-Refractive Index Detector (HPLC-RID, Agilent 1100 Series, USA), equipped with a manual injection quaternary pump (U.S.A) and Zorbax carbohydrate column (4.6x250 mm, 5 µm particle size)]. The antioxidant characteristics of chestnut bee pollen were prepared as described by Singleton and Rossi (17) and Ulusoy and Kolayli (18).

Experimental diets

A commercial pellet diet (including 45% crude protein, 20% crude fat, 11% ash, 3% crude fibre, 8.5% moisture, 12.5% nitrogen-free extract, and 5124 kcal/kg gross energy) was crushed and divided into three parts. Also, the bee pollen samples were divided into three portions of 10, 20 and 40 g to obtain doses of 1, 2 and 4%/kg in the commercial pellet diet (El-Asely et al., 2014). Each dose of the bee pollen was separately dissolved in 1 L of water. The water containing the bee pollen was added to the diet portions at a 1:1 ratio. The diets were reformed into pellets, spread to dry and stored at +4 °C for the feeding experiment. The experimental diets are referred to as the control (without bee pollen), BP-1 (1% bee pollen), BP-2 (2% bee pollen), and BP-4 (4% bee pollen).

Fish and experimental procedure

Rainbow trout (*Oncorhynchus mykiss*) fingerlings (~ 20 g) were purchased from a private farm (Keban, Elazig, Turkey) and monitored for health conditions through external and internal macroscopic examinations upon arrival. Fish were quarantined and bathed in 250 ppm formalin for 30 min (19). All fish were acclimatized for 30 days in 1000 L tanks during which were fed with the commercial pellet diet until satiation twice a day in two equal parts at 9.00 a.m. and 4.00 p.m.

After the acclimation period, a total number of 300 fish were stocked in 12 fiberglass tanks (100 L) at a density of 25 fish per tank, to have triplicates for each of the four experimental groups. Control group (C) was fed the basal diet without the bee pollen. The remaining groups were fed with 1% (BP-1), 2% (BP-2), and 4% (BP-3) bee pollen for 8 weeks. All experimental groups were fed with the abovementioned diets at 2.5% of biomass within 8 weeks. The biomass in each tank was weighed biweekly to adjust the feed amount.

The static water system with daily exchange was used in this study and the tanks were continuously aerated. Water exchange was done daily at a rate of 50% and 100% of the water was exchanged once a week. Water quality was monitored daily throughout the experiment. The water quality parameters containing temperature (16 ± 1 °C), dissolved oxygen (7.7 mg L^{-1}), and pH (7.4 ± 0.2), were measured daily during the experimental period.

Growth

On the 8th week of treatment, ten fish randomly from each experimental tank were collected to calculate growth performance including weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), and survival. The growth parameters were calculated as follows:

$WG\ (g) = Wt - W_0$;

$SGR\ (\%) = 100 \times (\ln Wt - \ln W_0)/t$;

$FCR = FI/(Wt - W_0)$.

(Wt and W_0: Final and initial weight of fish, respectively; t: experimental days; FI: Feed intake)

Sample collection

The fish blood was sampled (ten fish from each tank, a total of thirty fish per dietary treatment) for haematological and immunological assays at the end of the 8-week feeding trial. Fish were gently caught and anaesthetized in benzocaine solution (25 mg/L water). The blood samples were taken from the caudal vein with plastic syringes and immediately apportioned into two equal parts with (K_3EDTA) or without anticoagulant-coated tubes. The anticoagulant tubes containing blood samples were used immediately for the haematology [red blood cell (RBC) count, haemoglobin (Hb) and haematocrit (Ht) levels, and erythrocyte indices: mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC)] and immunology [white blood cell (WBC) count and types (lymphocytes (LYM), monocytes (MON), neutrophils (NEU), eosinophils (EOS), and basophil (BAS)), oxidative radical production (nitroblue tetrazolium (NBT) assay), phagocytic activity (PA) and phagocytic index (PI)] assays. The blood tubes without anticoagulant were allowed to clot at room temperature for 30 min, then centrifuged to collect the serum carefully at 5000 *g* for 10 min and reserved in new sterile eppendorf tubes at −20 °C until used for the other immunological analysis [total protein (TP) and immunoglobulin M (IgM) levels, serum lysozyme (LYZ) and myeloperoxidase (MPO) activities].

After the blood samples were collected, the liver, head kidney and spleen were carefully removed, washed with physiological saline (0.9 % NaCl) and stored at −40 °C un-

til the biochemical assays, which were performed within 1 month after extraction. The tissue was homogenised in a Teflon-glass homogeniser in a buffer containing 1.15% potassium chloride (KCl) at a 1:10 (w/v) ratio to the whole homogenate. The homogenate was centrifuged at 18,000 g at 4 °C for 30 min to determine malondialdehyde (MDA) level and superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities.

Haematological and immunological analysis

The RBC and WBC counts were simultaneously performed using a haemocytometer and the solution developed by Natt and Herrick (20). The LYM, MON, NEU, EOS, and BAS counts were conducted on May-Grunwald-Giemsa stained blood smears. The Ht levels were measured through a microhematocrit centrifugation technique (12.000 *g*, 5 min) in capillary glass tubes. The levels of Hb were determined using Drabkin's reagent at 540 nm (21). The erythrocyte indices (MCV, MCH, and MCHC) were calculated through the following formulas using the Ht, RBC, and Hb data (22-24).

MCV (μm^3) = Ht x 10/ RBC count

MCH (pg) = Hb x 10 / RBC count

MCHC (%) = Hb x 100/ Ht

To analyse radical oxygen production formed from phagocytes was used the NBT assay (25). The PA and PI were determined according to the method described by Siwicki and Anderson (26). The serum TP and IgM levels were determined, according to the protocol described by Siwicki et al. (25), Siwicki et al. (27), and Yonar (28). The serum LYZ activity was measured based on the sample ability to lyse Micrococcus luteus (29). The serum MPO activity was performed following the protocol provided by Quade and Roth (30) with a slight modification of Sahoo et al. (31).

Bacteria and challenge

A. salmonicida subsp. *salmonicida* (NCMB 1102) was used for the challenge test. *A. salmonicida* subsp. *salmonicida* was grown in blood agar for 24 h at 22 °C. The fresh culture was suspended in sterile PBS. The cell suspension was adjusted to an optical density (OD) 0.9 at 610 nm.

At the end of the feeding trial, the remaining fish from each tank (fifteen fish/tank) were challenged with pathogenic *A. salmonicida* subsp. *salmonicida*. A 0.1-ml dose of 24-h brain heart infusion agar (BHI) with the virulent bacterial pathogen of *A. salmonicida* subsp. *salmonicida* (1.2×10^8 cfu/ml) was given by intraperitoneal injection. The challenge dose of bacteria was selected based on the previous studies (7). The challenged fish were kept under observation for 14 days to record any abnormal clinical signs and the daily mortality rate. All groups were fed only the control diet. The cause of death was confirmed by re-isolating the organism from the liver and spleen of dead fish using conventional methods.

Relative percentage survival (RPS) was quantified using the following formula (32):

RPS (%) = (Number of surviving fish after challenge/ Number of fish injected with *A. salmonicida* subsp. *salmonicida*) ×100.

Oxidative stress and antioxidant activity in tissues

The MDA levels of tissues, an index of oxidative stress, were determined with the thiobarbituric acid (TBA) reaction (33). The tissue SOD, CAT, and GSH-Px activities were determined following the methods of Sun et al. (34), Aebi (35), and Beutler (369, respectively.

To calculate oxidant/antioxidant parameters the protein levels in the tissues were determined by the method described by Lowry et al. (37).

Ethics statement

All protocols in this experiment (Protocol number: 2014/14, 137) were accepted by the Animal Experimentation Ethics Committee (FUAEEC) of Firat University (Elazig, Turkey).

Statistical analysis

The SPSS 21 computer program was used for data analysis. All experimental data were expressed as arithmetic mean ± standard error (SE) of three replicates. Normality and homoscedasticity were analysed through the Kolmogorov–Smirnov and Bartlett's tests, respectively. One-way analysis of variance (ANOVA) was applied to evaluate the data. Multiple comparisons were performed with Duncan's post-hoc test. Differences were considered statistically significant at $p < 0.05$.

Results

Composition of bee pollen

The chemical composition and antioxidant characteristics of pollen are summarized in Table 1. The main components of the pollen were protein, starch, fat, mineral, and water. The chestnut bee pollen has approximately 16939.90 mg GAE/kg of total phenolic compounds, 1778.89 mg

Table 1. The chemical composition and antioxidant characteristics of chestnut bee pollen.

Chemical composition	
Protein (%)	20.19 ± 0.11
Fat (%)	3.06 ± 0.95
Moisture (%)	12.83 ± 0.88
Ash (%)	2.77 ± 0.03
Starch (%)	5.91 ± 1.42
pH	5.68 ± 0.01
Colour values	
L	58.8 ± 0.14
*a**	11.7 ± 0.05
*b**	50.1 ± 0.14
Main sugars with HPLC	
Fructose (%)	18.04 ± 0.03
Glucose (%)	14.29 ± 0.20
Galactose (%)	6.19 ± 0.23
Sucrose (%)	2.77 ± 0.17
Antioxidant characteristics	
Total Phenolic Content (mg GAE/kg)	16939.90 ± 125.80
Antiradical Activity (% inhibition)	95.17 ± 0.21
Total Flavonoid Content (mg catechin/kg)	1778.89 ± 93.88
β-carotene (mg/kg)	3.62 ± 0.001
Lycopene (mg/kg)	0.84 ± 0.001

GAE: Gallic acid equivalents

catechin/kg of total flavonoids content, 3.62 mg/kg of β-carotene, and 0.84 mg/kg of lycopene. The chestnut pollen also demonstrated high antioxidant activity, which was measured through antiradical activity. Furthermore, based on the results of HPLC analysis for pollen composition, pollen was ascertained to be main sugars such as fructose, glucose, galactose, and sucrose.

The effect of chestnut bee pollen on the growth performance

The effect of chestnut bee pollen on growth performance is shown in Table 2. The Wt, WG and SGR values were higher in all chestnut bee pollen-supplemented groups than in the control group ($p < 0.05$). On the other hand, it was found that the FCR was lower in all chestnut bee pollen-supplemented groups than the control group ($p < 0.05$). However, the growth parameters were found to be similar among chestnut bee pollen groups ($p > 0.05$). No mortality or any signs of disease were observed in all treatment groups. Fish survival was 100% in all treatments ($p > 0.05$).

The effect of chestnut bee pollen on the haematological values

Mean measured haematological values in the control and experimental groups are represented in Table 3. The RBC count, Hb concentration and Ht level were higher in all chestnut bee pollen-supplemented groups than in the control group ($p < 0.05$). However, the RBC count, Hb concentration and Ht level of chestnut bee pollen-supplemented groups were similar ($p > 0.05$). No statistical difference in the erythrocyte indices (MCV, MCH and MCHC) was observed among all experimental groups ($p > 0.05$).

The effect of chestnut bee pollen on the immunological values

The WBC counts and types of *O. mykiss* after chestnut bee pollen treatment are shown in Table 4. After chestnut bee pollen feeding for 8 weeks, the WBC counts were higher in the experimental groups compared to the control group ($p < 0.05$). In addition, the WBC counts were significantly higher in fed with 2 and 4% chestnut bee pollen than the group fed with 1% chestnut bee pollen. The percentage of LYM, MON, and NEU was higher in all chestnut bee pollen-supplemented groups than in the control group ($p < 0.05$).

The results of different dosages of chestnut bee pollen on the other immunological parameters are presented in Table 5. The immunological values were significantly increased in the experimental groups compared with the control group ($p < 0.05$). On the other hand, the immunological values of groups fed with 2 and 4% chestnut bee pollen were significantly higher than the group fed with 1% chestnut bee pollen ($p < 0.05$). Additionally, the groups fed with 2 and 3% chestnut bee pollen had the highest im-

Table 2. The growth performance of rainbow trout fed a control diet and diet supplemented with different levels of chestnut bee pollen after 8 weeks.

	Groups			
Parameters	**C**	**BP-1 (1%)**	**BP-2 (2%)**	**BP-3 (4%)**
W_0 (g)	20.45 ± 1.33 [a]	21.02 ± 1.19 [a]	20.84 ± 1.40 [a]	20.76 ± 1.08 [a]
Wt (g)	36.52 ± 1.71 [a]	41.66 ± 2.08 [b]	42.19 ± 1.98 [b]	42.75 ± 1.83 [b]
WG (g)	16.07 ± 1.45 [a]	20.64 ± 1.58 [b]	21.35 ± 1.69 [b]	21.99 ± 1.47 [b]
SGR (%)	1.03 ± 0.03 [a]	1.22 ± 0.02 [b]	1.26 ± 0.03 [b]	1.27 ± 0.02 [b]
FCR	1.32 ± 0.02 [b]	1.24 ± 0.02 [a]	1.23 ± 0.03 [a]	1.23 ± 0.03 [a]
Survival (%)	100	100	100	100

C: Control group, BP-1: Group fed with 1% chestnut bee pollen for 8 weeks, BP-2: Group fed with 2% chestnut bee pollen for 8 weeks, BP-3: Group fed with 4% chestnut bee pollen for 8 weeks. W_0: Initial weight of fish, Wt: Final weight of fish, WG: Weight gain, SGR: Specific growth rate, FCR: Feed conversion ratio. The values (n=30) are expressed as mean ± standard error. The mean values in the same line with different superscripts are significantly different ($p < 0.05$).

Table 3. The haematological values of rainbow trout fed a control diet and diet supplemented with different levels of chestnut bee pollen after 8 weeks.

	Groups			
Parameters	**C**	**BP-1 (1%)**	**BP-2 (2%)**	**BP-3 (4%)**
RBC	1.52 ± 0.02 [a]	1.64 ± 0.04 [b]	1.71 ± 0.05 [b]	1.72 ± 0.06 [b]
Hb	7.26 ± 0.41 [a]	7.58 ± 0.63 [b]	7.95 ± 0.52 [b]	7.97 ± 0.71 [b]
Ht	29.86 ± 1.33 [a]	33.47 ± 2.62 [b]	36.90 ± 1.98 [b]	37.00 ± 2.14 [b]
MCV	199.34 ± 12.04 [a]	206.10 ± 14.58 [a]	213.78 ± 13.20 [a]	213.96 ± 15.66 [a]
MCH	45.77 ± 2.83 [a]	44.29 ± 3.63 [a]	44.37 ± 2.11 [a]	44.72 ± 4.12 [a]
MCHC	23.41 ± 1.65 [a]	23.64 ± 2.55 [a]	22.23 ± 1.48 [a]	21.85 ± 1.96 [a]

RBC: Red blood cell (erythrocyte) count ($x10^6$), Hb: Haemoglobin concentration (g/dL), Ht: Haematocrit level (%), MCV: Mean corpuscular volume (μm^3), MCH: Mean corpuscular haemoglobin (pg), MCHC: Mean corpuscular haemoglobin concentration (%). The values (n=30) are expressed as mean ± standard error. The mean values in the same line with different superscripts are significantly different ($p < 0.05$).

Table 4. The WBC counts and types of rainbow trout fed control diet and diet supplemented with different levels of chestnut bee pollen after 8 weeks.

	Groups			
Parameters	**C**	**BP-1 (1%)**	**BP-2 (2%)**	**BP-3 (4%)**
WBC	22.83 ± 0.74 [a]	26.19 ± 0.66 [b]	31.43 ± 1.09 [c]	31.05 ± 0.84 [c]
LYM	58.33 ± 1.55 [a]	59.85 ± 1.69 [b]	59.45 ± 1.42 [b]	59.20 ± 1.83 [b]
MON	16.55 ± 0.00 [a]	17.56 ± 0.00 [b]	17.65 ± 0.00 [b]	17.34 ± 0.00 [b]
NEU	21.66 ± 0.03 [a]	22.33 ± 0.02 [b]	22.50 ± 0.03 [b]	22.85 ± 0.02 [b]
EOS	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
BAS	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

WBC: White blood cell counts ($x10^3$), LYM: Lymphocytes (%), MON: Monocytes (%), NEU: Neutrophils (%), EOS: Eosinophils (%), BAS: Basophil (%). The values (n=30) are expressed as mean ± standard error. The mean values in the same line with different superscripts are significantly different ($p < 0.05$).

Table 5. The immunological values of rainbow trout fed a control diet and diet supplemented with different levels of chestnut bee pollen after 8 weeks and the survival in fish challenged with A. salmonicida subsp. *salmonicida*.

	Groups			
Parameters	**C**	**BP-1 (1%)**	**BP-2 (2%)**	**BP-3 (4%)**
NBT	1.11 ± 0.09 [a]	1.24 ± 0.11 [b]	1.35 ± 0.10 [c]	1.36 ± 0.12 [c]
PA	33.50 ± 2.33 [a]	37.33 ± 2.10 [b]	42.60 ± 3.55 [c]	41.90 ± 3.10 [c]
PI	3.25 ± 0.60 [a]	5.41 ± 0.50 [b]	5.85 ± 0.72 [c]	5.88 ± 0.78 [c]
TP	22.13 ± 2.96 [a]	25.55 ± 1.95 [b]	28.99 ± 2.63 [c]	28.43 ± 2.10 [c]
IgM	10.78 ± 1.27 [a]	14.66 ± 1.73 [b]	18.02 ± 2.41 [c]	19.23 ± 1.92 [c]
LYZ	96.57 ± 5.20 [a]	122.87 ± 7.06 [b]	145.55 ± 6.17 [c]	143.89 ± 8.12 [c]
MPO	0.65 ± 0.05 [a]	0.81 ± 0.08 [b]	0.97 ± 0.10 [c]	0.98 ± 0.11 [c]
RPS	35.55 [a]	60.00 [b]	75.56 [c]	77.78 [c]

NBT: Nitroblue tetrazolium assay (mg/ml), PA: Phagocytic activity (%), PI: Phagocytic index, TP: Total protein level (mg/ml), IgM: Total immunoglobulin M level (mg/ml), BA: Serum bactericidal activity (% cfu/control), LYZ: Lysozyme activity (U/ml), MPO: Myeloperoxidase activity (Optic density), RPS: Relative percentage survivals (%). The values (n=30 for immunological analysis, n=45 for RPS) are expressed as mean ± standard error. The mean values in the same line with different superscripts are significantly different ($p < 0.05$).

munological values.

Disease resistance

Clinical signs of furunculosis were determined in infected fish after the challenge test. One or more clinical or internal symptoms were detected as furuncles in the surface of the skin, exophthalmos in the eyes, haemorrhage in the liver, inflammation in the tissues, darkened colour, and abnormal swimming behaviour.

The RPS rates of control and experimental groups fed with chestnut bee pollen are given in Table 5. Following the challenge, the control fish showed significantly lower survival and the groups fed with chestnut bee pollen had a significantly higher RPS rate than the control group ($p < 0.05$). Furthermore, the RPS rates of 2% and 4% chestnut bee pollen groups were significantly higher than the 1% group ($p < 0.05$). The highest resistance was determined in the 4% chestnut bee pollen group; however, there was no significant difference between the RPS rates of 2% and 4% chestnut bee pollen groups.

The effect of chestnut bee pollen on oxidative stress and antioxidant activity

The effects of chestnut bee pollen on the oxidative stress and antioxidant capacity in the tissues are represented in Table 6.

After 8 weeks, the tissue MDA levels showed a significant decrease in the groups fed with chestnut bee pollen compared to the control group. At the same time, the tissue MDA levels were significantly lower in the groups fed with 2 and 4% of chestnut bee pollen than in the 1% chestnut bee pollen group. However, there was no significant difference between the tissue MDA levels of 2 and 3% of chestnut bee pollen groups ($p > 0.05$).

On the other hand, there were significant increases in the tissue SOD, CAT, and GSH-Px activities of groups fed with chestnut bee pollen when compared to the control group. Among the groups fed with chestnut bee pollen, the tissue SOD, CAT and GSH-Px activities in the groups fed with 2 and 4% of chestnut bee pollen were significantly higher than the 1% chestnut bee pollen group ($p < 0.05$). However, there were no significant differences between the tissue antioxidant enzyme activities of groups fed with 2 and 4% of chestnut bee pollen ($p > 0.05$).

Discussion

Certain compounds found in the composition of bee pollen are responsible for biological activity, such as antioxidant, antibacterial, and activities. Among these, the most well-known are phenolic compounds. Phenolic compounds demonstrate a radical scavenging effect, in other words antiradical (9). Furthermore, previous studies carried out on bee products have shown protein components

Table 6. The oxidative stress and antioxidant status in fish fed control diet and diet supplemented with different levels of chestnut bee pollen after 8 weeks.

		Groups			
Tissues	**Parameters**	**C**	**BP-1 (1%)**	**BP-2 (2%)**	**BP-3 (4%)**
Liver	MDA	2.21 ± 0.39 ^{c}	2.02 ± 0.52 ^{b}	1.89 ± 0.50 ^{a}	1.87 ± 0.37 ^{a}
	SOD	3.05 ± 0.22 ^{a}	3.34 ± 0.60 ^{b}	3.70 ± 0.47 ^{c}	3.72 ± 0.76 ^{c}
	CAT	3.16 ± 0.38 ^{a}	3.44 ± 0.45 ^{b}	3.81 ± 0.61 ^{c}	3.82 ± 0.53 ^{c}
	GSH-Px	2.45 ± 0.41 ^{a}	2.69 ± 0.51 ^{b}	2.93 ± 0.63 ^{c}	2.95 ± 0.76 ^{c}
Head kidney	MDA	3.18 ± 0.46 ^{c}	2.86 ± 0.71 ^{b}	2.65 ± 0.53 ^{a}	2.67 ± 0.66 ^{a}
	SOD	2.61 ± 0.39 ^{a}	2.86 ± 0.60 ^{b}	3.03 ± 0.74 ^{c}	3.05 ± 0.59 ^{c}
	CAT	2.75 ± 0.44 ^{a}	3.07 ± 0.76 ^{b}	3.33 ± 0.41 ^{c}	3.38 ± 0.50 ^{c}
	GSH-Px	2.06 ± 0.29 ^{a}	2.45 ± 0.61 ^{b}	2.68 ± 0.50 ^{c}	2.71 ± 0.44 ^{c}
Spleen	MDA	2.94 ± 0.42 ^{c}	2.68 ± 0.33 ^{b}	2.49 ± 0.26 ^{a}	2.50 ± 0.42 ^{a}
	SOD	2.38 ± 0.26 ^{a}	2.59 ± 0.40 ^{b}	2.80 ± 0.52 ^{c}	2.83 ± 0.27 ^{c}
	CAT	2.92 ± 0.39 ^{a}	3.18 ± 0.31 ^{b}	3.47 ± 0.55 ^{c}	3.45 ± 0.34 ^{c}
	GSH-Px	2.12 ± 0.28 ^{a}	2.35 ± 0.22 ^{b}	2.61 ± 0.75 ^{c}	2.66 ± 0.53 ^{c}

MDA: Malondialdehyde level (nmol/mg protein), SOD: Superoxide dismutase activity (U/mg protein), CAT: Catalase activity (k/mg protein, k: the first-order rate constant) and GSH-Px: Glutathione peroxidase activity (U/mg protein). The values (n=30) are expressed as mean ± standard error. The mean values in the same line with different superscripts are significantly different ($p < 0.05$).

to possess radical scavenging and antioxidant activities. Therefore, these components are also named non-phenolic antioxidants (38). In the present study, compared to the controls, statistically significant differences were determined in the growth, haematological, immunological and antioxidant parameters of the groups fed diets containing chestnut bee pollen. These results show that chestnut bee pollen has a positive influence on the parameters investigated.

In this study, O. mykiss was fed with 1%, 2%, and 4% chestnut bee pollen, resulting in significantly increased Wt, WG, and SGR, and significantly decreased FCR. However, it should be noted that the growth parameters were similar among chestnut bee pollen groups. Our results indicated that supplement diets with chestnut bee pollen can enhance the growth of rainbow trout. In line with our finding, the Wt and SGR were significantly increased, and the FCR has significantly improved in Nile tilapia (*Oreochromis niloticus*) fed with bee pollen supplemental diets for 10, 20, and 30 days (13). Similarly, dietary honeybee pollen (2.5 %) significantly increased SGR, ADG (average daily gain) and FER (Feed Efficiency ratio), while significantly reducing FCR in Nile tilapia (*Oreochromis niloticus*) (39). Also, Ren et al. (40) showed that supplementation with 0, 10, 20, 30 and 40 g peony pollen significantly improved the growth performance in carp. On the other hand, the results of this study revealed that chestnut bee pollen improved the growth and did not negatively affect the growth of rainbow trout. This positive effect in the growth may be attributed to carotenoids detected in chestnut bee pollen, which could enhance nutrient utilization and growth performance (41) or may be due to the hormone-like functions of bee pollen as a flavonoid that can promote the growth of animals (42).

The haematological values are generally considered to evaluate the health, physiological status, stress, and disease conditions of fish under normal conditions (43,44). These parameters are reliable indicators that could be widely used in reflecting of nutritional status and the capacity for fish adaptation to the external environmental conditions (45). Also, the hematological parameters give information about the health status of fish by identifying any abnormalities caused using immunostimulants (46). The erythrocyte indices can be used as stress indicators to monitor the health status of fish and the etiological and morphological classification of anaemias (6). In the present study, increased RBC count, Hb concentration, and Ht level were observed in fish fed with chestnut bee pollen-supplemented diets after 8 weeks. However, no effect was found on the erythrocyte indices of rainbow trout fed with diets supplemented with different levels of chestnut bee pollen. Our data demonstrated that the dietary chestnut bee pollen positively affected the haematological parameters. Also, chestnut bee pollen did not cause anaemia when administered at the indicated doses and for the given time period to fish. These results showed that the fish was not under any stress caused by chestnut bee pollen.

It is known that the WBC play a major role in the immune system of fish, and they represent an important function in the cell immunity of fish (47,48). The different leukocyte counts are considerably influenced by the health state of fish and in many cases assist in the evaluation of the immune system. The different leukocyte counts are dependent on the fish species, physiological age, sex, season of the year and nutrition (49). On the other hand, the respiratory burst or oxidative burst is an indicator of the oxidative potential of reactive oxygen species produced by active phagocytic cells including granulocytes and monocyte/macrophages (50). Phagocytic cells in teleost fish are the key components of innate immune response that regulate immune function and play an important role in fighting diseases. Additionally, phagocytosis associated with respiratory burst activity is an important indicator of innate immunity in fish (51). The respiratory bursts from innate immune cells are determined using NBT assays, which measure in terms of respiratory burst activity of phagocytes is used to detect oxidative radical production (50). The results in the present study showed that the phagocytic

activity (PA and PI) and WBC count were significantly induced with dietary chestnut bee pollen. Also, the different leukocyte counts were significantly influenced by dietary chestnut bee pollen in rainbow trout. Similarly, El-Asely et al. (13) showed that the different leucocyte counts (neutrophils, monocytes, and lymphocytes) and phagocytic activity increased significantly in the fish fed with 1%, 2.5%, and 4% honey bee pollen for 30 days. These results may be explained by induction of the cellular immunity after the administration of chestnut bee pollen.

The humoral immune response to protect against pathogens such as bacteria and viruses is particularly important for disease resistance and it is a vital component of the nonspecific immune system in fish (529. Serum immunity parameters have been recognized as important elements for monitoring fish health 8539. Serum total protein, which includes albumin and globulin, plays an important role in the humoral immune response. The protein and globulin levels in serum are fractions that are vital for maintaining a healthy immune system. An increase in serum protein level indicates a stronger innate immune response in fish (46,54,55). IgM, the main antibody of fish, is a major component of the teleost humoral immune system and is described commonly as immunoglobulins in fish serum (56). This molecule plays a significant role in various immune processes, such as phagocytosis, opsonisation, and neutralization of pathogenic microorganisms, and toxins in fish (57,58). LYZ, an important component of the innate immune system, has an essential role in the diminution of disease by preventing bacterial pathogens (59). LYZ is found in the serum, mucus, and other body fluids and is responsible for the lysis of bacteria and activation of the complement system and phagocytes by acting as an opsonin (60). Both LYZ and IgM are indispensable for humoral immunity in fish. These are important indicators to evaluate fish health and immune function (61). MPO, a heme peroxidase, exists primarily in the cytoplasmic granules of fish neutrophils and contributes to the antimicrobial function by producing reactive oxygen species, such as hypohalous acid and other toxic oxidants produced from hydrogen peroxide and halides, and these oxidants can oxidize organic molecules and kill microbes (62). Our results show that the TP and IgM levels and the LYZ and MPO activities were significantly increased in all chestnut bee pollen-supplemented groups. In addition, these values were significantly higher in the group fed BP-2 and BP-3 diets compared to the BP-1 group. These results were similar to Cuesta et al. (63) findings that propolis, a honeybee product, increased serum humoral innate immune responses in gilthead sea bream. Also, Li et al. (64) stated that diets supplemented with flavonoids significantly increased serum humoral immune responses. Previous studies have well approved the impacts of plant-derived products on improving specific or non-specific immune humoral and cellular immune responses in various fish and shellfish species (7,65-70). Awad and Awaad (71) stated that the mode of action of those plants and their derivatives were attributed to the presence of many active components such as alkaloids, steroids, phenolics, tannins, terpenoids, saponins, glycosides, and flavonoids. Consistent with the above investigations, these increments in the TP and IgM levels and the LYZ and MPO activities could be attributed to phenols or flavonoids determined in chestnut bee pollen. Also, these increments in serum humoral immune responses could be correlated with enhanced phagocytic activity (72) or leucocyte number (6).

Experimental bacterial challenge test has often been used as a final indicator of fish health status after dietary treatments to evaluate the disease resistance of the host against pathogens (73). Additionally, the RPS is one of the most visual indices for assessing the immune effect in a challenge test (54). The present study revealed that after the challenge with *A. salmonicida* subsp. *achromogenes*, the chestnut bee pollen groups showed high survival when compared to the control group. Moreover, the best RPS rates were determined in the BP-2 and BP-3 groups. Those results on the RPS could be also related to the observed promotion of growth as well as to other activities related to immunity which are described above.

As is known, endogenous antioxidant enzymes such as SOD, CAT, and GSH-Px play an important role in scavenging harmful reactive oxygen derivatives used to evaluate non-specific immune responses (64,74). MDA as a main end product of lipid peroxidation, which is accepted to be a valuable indicator of the oxidative stress in cellular components, can be used as one of the indices of lipid peroxidation (75-81). Previous studies showed that plant flavonoids have strong antioxidant activity (64) and reduce the harmful effects of oxidative stress (7,9,82). In the present study, the dietary chestnut bee pollen significantly increased SOD, CAT, and GSH-Px enzyme activities while decreasing MDA levels in different tissue of rainbow trout. Similarly, supplementation of carp diets with peony pollen significantly increased intestinal CAT activity and total antioxidant capacity and decreased MDA level (40). Also, Dastan et al. (83) also reported the different concentrations of bee pollen increased total antioxidant status (TAS), while decreased total oxidant status (TOS), oxidative stress index (OSI), and the MDA levels in the liver, spleen, and heart of rainbow trout. Concerning our antioxidant defense parameters results, higher SOD, CAT, and GSH-Px activities, as well as lower MDA levels, were observed in association with chestnut bee pollen within the diet. This result can probably be explained by an increase in antioxidant capacity after pollen application. In addition to its direct antioxidant activity, this effect may also be related to the radical scavenging effect of bee pollen (9).

To conclude, findings in this feeding trial demonstrate that the administration of chestnut bee pollen with diet promotes the growth performance, haematological parameters, and immune responses of rainbow trout. Moreover, the chestnut bee pollen provided extraordinary protection against the challenge of *A. salmonicida subsp. salmonicida*. Taken together, these results show that the chestnut bee pollen has great potential as an immunostimulant in aquaculture and may enhance fish health and production in fish farming.

References

1. Harikrishnan R, Thamizharasan S, Devi G, Van Doan H, Ajith Kumar, TT, Hoseinifar SH, Balasundara C. Dried lemon peel enriched diet improves antioxidant activity, immune response and modulates immuno-antioxidant genes in Labeo rohita against Aeromonas sorbia. Fish Shellfish Immun 2020; 106: 675-684. doi: 10.1016/j.fsi.2020.07.040.
2. Abdel-Tawwab M, Abbass FE. Turmeric powder, Curcuma longa L., in common carp, Cyprinus carpio L., diets: growth perfor-

mance, innate immunity, and challenge against pathogenic Aeromonas hydrophila infection. J World Aquacult Soc 2017; 48(2): 303-312.
3. Saglam N, Yonar ME, Effects of sulfamerazine on selected haematological and immunological parameters in rainbow trout (Onchorhynchus mykiss, Walbaum, 1792). Aquacult Res 2009; 40 ; 395-404. doi: 10.1111/j.1365-2109.2008.02105.
4. Mohan K, Ravichandran S, Muralisankar T, Uthayakumar V, Chandirasekar R, Seedevi P, Abirami RG, Rajan DK. Application of marine-derived polysaccharides as immunostimulants in aquaculture: A review of current knowledge and further perspectives. Fish Shellfish Immun 2019; 86: 1177-1193. doi: 10.1016/j.fsi.2018.12.072
5. Yonar ME, Mişe Yonar S, Silici S. Protective effect of propolis against oxidative stress and immunosuppression induced by oxytetracycline in rainbow trout (Oncorhynchus mykiss, W.). Fish Shellfish Immun 2011; 31: 318-325. doi: 10.1016/j.fsi.2011.05.019
6. Mişe Yonar S. Growth performance, haematological changes, immune response, antioxidant activity and disease resistance in rainbow trout (Oncorhynchus mykiss) fed diet supplemented with ellagic acid. Fish Shellfish Immun 2019; 95: 391-395. doi: 10.1016/j.fsi.2019.10.056
7. Yonar ME, Mişe Yonar S, Ispir U, Ural MŞ. Effects of curcumin on haematological values, immunity, antioxidant status and resistance of rainbow trout (Oncorhynchus mykiss) against Aeromonas salmonicida subsp. achromogenes. Fish Shellfish Immunol 2019; 89: 83-90. doi: 10.1016/j.fsi.2019.03.038
8. Hoseinia SM, Hoseinifar SH, Van Doan H. Growth performance and hematological and antioxidant characteristics of rainbow trout, Oncorhynchus mykiss, fed diets supplemented with Roselle, Hibiscus sabdariffa. Aquaculture 2021; 530: 735827. doi: 10.1016/j.aquaculture.2020.735827
9. Eraslan G, Kanbur M, Silici S. Effect of carbaryl on some biochemical changes in rats: The ameliorative effect of bee pollen. Food Chem Toxicol 2009; 47: 86-91. doi: 10.1016/j.fct.2008.10.013
10. Zhao Y, Yan Y, Zhoua W, Chen D, Huang K, Yu S, Mi J, Lu L, Zenga X, Cao Y. Effects of polysaccharides from bee collected pollen of Chinese wolfberry on immune response and gut microbiota composition in cyclophosphamide-treated mice. J Funct Food 2020; 72: 104057. doi: 10.1016/j.jff.2020.104057
11. Themelis T, Gotti R, Orlandini S, Gatti R. Quantitative amino acids profile of monofloral bee pollens by microwave hydrolysis and fluorimetric high performance liquid chromatography. J Pharmaceut Biomed 2019; 173: 144-153. doi: 10.1016/j.jpba.2019.05.031
12. Eraslan G. Kanbur M, Silici S, Liman BC, Altınordulu S, Sarıca ZS. Evaluation of protective effect of bee pollen against propoxur toxicity in rat. Ecotoxicol Environ Saf 2009; 72: 931-937. doi: 10.1016/j.ecoenv.2008.06.008
13. El-Asely AM, Abbass AA, Austin B. Honey bee pollen improves growth, immunity and protection of Nile tilapia (Oreochromis niloticus) against infection with Aeromonas hydrophila. Fish Shellfish Immunol 2014; 40: 500-506. doi: 10.1016/j.fsi.2014.07.017
14. AOAC (Association of Official Analytical Chemists), Official method 920.153, Official Methods of Analysis of the Association of Official Analytical Chemists 17th ed, Association of Official Analytical Chemists, Gaithersburg, Maryland, USA, 2002.
15. AOAC (Association of Official Analytical Chemists), Official method 991.36, Official Methods of Analysis of the Association of Official Analytical Chemists 18th ed, Association of Official Analytical Chemists, Washington, DC, USA, 2005.
16. AOAC (Association of Official Analytical Chemists), Official method 960.52, Official Methods of Analysis of the Association of Official Analytical Chemists 15th ed, Association of Official Analytical Chemists, Virginia, USA, 1990.
17. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. Am J Enol Viticult 1965; 16: 144-158. doi: 10.5344/ajev.1965.16.3.144
18. Ulusoy E, Kolayli S. Phenolic composition and antioxidant properties of Anzer Bee pollen. J Food Biochem 2014; 38(1): 73-82. doi: 10.1111/jfbc.12027
19. Treves-Brown KM. Applied Fish Pharmacology, Dordrecht, Boston, Kluwer Academic Publishers, 2000.
20. Natt MP, Herrick CA. New blood diluents for counting the erythrocytes and leukocytes of the chicken. Poultry Sci 1952; 31: 735-738. doi: 10.3382/ps.0310735
21. Drabkin DL.The crystallographic and optical properties of the hemoglobin of man in comparison with those of other species. J Biol Chem 1946; 64: 703-723.
22. Saglam N. Ispir U, Yonar M.E. The effect of a therapeutic bath in malachite green on some haematological parameters of rainbow trout (Oncorhynchus mykiss, Walbaum, 1792). Fresen Environ Bull 2003; 12: 1207-1210.
23. Ispır U, Yonar ME, Barım Oz O. Effect of dietary vitamın E supplementation on the blood parameters of Nile tilapia (Oreochromıs niloticus). J Anim Plant Sci2011; 21(3): 566-569.
24. Mişe Yonar S, Yonar ME, Pala A, Sağlam N, Sakin F. Effect of trichlorfon on some haematological and biochemical changes in Cyprinus carpio: The ameliorative effect of lycopene. Aquac Rep 2020; 16: 100246. doi: 10.1016/j.aqrep.2019.100246
25. Siwicki AK, Anderson DP, Rumsey GL. Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against frunculosis. Vet Immunol Immunop 1994; 41: 125-129. doi: 10.1016/0165-2427(94)90062-0
26. Siwicki AK, Anderson DP. Nonspecific defense mechanisms assay in fish. II. Potential killing activity of neutrophils and macrophages, lysozyme activity in serum and organs and total immunoglobulin level in serum. In: Siwicki AK, Anderson DP, Waluga J (eds) Fish disease diagnosis and prevention methods. Olsztyn, Poland, , 1993, pp 105–112.
27. Siwicki AK, Studnicka M, Morand M, Pozet F, Terech-Majewska E. Comparative immunotoxicology – A new direction. Acta Vet Brno 1998; 67: 295-301. doi: 10.2754/avb199867040295
28. Yonar ME. The effect of lycopene on oxytetracycline-induced oxidative stress and immunosuppression in rainbow trout (Oncorhynchus mykiss, W.). Fish Shellfish Immun 2012; 32: 994-1001. doi: 10.1016/j.fsi.2012.02.012
29. Ellis, AE. Lysozyme Assays. In: Stolen JS, Fletcher TC, Anderson DP, Roberson BS, Van Muiswinkel WB (eds) Techniques in Fish Immunology, SOS Publications, Fair Haven, 1990, pp 101-103.
30. Quade MJ, Roth JA. A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Vet Immunol Immunop 1997; 58: 239-248. doi: 10.1016/s0165-2427(97)00048-2
31. Sahoo PK, Kumari J, Mishra BK. Non-specific immune responses in juveniles of Indian major carps. J Appl Ichthyol 2015; 21: 151-155. doi: 10.1111/j.1439-0426.2004.00606.x
32. Iswarya A, Vaseeharan B, Anjugam M, Gobi N, Divya M, Faggio C. β-1, 3 glucan binding protein based selenium nanowire enhances the immune status of Cyprinus carpio and protection against Aeromonas hydrophila infection. Fish Shellfish Immun 2018; 83: 61-75. doi: 10.1016/j.fsi.2018.08.057
33. Placer ZA, Cushman L, Johnson BC. Estimation of products of lipid peroxidation (Malonyl dialdehyde) in biological fluids. Anal Biochem 1966; 16: 359-364. doi: 10.1016/0003-2697(66)90167-9
34. Sun Y, Oberley,LW, Li Y. A simple method for clinical assay of superoxide dismutase. Clin Chem 1988; 34: 497-500.
35. Aebi H. Catalase. In: Bergmeyer HU (eds) Methods in enzymatic analysis. Academic Press, New York, 1983, pp 276–286.

36. Beutler E. Red cell metabolism. In: Beutler E (eds) A manual of Biochemical Methods. Grune and Stratton, New York, 1975, pp 71-73.
37. Lowry OH, Rosenberough NJ, Farr AL, Randal RJ. Protein measurement with folinphenol reagent. J Biochem 1951; 193: 265-275.
38. Campos MG, Webby RF, Markham KR, Mitchell KA, Da Cunha AP. Age-induced diminution of free radical scavenging capacity in bee pollens and the contribution of constituent flavonoids. J Agric Food Chem 2003; 51: 742-745. doi: 10.1021/jf0206466
39. Abbass AA, El-Asely AM, Kandiel, MMM. Effects of dietary propolis and pollen on growth performance, fecundity and some hematological parameters of Oreochromis niloticus. Turkish J Fish Aquat Sci 2012; 12: 851-859. doi: 10.4194/1303-2712-v12_4_13
40. Ren H, Huang Y, Lin L. Effects of dietary supplementation with peony pollen on growth, intestinal function, fillet quality and fatty acids profiles of common carp. Aquac Nutr 2021; 27: 908-917. doi: 10.1111/anu.13235
41. Sánchez EGT, Fuenmayor CA, Mejía SMV, Diaz-Moreno C, Mahecha HS. Effect of bee pollen extract as a source of natural carotenoids on the growth performance and pigmentation of rainbow trout (Oncorhynchus mykiss). Aquaculture 2020; 514: 734490. doi: 10.1016/j.aquaculture.2019.734490
42. Baker ME. Flavonoids as hormones. A perspective from an analysis of molecular fossils. Adv Exp Med Biol 1998; 439: 249-267.
43. Mişe Yonar S, Yonar ME, Yöntürk Y, Pala A. Effect of ellagic acid on some haematological, immunological and antioxidant parameters of rainbow trout (Oncorhynchus mykiss). J Anim Physiol An N 2014; 98: 936-941. doi: 10.1111/jpn.12162
44. Vazirzadeh A, Dehghan F, Kazemeini R. Changes in growth, blood immune parameters and expression of immune related genes in rainbow trout (Oncorhynchus mykiss) in response to diet supplemented with Ducrosia anethifolia essential oil. Fish Shellfish Immun 2017; 69: 164-172. doi: 10.1016/j.fsi.2017.08.022
45. Abdel-Tawwab M, Adeshina I, Jenyo-Oni A, Ajani EK, Emikpe BO. Growth, physiological, antioxidants, and immune response of African catfish, Clarias gariepinus (B.), to dietary clove basil, Ocimum gratissimum, leaf extract and its susceptibility to Listeria monocytogenes infection. Fish Shellfish Immun 2018; 78: 346-354. doi: 10.1016/j.fsi.2018.04.057
46. Talpur AD, Ikhwanuddin M. Azadirachta indica (neem) leaf dietary effects on the immunity response and disease resistance of Asian seabass, Lates calcarifer challenged with Vibrio harveyi. Fish Shellfish Immun 2013; 34: 254-264. doi: 10.1016/j.fsi.2012.11.003
47. Magnadóttir B. Innate immunity of fish (overview). Fish Shellfish Immun 2006; 20: 137-151. doi: 10.1016/j.fsi.2004.09.006
48. 48. Thrall MA. Veterinary Hematology and Clinical Chemistry. Philadelphia, PA: Lippincott Williams and Wilkins, 2004.
49. Modrá H, Svobodová Z, Kolářová J. Comparison of differential leukocyte counts in fish of economic and indicator importance. Acta Vet Brno1998; 67: 215-226. doi: 10.2754/avb199867040215
50. Song SK, Beck BR, Kim D, Park J, Kim J, Kim HD, Ringø E. Prebiotics as immunostimulants in aquaculture: A review. Fish Shellfish Immun 2014; 40; 40-48. doi: 10.1016/j.fsi.2014.06.016
51. Ispir U, Yonar ME. Effects of levamisole on phagocytic activity of rainbow trout (Oncorhynchus mykiss W.). Acta Vet Brno 2007; 76: 493-497. doi:10.2754/avb200776030493
52. Zhang X, Sun Z, Cai J, Wang J, Wang G, Zhu Z, Cao F. Effects of dietary fish meal replacement by fermented moringa (Moringa oleifera Lam.) leaves on growth performance, nonspecific immunity and disease resistance against Aeromonas hydrophila in juvenile gibel carp (Carassius auratus gibelio var. CAS III). Fish Shellfish Immun 2020; 102: 430-439. doi: 10.1016/j.fsi.2020.04.051
53. Kong Y, Gao C, Du X, Zhao J, Li M, Shan X, Wang G. Effects of single or conjoint administration of lactic acid bacteria as potential probiotics on growth, immune response and disease resistance of snakehead fish (Channa argus). Fish Shellfish Immun 2020; 102: 412-421. doi: 10.1016/j.fsi.2020.05.003
54. Wang E, Chen X, Wang K, Wang J, Chen D, Geng Y, Lai W, Wei X. Plant polysaccharides used as immunostimulants enhance innate immune response and disease resistance against Aeromonas hydrophila infection in fish. Fish Shellfish Immunol 2016; 59: 196-202. doi: 10.1016/j.fsi.2016.10.039
55. Reda RM, Selim KM, Mahmoud R, El-Araby IE. Effect of dietary yeast nucleotide on antioxidant activity, non-specific immunity, intestinal cytokines, and disease resistance in Nile Tilapia. Fish Shellfish Immun 2018; 80: 281-290. doi: 10.1016/j.fsi.2018.06.016
56. Guzmán-Villanueva LT, Tovar-Ramírez D, Gisbert E, Cordero H, Guardiola FA, Cuesta A, Meseguer J, Ascencio-Valle F, Esteban MA. Dietary administration of β-1,3/1,6-glucan and probiotic strain Shewanella putrefaciens, single or combined, on gilthead seabream growth, immune responses and gene expression. Fish Shellfish Immun 2014; 39: 34-41. doi: 10.1016/j.fsi.2014.04.024
57. Cuesta A, Meseguer J, Esteban MA. Total serum immunoglobulin M levels are affected by immunomodulators in seabream (Sparus aurata L.). Vet Immunol Immunopathol 2004; 101: 203-210. doi: 10.1016/j.vetimm.2004.04.021
58. Magnadottir B. Immunological control of fish diseases. J Mar Biotechnol 2010; 12: 361-379. doi: 10.1007/s10126-010-9279-x
59. Li S. Wang A, Li Z, Zhang J, Sang C, Chen N. Antioxidant defenses and non-specific immunity at enzymatic and transcriptional levels in response to dietary carbohydrate in a typical carnivorous fish, hybrid grouper (Epinephelus fuscoguttatus ♀ × E. lanceolatus ♂). Fish Shellfish Immun 2020; 100: 109-116. doi: 10.1016/j.fsi.2020.03.015
60. Adel M. Pourgholam R, Zorriehzahra J, Ghiasi M. Hemato – Immunological and biochemical parameters, skin antibacterial activity, and survival in rainbow trout (Oncorhynchus mykiss) following the diet supplemented with Mentha piperita against Yersinia ruckeri. Fish Shellfish Immun 2016; 55: 267-273. doi: 10.1016/j.fsi.2016.05.040
61. Kong Y, Tian J, Niu X, Li M, Kong Y, Li R, Chen X, Wang G. Effects of dietary quercetin on growth, antioxidant capacity, immune response and immune-related gene expression in snakehead fish, Channa argus. Aquac Rep 2022: 26; 101314. doi: 10.1016/j.aqrep.2022.101314
62. Wang H, Zhou, L, Yang M, Luo X, Li Y, Dan X. Identification and characterization of myeloperoxidase in orange-spotted grouper (Epinephelus coioides). Fish Shellfish Immun 2018; 72: 230-236. doi: 10.1016/j.fsi.2017.10.063
63. Cuesta A, Rodriguez A, Esteban MA, Meseguer J. In vıvo effects of propolis, a honeybee product, on gilthead seabream innate immun responses. Fish Shellfish Immun 2005; 18: 71-80. doi: 10.1016/j.fsi.2004.06.002
64. Li M, Zhu X, Tian J, Liu M, Wang G. Dietary flavonoids from Allium mongolicum Regel promotes growth, improves immune, antioxidant status, immune-related signaling molecules and disease resistance in juvenile northern snakehead fish (Channa argus). Aquaculture 2019; 501: 473-481. doi: 10.1016/j.aquaculture.2018.12.011
65. Sahu S, Das BK, Mishra BK, Pradhan J, Sarangi N. Effects of Allium sativum on the immunity and survival of Labeo rohita infected with Aeromonas hydrophila. J Appl Ichthyol 2007: 23; 80-86. doi: 10.1111/j.1439-0426.2006.00785.x
66. Sahu S, Das BK, Mishra BK, Pradhan J, Samal SK, Sarangi N. Effect of dietary Curcuma longa on enzymatic and immunological profiles of rohu, Labeo rohita (Ham.), infected with Aeromonas hydrophila. Aquac Res 2008; 39: 1720-1730. doi: 10.1111/j.1365-

2109.2008.02048.x

67. Mahmoud HK, Al-Sagheera AA, Redab FM, Mahgoub SA, Ayyata MS. Dietary curcumin supplement influence on growth, immunity, antioxidant status, and resistance to Aeromonas hydrophila in Oreochromis niloticus. Aquaculture 2017; 475: 16-23. doi: 10.1016/j.aquaculture.2017.03.043
68. NyaEJ, Austin B. Use of garlic, Allium sativum, to control Aeromonas hydrophila infection in rainbow trout, Oncorhynchus mykiss (Walbaum). J Fish Dis 2009; 32: 963-970. doi: 10.1111/j.1365-2761.2009.01100.x
69. Nya EJ, Austin B. Use of dietary ginger, Zingiber officinale Roscoe, as an immunostimulant to control Aeromonas hydrophila infections in rainbow trout, Oncorhynchus mykiss (Walbaum). J Fish Dis 2009; 32: 971-977. doi: 10.1111/j.1365-2761.2009.01101.x
70. Dügenci SK, Arda N, Candan A. Some medicinal plants as immunostimulant for fish. J Ethnopharmacol 2003; 88: 99-106. doi: 10.1016/s0378-8741(03)00182-x
71. Awad E, Awaad A. Role of medicinal plants on growth performance and immune status in fish. Fish Shellfish Immunol 2017; 67: 40-54. doi: 10.1016/j.fsi.2017.05.034
72. Jagruthi C, Yogeshwari G, Anbazahan SM, Mari LSS, Arockiaraj J, Mariappan P, Sudhakar GRL, Balasundaram C, Harikrishnan R. Effect of dietary astaxanthin against Aeromonas hydrophila infection in common carp, Cyprinus carpio. Fish Shellfish Immunol 2014; 41: 674-680. doi: 10.1016/j.fsi.2014.10.010
73. Gobi N, Ramya C, Vaseeharan B, Malaikozhundan B, Vijayakumar S, Murugan K, Benelli G. Oreochromis mossambicus diet supplementation with Psidium guajava leaf extracts enhance growth, immune, antioxidant response and resistance to Aeromonas hydrophila. Fish Shellfish Immun 2016; 58: 572-583. doi: 10.1016/j.fsi.2016.09.062
74. Sakin F, Ispir Ü, Mişe Yonar S, Yonar ME, Taysi R. Effect of short-term cypermethrin exposure on oxidant-antioxidant balance in the whole body of rainbow trout fry (Oncorhynchus mykiss). Fresen Environ Bull 2011; 20(10a): 2806-2809.
75. Ural MŞ, Yonar ME, Mişe Yonar S. Protective effect of ellagic acid on oxidative stress and antioxidant status in Cyprinus carpio during malathion exposure. Cell Mol Biol 2015; 61(5): 58-63. doi: 10.14715/cmb/2015.61.5.10
76. Köprücü K, Yonar ME, Özcan, S. Effect of dietary n-3 polyunsaturated fatty acids on antioxidant defense and sperm quality in rainbow trout (Oncorhynchus mykiss) under regular stripping conditions. Anim Reprod Sci 2015; 163: 135-143. doi: 10.1016/j.anireprosci.2015.10.008
77. Mişe Yonar S, Sakin F, Yonar ME, Ispir Ü, Kirici M. Oxidative stress biomarkers of exposure to deltamethrin in rainbow trout fry (Oncorhynchus mykiss). Fresen Environ Bull 2011; 20(8): 1931-1935.
78. Yonar ME, Ispir U, Mişe Yonar S, Kirici M. Effect of copper sulphate on the antioxidant parameters in the rainbow trout fry, Oncorhynchus mykiss. Cell Mol Biol 2016; 62: 55-58. doi: 10.14715/cmb/2016.62.6.10
79. Sakin F, Mişe Yonar S, Yonar ME, Saglam N. Changes in selected immunological parameters and oxidative stress responses in different organs of Oncorhynchus mykiss exposed to ivermectin. Rev Chem 2012; 63: 989-995.
80. Yonar ME, Mişe Yonar S, Pala A, Silici S, Saglam N. Trichlorfon-induced haematological and biochemical changes in Cyprinus carpio: Ameliorative effect of propolis. Dis Aquat Organ 2015; 114: 209-216. doi: 10.3354/dao02866
81. Seker E, Ispir U, Mise Yonar S, Yonar ME, Turk C. Antioxidant responses of rainbow trout (Oncorhynchus mykiss) gills after exposure to hydrogen peroxide. Fresen Environ Bull 2015; 24(5a): 1837-1840.
82. Yonar ME, Mişe Yonar S, Ural MŞ, Silici S, Düşükcan M. Protective role of propolis in chlorpyrifos-induced changes in the haematological parameters and the oxidative/antioxidative status of Cyprinus carpio carpio. Food Chem Toxicol 2012; 50: 2703-2708. doi: 10.1016/j.fct.2012.05.032
83. Dastan SD, Gulhan MF, Selamoglu Z, Dastan T. The determination of different effective concentration of ethanolic extract of bee pollen on biochemical analysis in liver, spleen and heart tissues of rainbow trout, Oncorhynchus mykiss (Walbaum, 1792). Iran J Fish Sci 2017; 16: 326-340.