scopeSscience

MARINE REPORTS 3(1) (2024) : 63-76
DOI: 10.5281/zenodo.12518162
marep@scopesscience.com

MARINE REPORTS

MARINE REPORTS
e-ISSN: 2822-5155
Journal homepage: https://scopesscience.com/index.php/marep/

Received: 27 May 2024; Received in revised form: 17 June 2024
Accepted: 24 June 2024; Available online: 25 June 2024

RESEARCH PAPER

Citation: Çoban N., Haga Y., Maita M., Futami K., Yılmaz S., Ergün S., Yiğit M., Seong T., Oktay O., & Katagiri T. (2024). Effects of humic acid supplemented feeds on growth performance, hematological parameters and antioxıdant capacity on common carp (*Cyprinus carpio*). *Marine Reports, 3*(1), 63-76. https://doi.org/10.5281/zenodo.12518162

EFFECTS OF HUMIC ACID SUPPLEMENTED FEEDS ON GROWTH PERFORMANCE, HEMATOLOGICAL PARAMETERS AND ANTIOXIDANT CAPACITY ON COMMON CARP (*Cyprinus carpio*)

Nergiz ÇOBAN[1], Yutaka HAGA[1], Masashi MAITA[1], Kunihiko FUTAMI[1], Sevdan YILMAZ[2], Sebahattin ERGUN[2], Murat YIGIT[3], Taekyoung SEONG[4], Ozan OKTAY[1] and Takayuki KATAGIRI[1*]

[1] Tokyo University of Marine Science and Technology, Tokyo, Japan*
[2] Department of Aquaculture, Faculty of Marine Sciences and Technology, Çanakkale Onsekiz Mart University, Çanakkale, Turkey
[3] Department of Fisheries Industry Engineering, Faculty of Marine Sciences and Technology, Çanakkale Onsekiz Mart University, Çanakkale, Turkey
[4] Graduate School of Fisheries Science and Environmental Studies, Nagasaki University, Nagasaki - Japan

**Corresponding author: takakata@kaiyodai.ac.jp, +81-3-5463-0546, ORCID: 0009-0006-8468-6223*

Abstract

The present study investigated the impact of humic acid on the growth, blood parameters, and antioxidant capacity of common carp (*Cyprinus carpio*). Fish were fed twice a day until saturation with increasing doses of humic acid at 0.0, 0.2, 0.4 and 0.8%. In a 60-day feeding treatment, FW, WG and SGR values (41.37±0.87, 23.26±1.01, 1.37±0.05 respectively) of the group with 0.4% humic acid had statistically greater values compared to the control group ($p < 0.05$). RBC (red blood cell), WBC (white blood cell) and Hct (haematocrit) parameters were evaluated for haematological analyses, the WBC value of the group fed 0.2% and 0.4% humic acid (20.62±3.74, 21.43±1.50 respectively) increased significantly ($p < 0.05$). However, serum biochemistry parameters did not show any statistical difference ($p > 0.05$). The SOD analysis evaluated to determine the antioxidant capacity showed that the group fed 0.4% and 0.8% humic acid had higher values than the control (72.78±3.38, 71.63±5.01 respectively). Our results indicate that 0.4% humic acid as a feed additive for carp can improve growth parameters, antioxidant capacity, and general health.

Keywords: Common carp, humic acid, hematological parameters, antioxidant, fish feed

Introduction

Aquaculture has become one of the world's fastest-growing animal food production industries due to the high nutritional value of aquatic organisms and the general increase in the awareness of healthy nutrition (Atamanalp et al., 2021). Therefore, global fisheries and aquaculture production will play an increasingly important role in providing food and nutrition in the future. In 2020, the common carp (*Cyprinus carpio*) was the fourth most cultivated species in the world and comprised 8.6% of the production of the leading aquaculture species (FAO, 2022). Carp cultivation in the Asian region is increasing due to its rich nutritional content and delicious taste (Zhang et al., 2021; Wang et al. 2015). However, intensive aquaculture to meet the demand and maximize profitability results in increases in stocking density and subsequent deterioration in water quality, stressed fish, and the danger of pathogens (Abdel-Latif & Khafaga, 2020). The sustainability of the global aquaculture industry is severely constrained by unfavorable conditions and the spread of infectious diseases, resulting in severe economic losses for farmers. Therefore, stimulating the fish immune system by prophylactic approaches is becoming increasingly important (Meinelt et al., 2008). With the increasing development of resistance to chemical substances used for therapeutic purposes, the importance of animal welfare and increasing social awareness of environmental protection, alternative treatment options, and immunostimulants that do not leave residues in the environment have become one of the main topics of study in aquaculture (Lieke et al., 2019). These low-cost alternatives aiming to improve feed quality and health performance include additives such as phytogenic compounds, enzymes, essential oils, organic acids, probiotics, and prebiotics (Bharati et al., 2019; Alemayehu et al., 2018). Humic substances, the main component of natural organic substances, result from biomolecules' physical, chemical, and microbiological transformation.. These ubiquitous substances constitute 95% of dissolved organic matter in aquatic ecosystems (Lieke et al., 2019; Thurman, 1985; Haitzer et al., 1998; Steinberg, 2003) and are compatible with all forms of aquatic life (Gao et al., 2017). Humic substances are widely used in agriculture and biomedicine owing to their antiseptic, antifungal, antioxidant, antimicrobial and detoxifying properties (Rupiasih & Vidyasagar 2005). Humic substances are considered to be part of the natural environment of the fish due to their water origin. This means that the fish's defence systems identify these natural xenobiotics. This makes humic substances an ideal alternative additives (Lieke et al., 2019). Humic substances are divided into three main groups according to their water solubility and molecular weight at different pH levels (Pettit, 2004): humic acid, fulvic acid and humin (Lieke et al., 2019; Stevenson, 1982). Humic acids can show antiviral and anti-inflammatory properties, antioxidant activity, immune system stimulation, and metal chelation owing to the phenol, hydroxyl and carboxyl groups they contain (Deng et al., 2020; Gao et al., 2017). Quinones, which have fungicidal/bactericidal properties, is responsible for the production of reactive oxygen (ROS) species in humic acids, which are essential for healing wounds. Carboxylic acids, especially phenolic groups with free radical scavenging capacity, are responsible for humic acid's antioxidant and anti-inflammatory properties (Gomes de Melo et al., 2015). Also, via its metal chelating feature, by transition metal catalysis, humic acid can control lipid peroxidation and DNA fragmentation by preventing the formation of free radicals (Marcinčák et al., 2023; Melo et al., 2016). Due to its chemical constituents, which include proteins, vitamins, digestive enzymes, and different antimicrobial and immunostimulating chemicals, humic acid significantly increases animal production (Turan & Turgut, 2020). Due to their antiviral activity, they improve the pH in the digestive system and reduce the number of infections in the environment by preventing pathogens from attaching to the flora (Çetin et al., 2006). It has been reported that humic substances used in aquaculture improved feed intake and growth, increased resistance to diseases, improving the health and welfare of the fish, and faster recovery from disease damage in *C. carpio* (Heidrich, 2005; Kodama et al., 2007; Abdel-Wahab, 2012; Sharaf & Tag, 2011); *Carassius aurata* (Heidrich, 2005); *Dicenctarchus labrax*

(Soytaş, 2015); *Plecoglossus altivelis* (Nakagawa et al., 2009); *Oncorhynchus mykiss* (Yılmaz et al., 2018a; Yılmaz et al., 2018b); *Oerochromis niloticus* (Deng et al., 2020). However, there are only limited studies on the antioxidant properties of humic acid (Adekunle & Ajuwon 2010; Deng et al., 2020; Prokešová et al. 2021).

Recent aquaculture applications of organic acid additives such as humic acid have created a new and practical approach to strengthening immune systems for immunocompromised organisms in intensive farming environments, achieving faster growth in a short time, and maintaining overall health. The current study aimed to investigate the effects on growth performance and general health status of carp and the antioxidant capacity of different doses of humic acid used as a feed additive.

Material and Method

Experimental Diets

Humic acid (powder form) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). The feed content was calculated according to the oil and protein ratios required by carp, namely about 35% protein and 9% oil. A two-mm diameter feed was made. First, the powder form was mixed in a drum mixer for thirty min. During the oil mixing process, fish oil and soybean oil were put into a beaker, and humic acid was added to the oil mix and mixed with a mixer plate for 30 min. The process was continued by removing starch at the same rate from the groups to which humic acid was added (Table 1). Finally, it was dried in a freeze-dryer for one day.

For the treatment groups, increasing doses of humic acid were added to the feed at rates of 0.2% (02HA), 0.4% (04HA), and 0.8% (08HA). Four groups with triplicate were formed, including the control group (0HA-without humic acid).

Table 1. Dietary formulation (%) and proximate composition of diet (%)

Ingredients (%)	0HA	02HA	04HA	08HA
Fish meal [a]	10	10	10	10
Soybean meal	20	20	20	20
Corn gluten meal	25	25	25	25
Wheat flour	15	15	15	15
Soybean oil	2	2	2	2
Fish oil [b]	4	4	4	4
Cellulose	4	4	4	4
Alpha starch	15	14.8	14.6	14.2
Humic acid	0	0.2	0.4	0.8
MCP	1	1	1	1
Mineral premix [c]	2	2	2	2
Vitamin premix [d]	2	2	2	2
Feed Proximate Analysis (%)				
Protein	35.77±0.03	35.56±0.03	35.71±0.30	36.15±0.18
Lipid	9.34±0.10	9.46±0.14	9.09±0.24	9.50±0.03

Moisture	4.04±0.01	5.32±0.05	5.77±0.03	3.67±0.23
Ash	5.14±0.09	5.04±0.01	4.98±0.03	5.23±0.01

[a] Fishmeal (Prime grade Peruvian anchovy meal, Diamante Co., Peru)
[b] Highcarol-E (Kanematsu Shintoa Foods Co. Ltd, Tokyo, Japan).
[c] Mineral mixture compostition (g/kg^{-1}): Sodium chloride 50; magnesium sulphate 745; iron (III) cirtrate n-hydrate 125; trace element mix 50; cellulose 30 [trace element mixture contains (g kg^{-1}): zinc sulphateheptahydrate 353; manganese sulphate 162; copper (II) sulphate pentahydrate 31; aluminum chloride hexahydrate 10; cobalt chloride hexahydrate 10; cobalt chloride 3; potassium iodate 1; cellulose 440.
[d] Vitamin mixture composition (unit kg^{-1}): Vitamin D3, 2,420,000 IU; Vitamin K3, 6,050 mg; thiamin, 3,025 mg; riboflavin, 3,630 mg; pyridoxine, 2,420 mg; cyanocobalamine, 6 mg; l-ascorbic acid, 368,900 mg; nicotinic acid, 24,200 mg; d-pantothenic acid, 6,050 mg; inositol, 121,000 mg; dbiotin, 363 mg; folic acid, 908 mg; para-aminobenzoic acid, 3025 mg.

Fish and Experimental Design

Common carps (20-50 g) were obtained from the Yoshida Research Station, Tokyo University of Marine Science and Technology, Shizuoka, Japan and transferred to a recirculating freshwater system. The health status of fish, normal physical form and coloration, the formation of cysts or spots on the body and gills, behavioral movements such as swimming and feeding reflexes were visually checked. Prior to the feeding trial, experimental fish were hand-fed twice a day using a commercial diet for adaptation period. For the humic acid group, 216 fish were used for the feeding trial with four treatment groups in a triplicate-random design. Experimental fish with an initial mean weight of 18 g were randomly distributed into twelve fiberglass aquariums 18 fish in each tank. Throughout a 60-day feeding trial, fish were hand-fed to visual satiation two times a day (10.00 a.m., and 5.00 p.m.). A 12L:12D light-dark cycle photoperiod was applied during the trial. Water quality parameters throughout the experiments were recorded 21-22 °C temperature, 7.2 pH, 0.2–0.5 mg/L ammonium and 0.02–0.04 mg/L nitrite. 50 % of the water was changed every day.

Blood Sampling

At the end of the 60-day feeding treatment, blood samples were taken for hematological and immunological analysis. Here, three fish (nine fish per group) from each tank were randomly caught and processed quickly after the fish were starved for one day. They were anesthetized with 35 mg/l ethyl-4-aminobenzoate (Wako, Japan). After blood collection from the caudal vein of each fish, blood samples were centrifuged to make serum at 5000 xg for 10 min. Serum samples were stored at -80 °C until analysis. In addition, blood was collected from nine different fish using heparinized syringes for hematological analysis.

Growth Performance

After 60 days, the fish, which were starved for one day, were weighed individually. Utilizing the data from initial weight, final weight, total feed consumption and protein ratio of feed, weight gain (WG), specific growth rate (SGR, % per day), feed conversion rate (FCR) and protein efficiency rate (PER) was calculated according to the formulas.

Haematological Parameters

Manual methods were used for RBC and WBC counts. The Natt-Herrick solution was first prepared (Natt and Herrick, 1952). Ten microliters of blood sample were added to 2 ml of Natt-Herrick solution (200×dilution). Then, the numbers of erythrocytes and leukocytes were counted using Burker hemocytometers at 400X magnification (McKnight, 1966; Yılmaz et al., 2016). Haematocrit analysis was performed using the microhematocrit method and haematocrit centrifuge (Tomy HC-12A). Here, blood samples were placed into heparinized glass haematocrit tubes and centrifuged at 12000 xg for 5 min. Measurements were calculated as a % (percentage) using a haematocrit reader.

Biochemical Analysis

The serum samples were analyzed for biochemical parameters in an automated chemical analyzer Hitachi 7020 (Hitachi, Tokyo, Japan) using commercial reagents obtained from Wako (Osaka, Japan). Measured parameters included aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea nitrogen (UN) (Urease-GIDH method), total cholesterol (TCHO) (Cholesterol oxidase-HMMPS method), high (HDL-C) and low (LDL-C) cholesterol. All samples were analyzed on the same day samples were collected.

Antioxidant analysis (SOD assay)

A commercial test kit (Dojindo S311 SOD, WST) was used for SOD analysis. Briefly, 200 µL of WST solution was added to 20 µL of serum, followed by the addition of 20 µL of enzyme solution. The samples were then incubated at 37 °C for 20 minutes. Then, readings were made at 450 nm. The results were evaluated as % inhibition.

Statistical Analysis

SPSS software was used for statistical evaluation of all analysis. For this, significance was set at $p < 0.05$. One-way analysis of variance (ANOVA) was used for the data and the resultant values were presented as Means ± Standard Error of Mean (SEM). In case of homogeneity of variances, Tukey's multiple comparison and Duncan test were used; otherwise, a Tamhane and Games-Howell post hoc test were applied.

Results

Growth Performance

All experimental groups were observed at each feeding and it was assured that all of them ate to satiation. During the treatment period, no visible signs of disease were visible in any of the tanks. Weight gain, specific growth rate, feed conversion rate, and protein efficiency rate were calculated for growth performance evaluation. According to these evaluations, the FW, WG, and SGR values of the 04HA group (with feed containing 0.4% humic acid) showed statistically higher values (41.37±0.87, 23.26±1.01, 1.37±0.05 respectively) compared to the control ($p <$ 0.05). While a statistical difference was observed in the 08HA group ($p < 0.05$), no difference was observed in the 02HA group ($p > 0.05$) in the FW value. No difference was observed between the groups in FCR and PER values ($p > 0.05$) (Table 2).

Table 2. Growth performance of common carp (*Cyprinus carpio*) when fed diets with varying levels of dietary humic acid (0%, 0.2%, 0.4%, and 0.8%) was observed after 60 days.

	0HA	**02HA**	**04HA**	**08HA**
IW (g)	17.72±0.09	17.57±0.15	18.10±0.32	18.22±0.09
FW (g)	35.77±1.02^{b}	36.12±1.08^{b}	41.37±0.87^{a}	40.35±0.92^{a}
WG (g)	18.04±1.06^{b}	18.55±0.93^{b}	23.26±1.01^{a}	22.13±0.57^{ab}
FCR	1.89±0.06	1.76±0.07	1.84±0.05	1.86±0.02
SGR (% d^{-1})	1.17±0.05^{b}	1.20±0.04^{ab}	1.37±0.05^{a}	1.33±0.02^{ab}

PER	1.54±0.52	1.70±0.07	1.61±0.04	1.55±0.01
Survival Rate (%)	100	100	100	100

Values (mean ± *SEM*, *n* = 3). Different letters in the same line indicate significant differences within groups ($p < 0.05$). IW (initial weight), FW (final weight), WG (weight gain), FCR (feed conversion ratio), SGR (specific growth rate), and PER (protein efficiency rate). (WG) = Final fish weight (g) - Initial fish weight (g), (SGR) = 100 (ln (final fish weight)) - (ln (initial fish weight))/experimental days, (FCR) = feed intake (g) /weight gain (g), (PER) = weight gain (g) /protein intake (g)

Haematological Variables

Haematocrit, RBC and WBC values were evaluated. Although the RBC value was quantitively higher in the 02HA and 04HA groups, RBC and haematocrit values were not statistically different between the groups. The WBC values of the 02HA and 04HA groups showed higher values compared to the other groups ($p < 0.05$). In addition, There was a statistical decrease in the 08HA group compared to all other groups ($p < 0.05$) (Table 3).

Table 3. Haematological variables of common carp (*Cyprinus carpio*) when fed diets with varying levels of dietary humic acid (0%, 0.2%, 0.4%, and 0.8%) was observed after 60 days.

	0HA	**02HA**	**04HA**	**08HA**
RBC (10^6/mm^3)	3.82±0.15	5.43±1.06	4.98±0.62	4.45±0.18
WBC (10^3/mm^3)	14.37±2.62^{ab}	20.62±3.74^{a}	21.43±1.50^{a}	8.35±1.65^{b}
Hct (%)	36.33±1.86	37.22±1.72	36.22±1.35	36.33±1.15

Values are mean ± *SEM* (*n* = 9). Different letters in the same line indicate significant differences within groups ($p < 0.05$). Variables are RBC (red blood cell) count, WBC (White blood cell), Hct (haematocrit). WBC= leukocyte count x 200/ (9x0.1x1 000), RBC= erythrocyte count x 200x5/(0.1x1 000 000)

Serum Biochemical Variables

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea nitrogen (UN), total cholesterol (T-CHO), high (HDL-C) and low (LDL-C) cholesterol for serum biochemistry results were evaluated. According to the results shown in Table 4, no statistical difference was observed between the groups ($p > 0.05$).

Table 4. Biochemical parameters of common carp (*Cyprinus carpio*) when fed diets with varying levels of dietary humic acid (0%, 0.2%, 0.4%, and 0.8%) was observed after 60 days.

	0HA	**02HA**	**04HA**	**08HA**
AST (U/L)	336±84.03	516±196.22	365.33±97.33	284±34.02
ALT (U/L)	9.33±1.33	16±4.00	13.33±1.33	12±2.31
ALP (U/L)	137.33±9.61	138.67±16.38	128±6.11	137.33±19.91
UN (mg/l)	3.6±0.23	2.67±0.13	3.33±0.13	3.6±0.83
T-CHO (mg/dl)	171.04±13.44	144.25±11.69	168.41±13.64	176.08±15.57
HDL-C (mg/dl)	124.13±11.74	109.6±7.94	122.93±7.08	129.2±8.4

LDL-C (mg/dl)	106.27±9.96	93.07±6.37	98.13±5.28	109.73±8.44

Values are mean ± *SEM* ($n = 9$). Different letters in the same line indicate significant differences within groups ($p < 0.05$). Variables are GOT (glutamic oxaloacetic transaminase), GPT (glutamic pyruvic transaminase), ALP (alkaline phosphatase), T-COL (total cholesterol), HDL-C (high-density lipoprotein cholesterol) and LDL-C (low-density lipoprotein cholesterol)

Antioxidant Capacity
SOD (superoxide dismutase) analysis was evaluated to determine the antioxidant capacity of humic acids. All treatment groups showed an increase compared to the control. The groups containing 0.4% and 0.8% humic acid showed statistically high values compared to the control ($p < 0.05$). No statistical change was observed in the 02HA group ($p > 0.05$) (Table 5).

Table 5. Antioxidant parameter of common carp (*Cyprinus carpio*) when fed diets with varying levels of dietary humic acid (0%, 0.2%, 0.4%, and 0.8%) was observed after 60 days.

	0HA	**02HA**	**04HA**	**08HA**
SOD (% inhibition)	51.81 ± 1.35^{b}	62.79 ± 5.91^{ab}	72.78 ± 3.38^{a}	71.63 ± 5.01^{a}

Values are mean ± *SEM* ($n = 9$). Different letters in the same line indicate significant differences within groups ($p < 0.05$). Variable is SOD (superoxide dismutase)

Discussion
The search for alternative additives to enhance growth and improve the health and welfare of fish has been the recent focus of aquaculture. Alternative additives such as organic acids increase mineral absorption by reducing feed and intestinal pH. It also prevents the colonization of harmful bacteria in the intestines and increases the utilization of nutrients. In addition, the hygienic quality of feed increases in an acidic environment, and the loss of nutritional value decreases (Ng & Koh, 2016).

Humic acids adhere to the mucous epithelium in the digestive system and form protective films against infections and pathogens, thus protecting the intestinal flora and helping to improve the digestion of nutrients (Yılmaz et al., 2018b; Islam et al., 2005). Hence, humic acids provide a higher food conversion rate, significantly improving growth performance parameters (Islam et al., 2005).

In our study, FW, WG, and SGR values showed a statistically significant increase in the group fed 0.4% humic acid. Consistent with the positive effects of growth parameters obtained in our study on humic acid, Abdel-Wahab et al. (2012) reported that carp fed with three different doses of humic acid (0.4, 0.8, and 1%) showed the highest value of body weight gain and condition factor in high dose groups. In a 55-day study conducted by Turan and Turgut in 2020, they found that Carassius auratus fish showed significant improvements in FCR values when fed with leonardite at a rate of 25g kg^{-1} and 5g kg^{-1}, a substance containing a high amount of humic acid. In *C. carpio* fed with humic acid (1%, 3%, 5%) additive for 21 days, all groups showed an increase compared to the control, while the highest body weight gain was observed in the 1% supplemented group (Rousdy & Wijayanti, 2015). In addition, although positive effects on growth were observed in studies on *C. carpio* (Sharaf & Tag, 2011) and sturgeon (Vasiliev et al., 2020), in studies on *Dicentrarchus labrax* (Soytaş, 2015) and *Oncorhynchus mykiss* (Yılmaz et al., 2018a) no contribution of humic substances on growth was observed. Also, There was no statistical difference in the growth parameters of *Clarias gariepinus* fed with commercial humic substances (1%, 3%, 6%) (Prokešová et al., 2021). These different findings show that the effect of humic acid may depend on the species, time, and doses used. Similar to other studies evaluating the effect of humic acid on carp, in our study humic acid addition to feed contributed to WG and SGR of carp. The digestibility and transfer of nutrients affect feed utilization and growth in fish (Esmaeili et al., 2017). Thus, additives such as humic acid, which increase nutrient digestion, can stimulate digestive enzymes and contribute to growth.

Hematological analyses are the most important techniques used to determine general fish health and physiological state and are considered indicators of diseases, nutritional deficiencies, and stress caused by adverse environmental conditions (Witeska et al., 2016). These values change during periods of infection and are widely used to detect the adverse effects of diseases (İspir et al., 2004). RBC is responsible for carrying oxygen to the gills and tissues. Elevated RBC values contribute to the health and growth of the fish by providing better oxygen delivery to the tissues (Esmaeili, 2021). Decreases in RBC and Hct ratios are indicators of anemia (Blaxhall & Daisley, 1973). In addition, since there is a decrease in erythrocytes in fish exposed to pathogens, they are an essential criterion for understanding the health status of fish (Yılmaz, 2017). WBC, a necessary part of innate immunity, plays a vital role in body defense and combating disease agents by phagocytosis (Yılmaz, 2017).

Humic acid improves immunity and stress management via its many beneficial effects, including antibacterial, antiviral, and anti-inflammatory properties (Islam et al., 2005). In this study, RBC and hematocrit values did not differ between the groups, but WBC values increased significantly in the 0.2% and 0.4% humic acid groups. Similar results were seen in feeding studies on *O. mykiss* when 0.1, 0.2, and 0.4% rates of commercial FARMARIN composed of humic substance components (Yılmaz et al., 2018a) and 0.3, 0.6 and 1.2% rates of humic acid sodium salt (Yılmaz et al., 2018b) were added to the feeds, no significant changes were observed in hematological values such as RBC, Hct and Hb. However, Rousdy and Wijayanti (2015) reported a significant increase in erythrocyte and hematocrit values with 1% humic acid, while the highest leukocyte values were observed in feed containing 3% humic acid. In the humic acid (0.25, 0.50, 0.75, and 1.5%) study on *D. labrax*, the RBC, Hb and Hct values of the 0.50% group were greater than the groups infested with *Amyloodinium* spp (Soytaş 2015). However, the haematological values decreased in the groups that received higher doses of humic acid, as previously documented by Soytaş (2015).

In these previous studies, conditions such as fish species used, treatment doses applied, and environmental factors may have caused differences in haematological values. In our research, the high WBC values of the group containing 0.2% and 0.4% humic acid may indicate that the doses used can improve immune defense and respond better to stress factors. It was also observed that the RBC values of the 02HA and 04HA groups were quantitively higher than the control, although not significantly so. Notably, when compared with the growth performance of the 04HA group, this may support the linear relationship between RBC increase and growth.

Serum biochemistry parameters are valuable indicators of the health status of animals fed with feed additives (Yılmaz et al., 2018b). The serum enzymes AST, ALT, and ALP are indicators of damage to tissues and organs (Campbell et al., 2004). These liver enzymes are evaluated for diagnosing liver problems (Campbell et al., 2004; Hart et al., 2010). AST, ALT, ALP, UN, T-CHO, HDL-C, and LDL-C were evaluated in serum biochemistry analysis in our study, with no significant changes in experimental groups.

Consistent with our study of humic acid treatment, no significant changes were observed in *C. gariepinus* serum biochemistry parameters following the feeding of humic additives (1, 3, and

6%) (Prokešová et al., 2021). Although there was no statistically significant change in AST, ALT, CHO, and ALP values in *O. mykiss*, the triglyceride level decreased compared to the control (Yılmaz et al., 2018b). While the CHO value did not change in the study on *D. labrax*, GLU, TPROT, ALB, and GLO parameters were lowest in the group containing 0.75% humic acid additive (Soytaş, 2015). Given the wide range of experimental variables seen in previous studies, it is safe to conclude that adding humic acid to feed does not adversely affect the general health of the fish and does not cause any damage to internal organs.

Antioxidants are substances that protect cells from damage caused by free radicals and prevent oxidative damage by neutralizing electrical charges (Goel & Dhingra, 2021). Humic substances can effectively eliminate free radicals by utilizing functional groups, such as hydroxyl and phenolic hydroxyl, which they contain (Deng et al., 2020). They also induce biotransformation enzyme activities and affect antioxidant defense in fish (Yurchenko & Morozov, 2022). Improvement in defense system enzymes such as SOD, CAT, and GST, needed to maintain the complex fish immune system, may increase the possibility of overcoming environmental stress (Deng et al., 2020). To determine the antioxidant capacity of humic acid, the SOD enzyme, one of the important components of the antioxidant defense system, was evaluated. Our SOD analysis found all treatment groups having quantitively higher values than the control, while the antioxidant capacity of the 04HA and 08HA groups were significantly higher.

Studies on feeding-related antioxidant values of humic substances in fish are limited. Adekunle and Ajuwon (2010), exposed *Clarias gariepinus* to different levels of humic acid (100, 250, 500, and 1000 mg/l). In SOD, CAT, and GSH analyses performed to evaluate antioxidant levels, all treatment groups decreased compared to the control. In a study where sodium humate was added to *Oreochromis niloticus* (GIFT) diets at 0.1, 0.2, 0.4, and 0.6%, no significant relationship was recorded in SOD, CAT, GPx, and ACP activities, although an increase in GSH and AKP activity was observed as the doses increased. (Deng et al., 2020). Although GSH and GSSG ratios of fish fed for 56 days with 1, 3 and 6% humic acid were not statistically significant, the GSH/GSSG ratio was higher with 3% humic acid compared to the other groups (Prokešová et al., 2021).

Many studies have reported that an increase in SOD and CAT enzymes is directly proportional to increasing immune response (Zhou et al., 2010; Park et al., 2016). Thus, an improvement in antioxidant enzymes levels may increase the probability that fish can overcome environmental stress (Deng et al., 2020). Our results show that a feed additive of 0.4% and 0.8% humic acid can improve the antioxidant defense activity and enhance antistress capability. The positive effects of humic substances on animal health are well known. These alternative additives can reduce the physiological problems caused by stress and strengthen immune responses. However, some studies have failed to document these positive effects. Although the alternative additives we used are similar to other studies, the literatures are replete with great variability in methods, such as; different species and sizes of fish, different doses administered, dosing technique, feed composition, experimental physical conditions and experimental season. Accordingly, the findings obtained in our study cannot be fully compared with the findings in the literature.

Conclusion

In addition to the fact that humic acid increases growth and reaches market size quickly, strengthens immunity, and provides resistance to diseases, its eco-friendly, easily accessible, and low rates of effect, reducing antibiotic and chemical costs, makes humic acid a sustainable and economical alternative. In our 60-day study, the groups containing 0.2% and 0.4% humic acid with high WBC values had a better immune response. Notably, the 0.4 % humic acid group displayed solid increases in WBC and SOD enzymes, with a concomitant decrease in oxidative stress effects and stimulated immune response. The growth performance, hematological value, and antioxidant capacity in the 0.4 % humic acid group contributed to the growth and health of the fish. Therefore, incorporating humic acid into fish feed can promote fish health and growth while maintaining ecological and economic sustainability.

Acknowledgements

The present study was produced from a Doctoral Dissertation (Ph.D.) under the supervision of Assoc. Prof. Takayuki KATAGIRI. Tokyo University of Marine Science and Technology (Japan) is acknowledged for the use of research facilities and laboratory equipments provided for this study.

Ethical approval

The animal study protocol was approved by the "Animal Ethics Committee" of Tokyo University of Marine Science and Technology, Japan.

Informed consent

Not available

Data availability statement

The authors declare that data can be provided by corresponding author upon reasonable request.

Conflicts of interest

There is no conflict of interests for publishing this study.

Funding organizations

No funding is available for this study.

Contribution of authors

Nergiz Çoban: : Investigation, methodology, formal analysis, writing original draft
Yutaka Haga: : Validation, Review and editing
Masashi Maita: : Validation, Review and editing
Kunihiko Futami: : Validation, Review and editing
Sevdan Yilmaz: : Data curation, Investigation, Methodology
Sebahattin Ergün: : Validation, Review and editing
Murat Yigit: : Validation, Review and editing
Taekyoung Seong : Formal analysis
Ozan Oktay : Formal analysis
Takayuki Katagiri : Investigation, Methodology, Project administration, Supervision, Validation, Review and editing

All authors have read and agreed to the published version of the manuscript.

References

Abdel-Latif, H. M. R., & Khafaga, A. F. (2020). Natural co-infection of cultured Nile tilapia *O. niloticus* with *Aeromonas hydrophila* and *Gyrodactylus cichlidarum* experiencing high mortality during summer. *Aquaculture Research, 51*, 1880-1892. https://doi.org/10.1111/are.14538

Abdel-Wahab, A. M., El-Refaee, A. M. E., & Ammar, A. A. (2012). Effects of humic acid as feed additive in improvement of nonspecific immune response and disease resistance in common carp (*C. carpio*). *Egyptian Journal for Aquaculture, 2-1*.

Adekunle, I., & Ajuwon, O. R. (2010). Influence of humic acid derived from composted wastes of Nigeria origin on oxidative and antioxidant status of African mud catfish (*C. gariepinus*). *Pakistan Journal of Biological Sciences, 13*(17), 821-827. https://doi.org/10.3923/pjbs.2010.821.827

Alemayehu, T. A., Geremew, A., & Getahun, A. (2018). The Role of functional feed additives in tilapia nutrition. *Fisheries and Aquaculture Journal, 9*. https://doi.org/10.4172/2150-3508.1000249

Atamanalp, M., Ucar, A., Kocaman, E. M., & Alak, G. (2021). Evaluation of brown trout (*Salmo trutta fario*) fillets' shelf life: Fed with a humic supplemented diet. *Food Packaging and Shelf Life.* https://doi.org/10.1016/j.fpsl.2021.100675

Blaxhall, P.C., & Daisley, K.W. (1973). Routine hematological methods for use with fish blood. *Journal of Fish Biology, 5*, 771-781. https://doi.org/10.1111/j.1095-8649.1973.tb04510.x

Campbell, T. W., Thrall M. A., Baker D. C., DeNicola, D., Fettman M. J., Lassen E. D., Rebar A., & Weiser, G. (Eds). (2004). *Veterinary Hematology and Clinical Chemistry*, (1st ed.). Lippincott Williams and Wilkins Pennsylvania.

Çetin, N., Çetin, E., & Güçlü, B. K. (2006). The effect of humate and organic acids added to the ration on some haematological parameters in laying hens. *Ankara Üniversitesi Veteriner Fakültesi Dergisi, 53*, 165-168.

Deng J., Lin, B., Zhang, X., Guo, L., Chen, L., Li, G., Wang, Q., Yu, C., & Mi, H. (2020). Effects of dietary sodium humate on growth, antioxidant capacity, non-specific immune response, and resistance to *Aeromonas hydrophila* in genetic improvement of farmed tilapia (GIFT, *O. niloticus*). *Aquaculture, 520*, 734788. https://doi.org/10.1016/j.aquaculture.2019.734788

Esmaeili, M., Abedian Kenari, A., & Rombenso, A. (2017). Effects of fish meal replacement with meat and bone meal using garlic (*Allium sativum*) powder on growth, feeding, digestive enzymes and apparent digestibility of nutrients and fatty acids in juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792). *Aquaculture Nutrition, 23*, 1225–1234. https://doi.org/10.1111/anu.12491

Esmaeili, M. (2021). Blood performance: A new formula for fish growth and health. *Biology, 10*, 1236. https://doi.org/10.3390/biology10121236

FAO, (2022). The state of world fisheries and aquaculture 2022. Towards blue transformation. Rome, FAO. https://doi.org/10.4060/cc0461en.

Gao, Y., He, J., He, Z., Li, Z., Zhao, B., Mu, Y., Lee, J. Y., & Chu, Z. (2017). Effects of fulvic acid on growth performance and intestinal health of juvenile loach *Paramisgurnus dabryanus* (Sauvage). *Fish Shellfish Immunology, 62*, 47–56. https://doi.org/10.1016/j.fsi.2017.01.008

Goel, P., & Dhingra, M. (2021). Humic substances: Prospects for use in agriculture and medicine. In book: Humic Substances (Abdelhadi Makan, Ed) (pp. 184). https://doi.org/10.5772/intechopen.99651

Gomes de Melo, B. A., Motta, F. L., Helena, M., & Santana, A. (2015). Humic acids: Structural properties and multiple functionalities for novel technological developments. *Materials Science and Engineering, 62*, 967–974. https://doi.org/10.1016/j.msec.2015.12.001

Haitzer, M., Hoss, S., Traunspurger, W., & Steinberg, C. E. W. (1998). Effects of dissolved organic matter (DOM) on the bioconcentration of organic chemicals in aquatic organisms-A review. *Chemosphere, 37*, 1335–1362. https://doi.org/10.1016/S0045-6535(98)00117-9

Hart, S. D., Bharadwaj, A. S., & Brown, P. B. (2010). Soybean lectins and trypsin ınhibitors but not oligosaccharides or the ınteractions of factors ımpact weight gain of rainbow trout (*O. mykiss*). *Aquaculture, 306*, 310–314. https://doi.org/10.1016/j.aquaculture.2010.03.027

Heidrich, S. (2005). Prophylaktischer und therapeutischer einsatz von braunkohle-huminstoffen in der nutz- und zierfischzucht. Inaugural-dissertation veterinärmedizinische fakultät, universität leipzig.

Islam, K., Schuhmacher, A., & Gropp, J. (2005). Humic acid substances in animal agriculture. *Pakistan Journal of Nutrition, 4*(3), 126–134. https://doi.org/10.3923/pjn.2005.126.134

Kodama, H., Denso, & Nakagawa, T. (2007). Protection against atypical *Aeromonas salmonicida* Infection in carp (*C. carpio* L.) by oral administration of humus extract. *Journal of Veterinary Medical Science, 69*, 405-408. https://doi.org/10.1292/jvms.69.405

Lieke, T., Meinelt, T., Hoseinifar, S. H., Pan, B., Straus, D. L., & Steinberg, C. E. W. (2019). Sustainable aquaculture requires environmental-friendly treatment strategies for fish diseases. *Reviews in Aquaculture, 12*(2). https://doi.org/10.1111/raq.12365

Marcinčák, S., Semjon, B., Marcinčáková, D., Reitznerová, A., Mudroňová, D., Vašková, J., & Nagy, J. (2023). Humic substances as a feed supplement and the benefits of produced chicken meat. *Life, 13*(4), 927. https://doi.org/10.3390/life13040927

McKnight, I. M. (1966). A hematological study on the mountain whitefish, *Prosopium williamsoni*. *Journal of the Fisheries Research, 23*, 45-64. https://doi.org/10.1139/f66-005

Meinelt, T., Schreckenbach, K., Pietrock, M., Heidrich, S., & Steinberg, C. E. W. (2008). Humic Substances Part 1: Dissolved humic substances (HS) in aquaculture and ornamental fish breeding. *Environmental Science and Pollution Research, 15*(1), 17–22. http://dx.doi.org/10.1065/espr2007.08.448

Melo, B. A. G., Motta, F. L., & Santana, M. H. A. (2016). Humic acids: Structural properties and multiple functionalities for novel technological developments. *Materials Science and Engineering, 62*, 967–974. https://doi.org/10.1016/j.msec.2015.12.001

Nakagawa, J., Iwasaki, T., & Kodama, H. (2009). Protection Against *Flavobacterium psychrophilum* Infection (cold water disease) in ayu fish (*P. altivelis*) by oral administration of humus extract. *Journal of Veterinary Medical Science, 71*(11), 1487-1491. https://doi.org/10.1292/jvms.001487

Natt, M. P., & Herrick, C. A. (1952). A new blood diluent for counting the erythrocytes and leukocytes of the chicken. *Poultry Science, 31*(4), 735-738. https://doi.org/10.3382/ps.0310735

Ng W. K., & Koh C. B. (2016). The Utilization and mode of action of organic acids in the feeds of cultured aquatic animals. *Reviews in Aquaculture, 9*, 342–368. https://doi.org/10.1111/raq.12141

Park Y., Lee, S., Hong, J., Kim, D., Moniruzzaman, M., & Bai, S. C. (2016). Use of probiotics to enhance growth stimulate ımmunity and confer disease resistance to *Aeromonas salmonicida* in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture Research, 1-11*. https://doi.org/10.1111/are.13099

Pettit, R. E. (2004). Organic matter, humus, humate, humic acid, fulvic acid and humin: Their importance in soil fertility and plant health. Covington: CTI Research, (pp. 17). https://humates.com/wp-content/uploads/2020/04/ORGANICMATTERPettit.pdf

Prokešová, M., Bušová, M., Zare, M., Tran, H.Q., Kucerová, E., Ivanova, A. P., Gebauer, T., & Stejskal, V. (2021). Effect of humic substances as feed additive on the growth performance, antioxidant status, and health condition of African catfish (*C. gariepinus*, Burchell 1822). *Animals, 11*(8), 2266. https://doi.org/10.3390/ani11082266

Rousdy, D. W., & Wijayanti, N. (2015). Hematology profile and growth respons of carp (*C. carpio* Linn.) on administration of humic acid from kalimantan peat soil. Prosiding Semirata, Prosiding Semirata 2015 bidang MIPA BKS-PTN Barat, Universitas Tanjungpura Pontianak (pp. 135-144).

Rupiasih, N. N., & Vidyasagar P. B. (2007). Humic substances: structure, function and applications. *International Journal of Environment and Pollution, 5*(2), 39-47. https://www.researchgate.net/publication/225303930

Sharaf, M. M., & Tag, H. M. (2011). Growth performance, gill, liver and kidney histomorphology of common carp (*C. carpio*) fingerlings fed humic acid supplemented diets. *Egyptian Journal of Experimental Biology, 7*(2), 285-294. https://www.bibliomed.org/mnsfulltext/3/3-1432005411.pdf?1717857333

Soytaş, N. (2015). Effects on survival rate and some blood parameters of *Amyloodinium* sp. infestation at the sea bass (*D. labrax*) fed on humic acid added diet. (MsC), Graduate School of Natural and Applied Sciences, Çanakkale Onsekiz Mart University, Türkiye, pp. 64.

Steinberg, C. E. W. (2003). Ecology of humic substances in freshwaters: Determinants from geochemistry to ecological niches. *Springer Science & Business Media*, Heidelberg (pp. 440). https://doi.org/10.1007/978-3-662-06815-1

Stevenson, F. J. (1982). Humus chemistry: Genesis, composition, reactions. Wiley, New York (pp. 443). https://doi.org/10.1021/ed072pA93.6

Thurman, E. M. (1985). Organic geochemistry of natural waters. Nijhoff, M./Junk, W. Publishers, *Springer Science & Business Media* (pp. 497).

Turan, F., & Turgut, M. (2020). The Effect of leonardite as feed additive on growth of goldfish (*Carassius auratus* L.). *NESciences, 5*, 184-191. https://doi.org/10.28978/nesciences.832994

Vasiliev, A. A., Tarasov, P. S., Turenko, O. Y., Matsyupa, I. O., Sadygova, M. K., Bukhovets, V. A., Zaitsev, V. V., & Kokorev, V. A. (2020). The effectiveness of using humic acids for feeding sturgeons in the conditions of a ras (Recirculation aquaculture system). *Ecology, Environment and Conservation Journal, 26*(2), 910-913. http://www.envirobiotechjournals.com/EEC/v26i220/EEC-67.pdf

Wang, J. L., Meng, X., Lu, R. H., Wu, C., Luo, Y. T., & Yan, X. (2015). Effects of *Rehmannia glutinosa* on growth performance, immunological parameters and disease resistance to *Aeromonas hydrophila* in common carp (*C. carpio* L.). *Aquaculture, 435*, 293–300. https://doi.org/10.1016/j.aquaculture.2014.10.004

Witeska, M., Lugowska, K., & Kondera, E. (2016). Reference values of hematological parameters for juvenile *C. carpio*. *Bulletin of the European Association of Fish Pathologists, 36*(4), 169. https://www.researchgate.net/publication/306380872_Reference_values_of_hematological_parameters_for_juvenile_Cyprinus_carpio#fullTextFileContent

Yılmaz, S., Ergün, S., & Çelik, E. Ş. (2016). Effect of dietary spice supplementations on welfare status of sea bass *Dicentrarchus labrax* L. Proceedings of the National Academy of Sciences India Section B: *Biological Sciences, 86*, 229-237. https://doi.org/10.1007/s40011-014-0444-2

Yılmaz, S. (2017). The effect of dietary cinnamic acid or *Bacillus subtilis* feed on growth performance and some immune parameters in rainbow trout. Doctoral dissertation, Canakkale Onsekiz Mart University, Graduate School of Natural and Applied Sciences, Türkiye (pp. 210).

Yılmaz, S, Ergün, S., & Yiğit, M. (2018a). Effects of dietary FARMARIN® XP supplement on immunological responses and disease resistance of rainbow trout (*O. mykiss*). *Aquaculture, 496*, 211-220. https://doi.org/10.1016/j.aquaculture.2018.07.024

Yılmaz, S., Ergün, S., Çelik, E. Ş., & Yiğit, M. (2018b). Effects of dietary humic acid on growth performance, haemato-immunological and physiological responses and resistance of Rainbow trout, *O. mykiss* to *Yersinia ruckeri*. *Aquaculture Research, 49*(10), 3338–3349. https://doi.org/10.1111/are.13798

Yurchenko, V., & Morozov, A. (2022). Responses of hepatic biotransformation and antioxidant enzymes in Japanese medaka (*Oryzias latipes*) exposed to humic acid. *Fish Physiology and Biochemistry, 48*, 1-13. https://link.springer.com/article/10.1007/s10695-021-01034-4

Zhang, J., Huang, M., Feng, J., Chen, Y., Li, M., & Chang, X. (2021). Effects of dietary *Bacillus licheniformis* on growth performance, intestinal morphology, intestinal microbiome, and disease resistance in common carp (*C. carpio* L.). *Aquaculture International, 29*, 1343–1358. https://link.springer.com/article/10.1007/s10499-021-00701-w

Zhou, X., Tian, Z., Wang, Y., & Li, W. (2010). Effect of treatment with probiotics as water additives on tilapia (*Oreochromis niloticus*) growth performance and immune response. *Fish Physiology and Biochemistry, 36*, 501-509. https://doi.org/10.1007/s10695-009-9320-z