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Research Article

Microalgae feed additives improve growth, immunity, and resistance to *Vibrio anguillarum* infection in juvenile rainbow trout, *Oncorhynchus mykiss*

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Abstract

The present study was conducted to evaluate the effects of three different dietary microalgae on growth, intestinal histology, immune biomarkers, and resistance to a bacterial pathogen (*Vibrio anguillarum*) in juvenile rainbow trout, *Oncorhynchus mykiss*. Four experimental diets were prepared, including a basal diet (CON) and three diets containing *Chlorella* sp. (CHL), *Haematococcus* sp. (HAE), or *Schizochytrium* sp. (SCH), at 0.5% for each microalga which was supplemented in the basal diet. A total of 180 juvenile rainbow trout with an initial body weight of 12.16±0.01 g (mean±SD) were randomly distributed into 12 tanks and reared by the semi-recirculation system. After six weeks of the feeding trial, the weight gain (99.4%), specific growth rate (1.92%/day), and myeloperoxidase activity (5.08), of the fish fed the HAE were significantly higher than those of fish fed the other diets (p<0.05). The intestinal villus length of the fish fed the HAE diet (1.34 µm) was significantly higher than that of those fed the CHL (1.13 µm) and CON (1.14 µm) diets. The cumulative survival rates (CSR) were recorded for 27 days after intraperitoneal injection of the bacterial pathogen *V. anguillarum*. The CSR of the fish fed the HAE diet (75%) was significantly higher than that of those fed the other diets. It is suggested that *Haematococcus* sp. (0.5% inclusion in the diet) may improve weight gain, specific growth rate, intestinal villus length, and myeloperoxidase activity as well as increase the survival rate of juvenile rainbow trout against the *V. anguillarum* challenge.

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Introduction

Up to date, most fish farmers use large amounts of antibiotics to prevent high mortality from disease and/or as promoters of the immune system in fish. However, their indiscriminate use is harmful in several ways, especially in the environmental context. This can result in bacterial resistance to antibiotics, ecological disasters, and residues in the animals and humans who consume the fish (Dawood *et al.*, 2018; Han *et al.*, 2019; Yuan *et al.*, 2019). For these reasons, several countries have banned or restricted the use of antibiotics as growth promoters or immune system enhancers in animal feeds (Gamboa-Delgado and Márquez-Reyes, 2018; Lulijwa *et al.*, 2020; Zhou *et al.*, 2021). Thus, researchers are searching for antibiotic replacements in fish farming. The use of natural feed additives is a potential solution being researched to reduce the use of antibiotics in aquaculture (Cho and Lee, 2012; Bai *et al.*, 2015; Zhao *et al.*, 2020). Currently, feed additives such as probiotics, prebiotics, synbiotics, or natural immunostimulants are being increasingly tested. Immunostimulants such as yeast, bacteria, plants, and microalgae have been tested in aquaculture (Thépot *et al*., 2021).

Microalgae have been shown to improve the animal immune system (Milan *et al.*, 2021) with a high nutritional value due to nutrients such as proteins, polyunsaturated fatty acids, and polysaccharides (Christaki *et al.*, 2011), as well as antioxidants (Becker, 2013; Li *et al.*, 2015) and other bioactive compounds (Zhang *et al.*, 2020). All of this makes microalgae attractive live feed (Sirakov *et al.*, 2015) or feed additive in the production of aquaculture species. According to Kaparapu (2018), *Haematococcus* sp. and *Chlorella* sp. are the most common microalga species used as fish feed ingredients. *Haematococcus* sp. has been mainly used as a dietary supplement in salmonids for muscle pigmentation (Choubert *et al.*, 2009; Li and Liu, 2019). Recently, they have received more attention as dietary antioxidants due to their biological properties (Lorenz and Cysewski, 2000; Mularczyk *et al.*, 2020). *Chlorella* sp. contains a high percentage of protein (51-58%), and the remainder is made up of carbohydrates, lipids, and other nutrients (Panahi *et al.*, 2019) which facilitate digestion (Rani *et al.*, 2018). *Schizochytrium* sp., as heterotrophic algae, are a source of polyunsaturated fatty acids (n-3 LC-PUFA) that are becoming available in aquafeed, because they are rich in docosahexaenoic acid (DHA). Thus, they are being tested to replace fish oil and fishmeal, demonstrating better digestibility and growth performance in fish (Bélanger-Lamonde *et al.*, 2018; Allen *et al.*, 2019; Tibbetts, *et al.*, 2020). For example, experiments conducted using microalgae as feed additives have shown a significant improvement in the immune response, growth, feed utilization, and disease resistance in aquatic organisms by using *Haematococcus* sp. (Ju *et al.*, 2012; Sheikhzadeh *et al.*, 2012; Xie *et al.*, 2018; Xie *et al.*, 2020; Yu *et al.*, 2020), *Chlorella* sp. (Kim *et al.*, 2002; Xu *et al.*, 2014; Mahmoud *et al.*, 2020), and *Schizochytrium* sp. (Lyons *et al.*, 2017; Shah *et al.*, 2018; Xie *et al.*, 2019; Sarker *et al.*, 2020). *Haematococcus* sp. has been approved in Japan and Canada as a natural pigment for

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fish feeds. Also, the US Food and Drug Administration (FDA) approved its use as a color additive in salmonid feeds as well as direct human consumption. Astaxanthin and biomass of *Haematococcus* sp. can be used as nutrient ingredients in foods (human supplements). Moreover, both are permitted as feed colors for salmonid fish in the USA and Europe (Ambati *et al.*, 2014; de Carvalho *et al.*, 2022). Nowadays, several companies are producing these microalgae using new techniques to extract natural astaxanthin (Lorenz and Cysewski, 2000). *Chlorella* sp. has been utilized as a nutritional supplement for many years (Mahmoud *et al.*, 2020), possesses various biological and pharmacological properties, and is widely produced and used in aquaculture (Galal *et al.*, 2018). *Schizochytrium* sp. is suitable for large-scale heterotrophic cultivation under controlled conditions and utilized in feed due to long-chain polyunsaturated fatty acids (Xie *et al.*, 2019). As reported by Skalli *et al.* (2020), microalga supplementation in diets is emerging as a potential additive in high-value fish; however, limited studies have been conducted in salmonid species (Shah *et al.*, 2018; Yarnold *et al.*, 2019).

Rainbow trout is one of the most economically important freshwater salmonids (Sarker *et al.*, 2020). In 2020, the total production of rainbow trout reached 959.6 thousand tons consisting of 739.5 thousand tons of inland and 220.1 thousand tons of marine and coastal aquaculture production (FAO, 2022). However, intensified practices to obtain the maximum yield per unit area in rainbow trout production using intensive culture face problems such as stress and provide conditions for easy disease transmission (Lakwani *et al.*, 2022; Radosavljevic *et al.*, 2022). Therefore, this study was conducted to evaluate the efficacy of the selected microalgae (*Chlorella* sp., *Haematococcus* sp., and *Schizochytrium* sp.) as natural feed additives for juvenile rainbow trout in terms of growth performance, intestinal histology, immune biomarkers, and disease resistance to bacterial infection of *V. anguillarum*.

Materials and methods

Diets preparation and experimental design

Four experimental diets were formulated to be isonitrogenous (ca. 51%) and isolipidic (ca.13%) on the basis of the nutrient requirements of juvenile rainbow trout (National Research Council, 2011). Sardine fish meal, soybean meal, and poultry byproducts were used as the main protein sources and fish oil as the main lipid source. To evaluate the dietary microalgae as a functional feed additive, three different microalgae, including *Chlorella* sp. (CHL), *Haematococcus* sp. (HAE), or *Schizochytrium* sp. (SCH) were supplemented in a basal diet (5 g of each microalgae per kg diet). In the basal diet, the same amount of cellulose (5 g per kg diet) was supplemented to match the total volume of the test diets. All microalgae meals were obtained from the Korea Fisheries Resources Agency (FIRA), Republic of Korea and the ingredients were prepared in powder form through freeze-drying of each microalga. The formulation and proximate composition of the four experimental diets for juvenile rainbow trout are shown in Table 1.

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The procedures for diet preparation were followed, as described by Bai and Kim (1997).

Table 1: Formulation and proximate composition of the four experimental diets for juvenile rainbow trout.

Ingredients	Diets[1]			
	CON	CHL	HAE	SCH
Sardine fish meal	22.0	22.0	22.0	22.0
Dehulled soybean meal	17.5	17.5	17.5	17.5
Blood meal	4.70	4.70	4.70	4.70
Wheat gluten meal	5.00	5.00	5.00	5.00
Poultry byproduct	16.2	16.2	16.2	16.2
Meat and bone meal	9.20	9.20	9.20	9.20
Wheat flour (feed grade)	13.9	13.9	13.9	13.9
Fish oil	6.80	6.80	6.80	6.80
Mineral Mix[2]	2.00	2.00	2.00	2.00
Vitamin Mix[3]	2.00	2.00	2.00	2.00
Choline	0.04	0.04	0.04	0.04
Lecithin	0.20	0.20	0.20	0.20
Cellulose[4]	0.50	-	-	-
Chlorella sp.	-	0.50	-	-
Haematococcus sp.	-	-	0.50	-
Schizochytrium sp.	-	-	-	0.50
Total	100	100	100	100
Proximate composition (% of dry matter basis) of experimental diet[5]				
Moisture	5.52 ± 0.08	5.16 ± 0.08	6.87 ± 0.03	7.74 ± 0.03
Crude protein	51.3 ± 0.42	51.8 ± 0.21	49.9 ± 0.22	50.4 ± 0.12
Crude lipid	13.2 ± 0.15	12.2 ± 0.57	12.7 ± 0.12	14.1 ± 0.69
Crude ash	12.2 ± 0.16	12.2 ± 0.26	11.7 ± 0.07	11.9 ± 0.27
Gross energy (MJ/kg)[6]	20.4 ± 0.1	20.3 ± 0.1	20.1 ± 0.0	20.3 ± 0.2

[1]Diets represent CON = Control, CHL = *Chlorella* sp., HAE = *Haematococcus* sp. and SCH = *Schizochytrium* sp.; [2]Contains (as mg/kg in diets): NaCl, 437.4; $MgSO_4 \cdot 7H_2O$, 1379.8; $ZnSO_4 \cdot 7H_2O$, 226.4; Fe-Citrate, 299; $MnSO_4$, 0.016; $FeSO_4$, 0.0378; $CuSO_4$, 0.00033; Calcium iodate, 0.0006; MgO, 0.00135; $NaSeO_3$, 0.00025; [3]Contains (as mg/kg in diets): Ascorbic acid, 300; dl-Calcium pantothenate, 150; Choline bitatrate, 3000; Inositol, 150; Menadione, 6; Niacin, 150; Pyridoxine HCl, 15; Riboflavin, 30; Thiamine mononitrate, 15; dl-a-tocopherol acetate, 201; Retinyl acetate, 6; Biotin, 1.5; Folic acid, 5.4; Cobalamin, 0.06; [4]Cellulose, Sigma-Aldrich Korea Yongin, Republic of Korea; [5]Values are mean of triplicate samples (± SD). [6]Gross energy of the diets was calculated using the following values: crude protein 23.7 kJ/g, crude lipid 39.6 kJ/g, and NFE (nitrogen-free extract) 17.2 kJ/g (Pfalzgraff *et al.*, 2021).

All powdered ingredients were weighed and then mixed using an electronic industrial mixer (HYVM-1214, Hanyoung Food Machinery, Gyeonggi-do, Republic of Korea). The fish oil was slowly added to the mixer with the addition of approximately 30% filtered tap water to make a feed dough. The moistened mixture was pelleted into a 2-mm diameter die using a pelleting machine (SMC-12, SUN Engineering, Seoul, Republic of Korea). The pelletized diets were gently broken into small pellets by hands and were dried at room temperature for 48 h. Then, the experimental diets were stored at -20°C throughout the feeding trials. The proximate composition was determined for all diets following the standard methods of the Association of Official Analytical Chemists, 2005). The diet samples were dried to a constant weight at 105°C to determine their moisture content. The content of crude protein was determined by the Kjeldahl method (N x 6.25). The

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content of crude lipid in the diets was analyzed by the Soxhlet extraction method using the Soxtec system 1046 (Tecator AB, Hoganas, Sweden). Crude ash was determined by incineration at 550°C with a muffle furnace (DAIHAN, WiseTherm, Seoul, Korea).

Experimental fish and feeding trial

The experimental juvenile rainbow trout were purchased from a fish farm (Hyangchon, Chungju - Chungcheong, Republic of Korea) and transported to the Feeds and Foods Nutrition Research Center (FFNRC) at Pukyong National University, Busan, Republic of Korea, where the study was conducted. Fish were acclimated in an indoor semi-recirculating system receiving a constant water flow (2L.min^{-1}) for a week and fed with the basal diet. At the beginning of feeding trials, a total of 180 fish with an initial body weight of 12.16±0.01 g (mean±SD) were randomly distributed in 12 rectangular tanks (filled with 30-L of water) at the stocking density of 15 fish per tank. Each tank was randomly distributed into one of the three replicates of the four dietary treatments.

The water quality was monitored four times per day. Water temperature was maintained at 15.5±1°C (mean±SD). Dissolved oxygen concentration was maintained near saturation using an air stone equipped in each tank. Photoperiod was scheduled to be 12L: 12 D. The juveniles were fed three times per day (09:00, 12:00, and 17:00 h) at a rate of 3 – 3.5% of body weight per day throughout the six-week feeding trial. The tanks were cleaned daily to remove the uneaten feed and feces by siphoning. When mortality was detected, the feed amount was adjusted accordingly.

Sample collection and analysis

At the end of the feeding trial, all fishes (24-h post starvation) in each tank were weighed and counted to measure growth performance, including final biomass (FB), biomass gain (BG), final body weight (FBW), weight gain (WG), specific growth rate (SGR), and survival rate (SR). In addition, five fish from each tank were randomly selected from each tank, anesthetized with MS-222 (tricaine methanesulfonate; 100 ppm) to measure condition factor (CF), then they were dissected out to collect liver and viscera for calculation of hepatosomatic index (HSI) and viscerosomatic index (VSI), respectively, as indices of morphological changes in response to the dietary treatments. The aforementioned measurements were calculated following the equation provided below:

Weight gain (WG, %) = (FBW – IBW) / IBW × 100
Specific growth rate (SGR, %/day) = [ln (FBW - ln (IBW)] / day of feeding × 100
Survival rate (SR, %) = [(number of total fish – number of dead fish)] / number of total fish × 100
Condition factor (CF) = (FBW / total length^3) × 100
Hepatosomatic index (HSI, %) = Liver weight / FBW × 100
Viscerosomatic index (VSI, %) = Viscera weight / FBW × 100
Where the IBW indicated the initial body weight. The units of weight and length are g and cm, respectively.

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Three fish per tank were randomly collected, euthanized with the MS-222 (300 ppm), pulverized, and freeze-dried for 48 hours for whole-body proximate composition analysis (moisture, crude protein, crude lipid, and crude ash). The analysis was conducted at the FFNRC (Feeds & Foods Nutrition Research Center, Pukyong National University, Busan, Republic of Korea) following the standard methods of the AOAC (2005). Investigation of changes in the whole-body nutrient composition in response to dietary treatments can be used as the nutritional status indicator of tested animals. The nutritional status of aquatic animals is highly associated with exogenous (e.g., food availability, nutrient composition of food items, temperature, salinity, etc.) and endogenous factors (developmental stage, sexual maturity, hormones, etc.) (Bar and Volkoff, 2012). Thus, in the current study, the nutrient composition was investigated to understand whether supplementation of the different microalgae could affect the nutritional status of the juveniles at the end of the feeding trial.

The moisture content was measured by drying the samples in an oven (OF02G-4C, WiseVen®, Germany) at 105°C for 24h. The crude protein content was measured by the Kjeldahl method (N×6.25) using the Auto Kjeldahl system (Buchi B-324/435/412, Switzerland; Metrohm 8-719/806, Switzerland). The crude lipid content of the rainbow trout was measured by the Soxhlet extraction method using the Soxhlet 1046 system (Tacator AB, Hoganas, Sweden). The total ash content was measured by cremation in a muffle at 550 °C for 3 h (DAIHAN, WiseTherm®, Germany). The proximal analysis of the fish samples was performed in triplicate.

Blood samples were taken from the five pre-anesthetized juveniles through puncture of the caudal vein using non-heparinized syringes (1.0 mL) to measure the hematological and biochemical parameters. The collected blood samples were pooled and centrifuged at 5,000 x *g* for 10 min, and the serum supernatant was removed and stored at -70°C until analysis. The serum samples were used to analyze the aspartate transaminase (AST), alanine transaminase (ALT), glucose (GLU), and total protein (TP) using a chemical analyzer Fuji DRICHEM 3500i (Fuji Photo Film Ltd., Tokyo, Japan).

As indicates of non-specific immunity, lysozyme (LYZ) and myeloperoxidase (MOP) activities in serum were determined. The lysozyme levels were determined following the method reported by Hultmark *et al.* (1980), with some modification using a lysozyme test kit (Sigma-Aldrich, Cat. No. LY0100), where 0.1 mL of serum was added to 2 mL of *Micrococcus lysodeikticus* (0.2 mg/mL) and added to a 96-well Elisa plate. The absorbance was evaluated at a 450 nm wavelength in a microplate reader between 0 and 30 minutes. Determination of the total myeloperoxidase (MPO) activity was performed according to the method of Quade and Roth (1997). For this purpose, 20 µL of serum was diluted in 80 µL Hanks Balanced Salt Solution (HBSS) without Ca^{2+} or Mg^{2+} into a 96-well ELISA plate; and then, 35 µL of 3, 3', 5, 5' tetramethylbenzidine hydrochloride (TMB, 20 mM; Sigma-Aldrich) and H_2O_2 (5 mM) were subsequently added. The plate was

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incubated at 30°C for 2 min; then, 35 µL 4M H_2SO_4 was added to stop the color reaction, and the absorbance was read at 450 nm using the microplate reader.

The intestinal histological evaluation was performed as described previously by Lee *et al.* (2017). The previously sacrificed fishes were used to collect the intestine, which was fixed in formalin (10%, pH 7). Then, the samples were dehydrated and processed into paraffin blocks. The tissue blocks were sectioned (4 µm thickness) using a rotary microtone machine (CUT 4055, MicroTec, Germany) and placed in a water bath at 44°C. The dried slices were stained routinely with hematoxylin and eosin (H&E) for observation under a microscope (AX70 Olympus, Tokyo, Japan) equipped with a digital camera (DIXI Optics, Daejeon, Korea). Imaging software (Image J 1.32j, National Institute of Health, Bethesda, MD, USA) was used to analyze the image of the histological slides. Tissue sections were examined in terms of the villus lengths. The length of the villi was measured (µ) and compared. The villi were chosen according to their integrity and higher length.

For the challenge test experiment, five fish from each tank were randomly selected and injected intraperitoneally with 0.1 mL of bacterial pathogen suspension *V. anguillarum* at a concentration of 1 x 10^6 colony-forming units (CFU/mL). The water temperature was maintained at a 15.5±1°C (mean±SD) and dissolved oxygen concentration was maintained near saturation through aeration. The mortality of the fish in each tank was monitored and recorded daily for up to 27 days to calculate the cumulative survival rate. During the period of the challenge test, the fish were not fed.

Statistical analysis

All results were checked for normality (Shapir-Wilk) and homogeneity of variance (Levene's test). Then, they were subjected to a one-way analysis of variance (ANOVA) at a significance level of 0.05 (p<0.05). When the significance was detected, a post-hoc test using a Least Significant Difference (LSD) was used to compare means. All statistical analyses were performed with Statistical Product and Service Solutions (IBM SPSS Inc., Michigan Avenue, Chicago, IL, USA).

Results

Growth performances

The growth performances of the juvenile rainbow trout fed different experimental diets for six weeks are shown in Table 2. The fish fed the HAE diet were significantly higher in weight gain (WG) and specific growth rate (SGR) compared to the other experimental diets. However, there were no significant differences in the hepatosomatic index (HSI), viscerosomatic index (VSI), condition factor (CF), and survival rate (SR) among all experimental diets.

Whole-body proximate composition

The whole-body proximate composition of the fish fed with different experimental diets is shown in Table 3. The results showed that fish fed the four experimental diets for six weeks did not show significant differences in moisture, crude protein, crude lipid, and crude ash.

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Histology

The statistical significance of the intestinal villus length and the histological analysis of the juvenile rainbow trout fed with different experimental diets for six weeks are shown in Table 4. The histological sections of the intestine are shown in Figure 1. The villus length of the fish fed the HAE diet was significantly higher than those fed the CHL and CON diets. However, there were no significant differences in the villus length of the fish fed the HAE and SCH diets as well as the fish fed the CHL, SCH, and CON diets.

Table 2: Growth performances of juvenile rainbow trout fed with different experimental diets for six weeks[1].

Parameters[3]	Diets[2]			
	CON	CHL	HAE	SCH
Initial Biomass	182.25 ± 1.92	182.50 ± 1.80	182.50 ± 1.66	182.25 ± 1.48
Final Biomass	287.25 ± 42.29	281.50 ± 20.41	291.75 ± 18.46	282.50 ± 32.96
Biomass Gain	105.00 ± 41.34	99.00 ± 20.41	109.25 ± 19.07	100.25 ± 32.02
IBW	12.09 ± 0.13	12.22 ± 0.12	12.16 ± 0.11	12.20 ± 0.10
FBW	22.13 ± 1.00	21.19 ± 1.56	24.24 ± 0.70	20.90 ± 1.06
WG	83.1 ± 9.94[b]	78.3 ± 11.58[b]	99.4 ± 6.08 [a]	71.3 ± 10.01 [b]
SGR	1.68 ± 0.15 [b]	1.61 ± 0.19 [b]	1.92 ± 0.09 [a]	1.49 ± 0.16 [b]
HIS	1.14 ± 0.14	1.20 ± 0.18	1.10 ± 0.09	1.17 ±0.29
VSI	4.74 ± 0.96	4.74 ± 0.92	4.78 ± 0.81	4.81 ± 1.62
CF	1.02 ± 0.08	1.01 ± 0.06	1.01 ± 0.09	0.99 ± 0.09
SR	93.3 ± 15.18	86. 7 ± 7.45	84.4 ± 7.26	95. 6 ± 12.80

[1]Values are means from triplicate groups of rainbow trout where the values in each row with different superscripts are significantly different (±SD; p<0.05); [2]Diets represent CON=Control, CHL=*Chlorella* sp., HAE=*Haematococcus* sp. and SCH = *Schizochytrium* sp; [3]WG: Weight gain (%); SGR: Specific growth rate (% day^{-1}); HSI: Hepatosomatic index (%); VSI: Viscerosomatic index (%); CF: Condition factor; SR: Survival rate (%).

Table 3: Whole-body proximate composition (%; dry matter basis) of juvenile rainbow trout fed with different experimental diets for six weeks [1].

Parameters	Diets[2]			
	CON	CHL	HAE	SCH
Moisture	76.7 ± 0.04	76.1 ± 0.39	75.4 ± 0.14	75.5 ± 0.13
Crude protein	16.7 ± 0.13	17.1 ± 0.09	16.8 ± 0.05	16.7 ± 0.06
Crude lipid	4.00 ± 0.07	4.15 ± 0.01	5.36 ± 0.14	4.98 ± 0.10
Crude ash	2.64 ± 0.05	2.74 ± 0.03	2.66 ± 0.07	2.48 ± 0.10

[1]Values are means from triplicate groups of rainbow trout where the values in each row with different superscripts are significantly different (±SD; p<0.05); [2]Diets represent CON=Control, CHL=*Chlorella* sp., HAE=*Haematococcus* sp. and SCH=*Schizochytrium* sp.

Table 4: Intestinal histology of juvenile rainbow trout fed with different experimental diets for six weeks[1].

	Diets[2]			
	CON	CHL	HAE	SCH
Villus length (µm)	1.14 ± 0.03 [b]	1.13 ± 0.05 [b]	1.34 ±0.09 [a]	1.29 ± 0.04 [ab]

[1]Values are means from triplicate groups of rainbow trout where the values in each row with different superscripts are significantly different (±SD; p<0.05).; [2]Diets represent CON=Control, CHL=*Chlorella* sp., HAE=*Haematococcus* sp. and SCH=*Schizochytrium* sp.

Serum biochemical parameters

The serum biochemical parameters of the juvenile rainbow trout that were fed the experimental diets are shown in Table 5. There were no significant differences in the serum aspartate aminotransferase (AST),

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alanine aminotransferase (ALT), glucose (GLU) and total protein (TP) contents among the fish fed the experimental diets for six weeks.

Nonspecific immune responses

The non-specific immune responses of the fish fed diets with the four experimental diets are shown in Figure 2. The fish fed the HAE diet showed significantly higher MPO activity than that of those fed the other experimental diets. However, there were no significant differences in the lysozyme (LYZ) activities among the different treatments.

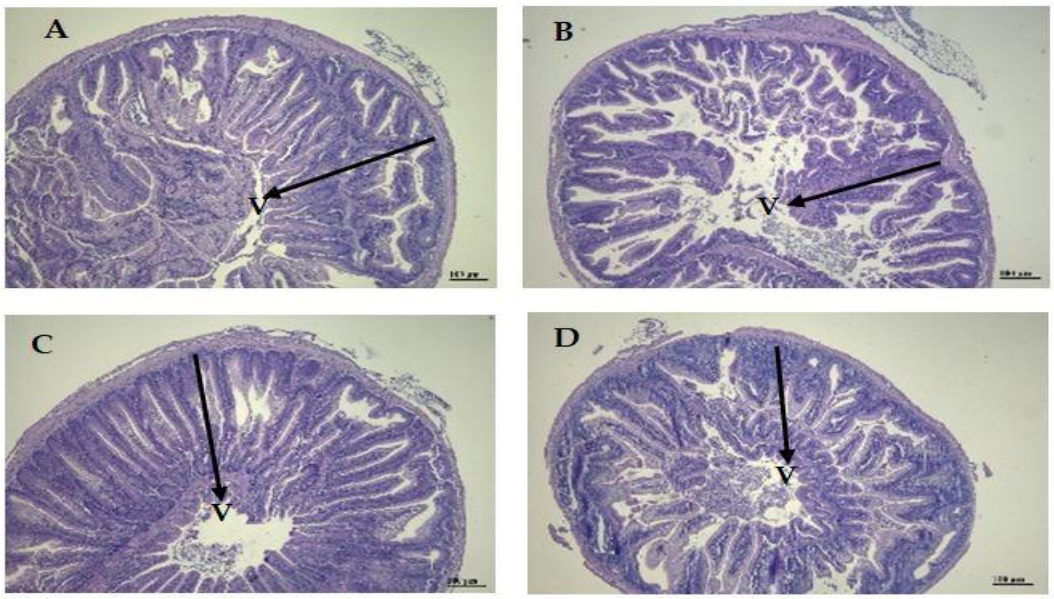

Figure 1: Histological changes of the anterior intestine of rainbow trout fed four experimental diets for six weeks (Original magnification×40). Diets represent A=Control (CON), B=*Chlorella* sp. (CHL), C=*Haematococcus* sp. (HAE) and D=*Schizochytrium* sp. (SCH).

Table 5: Serum biochemical parameters of juvenile rainbow trout that were fed the experimental diets for six weeks[1].

Parameters[3]	Diets[2]			
	CON	CHL	HAE	SCH
AST	384 ± 52.84	435 ± 70.73	456 ± 118.88	529 ±103.51
ALT	8.67 ± 1.82	8.67 ± 0.95	6.33 ± 1.25	8.00 ± 1.29
GLU	82.3 ± 4.99	73.7 ± 2.94	83.3 ± 1.82	70.3 ± 5.18
TP	3.47 ± 0.53	3.70 ± 0.47	3.70 ± 0.17	5.40 ± 0.26

[1]Values are means from triplicate groups of rainbow trout where the values in each row with different superscripts are significantly different (±SD; $p<0.05$); [2]Diets represent CON=Control, CHL=*Chlorella* sp., HAE=*Haematococcus* sp., and SCH=*Schizochytrium* sp.; [3]AST: Aspartate transaminase (U/L); ALT: Alanine transaminase (U/L); GLU: Glucose (mg/dL); TP: Total protein (g/L).

Challenge test against Vibrio anguillarum

The cumulative survival rates (CSR) of the juvenile rainbow trout injected with *V. anguillarum* for 27 days after six weeks of the feeding trial are shown in Figure 3. During the challenge test, the first mortality was observed on the second day from the fish fed the SCH diet, on the sixth day from fish fed the CON diet, on the thirteenth day from the fish fed the CHL diet, and sixteenth day from the fish fed the HAE diet. After 27 days of the challenge test, the

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CSR of the fish fed the HAE diet was significantly higher than that of those fed the other diets. Moreover, the CSR of the fish fed the CHL and SCH diets was significantly higher than that of those fed the CON diet. However, there were no significant differences in the CSR among the fish fed the CHL and SCH diets.

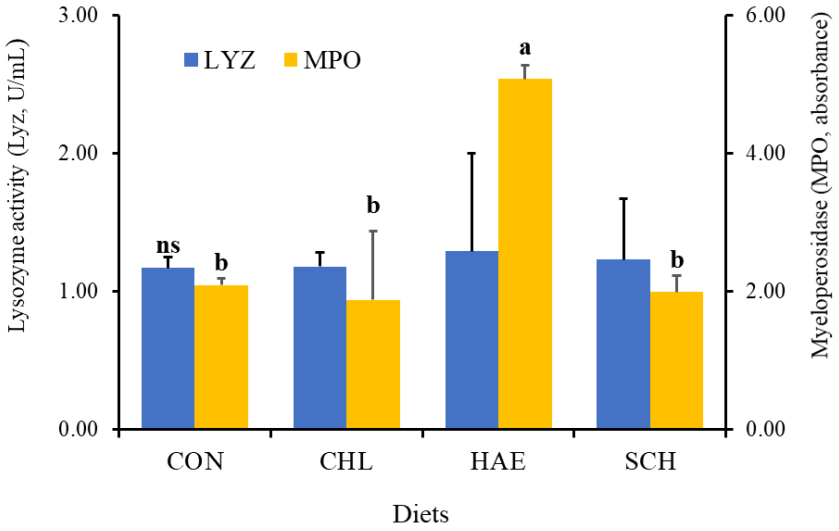

Figure 2: Non-specific immune responses of juvenile rainbow trout fed different experimental diets (CON=control, CHL=*Chlorella* sp., HAE=*Haematococcus* sp., and SCH=*Schizochytrium* sp.) for six weeks. Alphabet letters a and b indicate a significant difference among the diets within the result of the myeloperoxidase. The word 'ns' represents no significant difference among the diets within the result of the lysozyme.

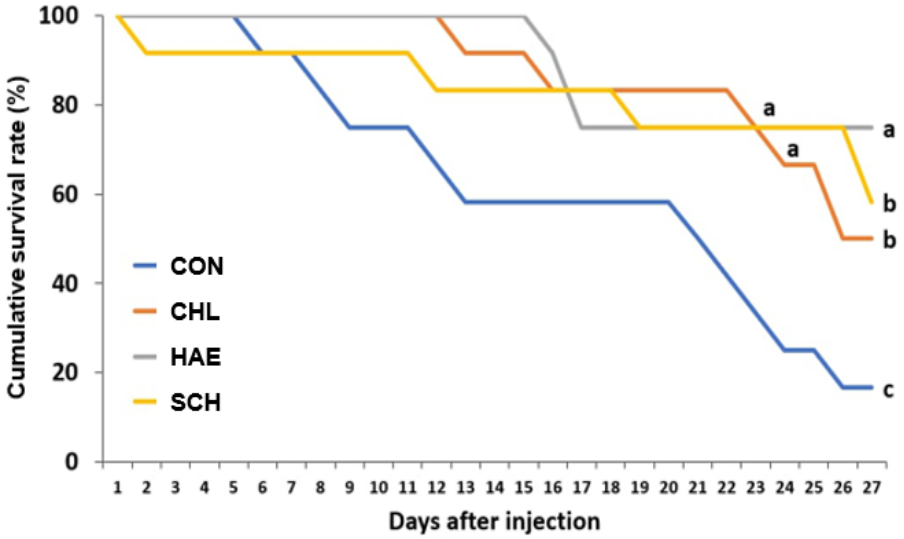

Figure 3: The cumulative survival rate after challenge with *Vibrio anguillarum* for 27 days in juvenile rainbow trout that were fed the four experimental diets for six weeks. Lines represent CON=Control, CHL=*Chlorella* sp., HAE=*Haematococcus* sp., and SCH=*Schizochytrium* sp. Different letters (a, b, c) are significantly (p<0.05) different according to LSD test.

Discussion

This study evaluated the effect of dietary microalgae on growth performances, whole-body proximate composition, serum biochemical parameters, non-specific immune responses, and intestinal histology

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in rainbow trout juveniles. During the experimental period, all the experimental diets containing microalgae were well-accepted by the juvenile rainbow trout. The findings of the present study indicated that the inclusion of 0.5% HAE significantly improved the weight gain (WG) and the specific growth rate (SGR). Previous research demonstrated that the inclusion of microalgae in fish diets could improve the growth performance of fish (Ju *et al.*, 2012; Galal *et al.*, 2018: Xi *et al.*, 2022). Li *et al.* (2014) obtained similar results with inclusion levels of 0.28, 0.56, and 1.12 g/100g ¯in the diets of yellow croaker, *Pseudosciaena crocea.* The same results were observed for other carnivorous fish such as golden pompano, *Trachinotus ovatus,* and spotted sea bass, *Lateolabrax macula* at the levels of 0.3% and 0.4-1.0%, respectively (Xie *et al.*, 2019; Yu *et al.*, 2020). The significant improvement in the WG and SGR of fish fed the HAE diet demonstrated that *Haematococcus* sp. could be used as a feed additive. On the other hand, in the present study, the inclusion of the microalgae *Chlorella* sp. and *Schizochytrium* sp. in the diets of juvenile rainbow trout did not show significant differences concerning the growth performances. Similar results were observed by Lyons *et al.* (2017) and Hossain *et al.* (2017), when fish were fed diets supplemented with *Schizochytrium* sp. and *Chlorella* sp, respectively. However, *Chlorella* sp. improved the WG and SGR in rainbow trout, *O. mykiss* (Chen *et al.*, 2021), juvenile olive flounder, *Paralichthys olivaceus* (Kim *et al.*, 2002), and tilapia, *O. niloticus* (Galal *et al.*, 2018). These results suggest that microalgae *Haematococcus* sp. could be used as a feed additive that positively affects rainbow trout growth. This may be due to the polyunsaturated fatty acid compounds contained in these algae. Moreover, *Haematococcus* sp. contains bioactive compounds such as astaxanthin which have been shown to promote the health and growth performance of animals (Shah *et al.*, 2016).

Furthermore, we did not observe significant differences in hepatosomatic index (HSI), viscerosomatic index (VSI), condition factor (CF), and survival rate (SR). Similar to this study, the inclusion of microalgae *Schizochytrium* sp. as a substitute for fish oil in shrimp feed did not result in significantly different SR compared to the control (Allen *et al.*, 2019). Moreover, the supplementation of diets with *Haematococcus* sp. in large yellow croaker (Li *et al.*, 2014), and in spotted sea bass (Yu *et al.*, 2020), showed no differences in SR among the treatment groups. Xie *et al.* (2019), reported that the inclusion of 3% *Schizochytrium* sp. in the golden pompano diet had no significant effects on the HSI, VSI, and CF. In another recent trial by Xie *et al.* (2020), golden pompano fed a diet supplemented with *Haematococcus* sp. were found to have no significant differences in HSI, VSI, and CF. The liver, viscera, and healthy index of fish shown by the HSI, VSI, and CF are important tools to assess the physiology and biological conditions of fish. Our findings could indicate that the inclusion of microalgae in rainbow trout diet did not affect the liver, viscera, and overall health status of fish. It may also be attributed to the fact that the experimental diets were well-accepted by the fish during the feeding trial.

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In this experiment, no significant differences were observed in the whole-body proximate composition of juvenile rainbow trout fed the three different microalgae. Our results were in accordance with the studies that used dietary *Chlorella ellipsoidea* in juvenile olive flounder (Kim *et al.*, 2002), and dietary *Schizochytrium* sp. in golden pompano diet (Xie *et al.*, 2019). Moreover, the whole-body composition of several fish fed with *Haematococcus* sp. was not significantly different among the treatment groups (Sirakov *et al.*, 2015; Xie *et al.*, 2020; Yu *et al.*, 2020). However, in the present study, the crude lipid in juvenile rainbow trout whole body fed with 0.5% *Haematococcus* sp. was higher than other dietary groups. A similar result was reported by Li *et al.* (2014), with the addition of *H. pluvialis* in the large yellow croaker diet. These authors reported that *Haematococcus* sp. is rich in lipids that can add to the energy reserves of fish and consequently lead to more lipid bioaccumulation. This can also explain our results for the higher WG for those fish fed diets supplemented with *Haematococcus* sp.

The findings from this study showed that the intestinal villus lengths of the fish fed the 0.5% HAE diet were significantly higher than that of those fed the CHL and CON diets. However, there were no significant differences in the villus length of fish that were fed the HAE and SCH diets, as well as the fish that were fed the CHL, SCH, and CON diets. In agreement with the present study, dietary *Haematococcus* sp. has been shown to increase the intestinal villus length of golden pompano (Zhao *et al.*, 2021). On the other hand, Xie *et al.* (2019) showed that golden pompano fed with *Schizochytrium* sp.-supplemented diets had a longer villus length than those fed with the control diet. Moreover, in rainbow trout fed diets supplemented with *Scenedesmus* sp., there were no changes in the villus length between dietary groups (Skalli *et al.*, 2020). The villus length can help to digest, absorb, and assimilate nutrients in the gastrointestinal tract of fish. An increase in the villus length could better explain the growth and feed utilization results in our study. Generally, these results could suggest that dietary supplementation with *Haematococcus* sp. and *Schizochytrium* sp. could promote early intestinal development, which improves absorption ability and intestinal digestion. The structure and morphology of the intestine are crucial for the maintenance of normal intestinal functions and nutrient absorption. The longer villus length leads to better nutrient absorption and reflects the function of the intestinal wall (Choi *et al.*, 2022).

In the present study, there were no significant differences in the levels of AST, ALT, glucose (GLU), and total protein (TP) between the four different diets. These results were similar to a previous study using dietary *Haematococcus pluvialis* in large yellow croaker (Li *et al.*, 2014). Abdel-Tawwab *et al*. (2022), reported that no significant changes were observed in glucose, ALT, and ALT of common carp, *Cyprinus carpio* fed diets supplemented with *Chlorella pyrenoidosa*. Moreover, similar results were reported by Sheikhzadeh *et al.* (2012), as the AST and ALT levels in rainbow trout fed diets supplemented with *H. pluvialis* were

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similar to those fed diets without algae. These authors stated that the TP content did not show any significant differences compared with the control. Similarly, Yu *et al.* (2020), fed *Haematococcus* sp. to spotted sea bass and observed no significant differences compared with the basal diet. Moreover, the TP and GLU were not significantly different in the olive flounder fed diets supplemented with *Chlorella* sp. (Kim *et al.*, 2002). ALT and AST are sensitive measures of hepatotoxicity and histopathological changes. The findings of our experiment and previous studies could indicate that *H. pluvialis* at 1–10 g/kg feed doses is a safe supplement (Sheikhzadeh *et al.*, 2012). *H. pluvialis* contains astaxanthin which is considered a super anti-oxidant (Shah *et al.*, 2016). Astaxanthin has the potential to improve the health of fish, especially by providing better liver functions (Li *et al.*, 2014).

Myeloperoxide (MPO) is an enzyme involved in innate immune defense, producing hypochlorous acid, a toxic molecule to pathogenic bacteria. MPO belongs to the peroxidase superfamily and are cationic leukocyte haloperoxidases with potent microbicidal and detoxifying activities. Due to the fact that MPO plays an important role in innate immunity, MPO activity has been often used as a key biomarker when immunostimulants such as phytochemicals, probiotics, prebiotics, and microalgae are applied in aquaculture settings (Ibrahim *et al.*, 2022). In the current study, the contents of the MPO activity in fish fed with the dietary HAE were significantly different from the other treatments. However, there were no significant differences in the MPO activity in the fish fed the CHL, and SCH diets compared with the CON diet. These results support the findings on European sea bass, *Dicentrarchus labrax*, gibel carp, and olive flounder. Microalgae supplementation in the diets of these fish has been shown to enhance the non-specific immune responses and, therefore, has been effectively used in aquaculture as an immunostimulant (Kim *et al.*, 2015; Wang *et al.*, 2015; Messina *et al.*, 2019). In contrast to our results, in another study, when rainbow trout were fed microalgae *Scenedesmus* sp., there were no significant effects on the MPO levels measured in the serum (Skalli *et al.*, 2020). *H. pluvialis* can neutralize and scavenge reactive oxygen species by improving the antioxidant defense system (Sirakov *et al.*, 2015). Moreover, *H. pluvialis* can eliminate reactive oxygen species to avoid producing more antioxidant enzymes in the liver (Yu *et al.*, 2020). The MPO is an important enzyme for microbicidal activity that utilizes the reactive oxygen radical to produce hypochlorous acid which is potent in killing pathogens and is a key component of neutrophil granules involved in killing engulfed microorganisms (Kim *et al.*, 2015; Wang *et al.*, 2015). On the other hand, in the present study, there were no significant differences in the lysozyme (LYZ) activities among the different treatments. These results were similar to a previous experiment conducted on Nile tilapia fed a diet containing 1.2% *Schizochytrium* sp. meal, the lysozyme concentration did not differ significantly between the dietary groups. These authors reported that the inclusion of algae in the diet did not significantly influence the immune

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response of Nile tilapia (De Souza *et al.*, 2020). However, in the results reported by Li *et al.* (2014), the lysozyme increased with the increase in the *H. pluvialis* levels in large yellow croaker feed. These authors reported that fish innate immune response was affected by *H. pluvialis*, and it improved the complement response. The immune responses of algae-fed fish can vary, depending on the algae species and concentrations used (De Souza *et al.*, 2020).

In this study, after 27 days of the challenge test, we found the cumulative survival rates of the fish fed the HAE diet were significantly higher than those of the fish fed the other diets. Moreover, the cumulative survival of the fish fed the CHL and SCH diets was significantly higher than that of those fed the CON diet. This result agrees with the observation by Yu *et al.* (2020), as the survival rate of the spotted sea bass fed *H. pluvialis* and challenged with *Vibrio harveyi* was higher compared to the control group. In a similar report by Galal *et al.* (2018), *O. niloticus* was fed *Chlorella vulgaris* and challenged with *Aeromonus sobria*; the results showed a lower mortality rate compared to the control group. These authors indicated that dietary *C. vulgaris* may protect fish against disease via the improvement of immune responses, which increases the resistance to bacterial infection. Similarly, Chen *et al.* (2022), dietary administration of *Chlorella sorokiniana* meal significantly increased the survival rate of juvenile rainbow trout infected with *Aeromonas salmonicida*. *Schizochytrium* sp., in the diet of golden pompano increased the resistance to pathogens, the innate antiviral capacity, and the innate immunity (Xie *et al.*, 2019). These findings suggest that dietary *Haematococcus* sp., *C. vulgaris*, and *Schizochytrium* sp. improve disease resistance in fish. *H. pluvialis* can enhance the non-specific immunity of fish by activating the complement system (Zhao *et al.*, 2021). Some algae species might contain bioactive substances involved in the regulation of the immune response (Xu *et al.*, 2014). *H. pluvialis* has anti-inflammatory properties and potently suppresses inflammatory responses in the gut. The astaxanthin content in *Haematococcus* sp. is an excellent anti-inflammatory agent that prevents the onset of inflammation in biological systems, scavenges oxygen radicals in cells, acts as a viable hepatoprotective agent, and plays an important role in animal health as an antioxidant (Zhao *et al.*, 2023). Our study and previous findings suggest that algae could be used as a novel immunostimulant to provide better protection for fish against some infectious diseases.

The results of the present study indicated that dietary inclusion of 0.5% *Haematococcus* sp. could improve weight gain, specific growth rate, intestinal villus length, and myeloperoxidase activity in rainbow trout. Moreover, it was shown that *Haematococcus* sp. could be an immunostimulant feed additive by improving the disease resistance of rainbow trout against pathogenic bacteria. Due to the low incorporation rate and reasonable commercial price, we conclude that microalgae *Haematococcus* sp. has the potential to be used as a functional feed additive in fish. Further studies are required to investigate the inclusion of this

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microalgae at higher levels as a feed additive or ingredient and its effects on the growth performance and immune parameters of fish and shrimp species. The longer experimental periods and additional physiological and biochemical parameters to better understand the effect of microalgae-based diets on fish health, immune responses and overall performance should be considered for future studies.

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Confilicts of interest

The authors have no conflicts of interest to declare.

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