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RESEARCH

Influence of astaxanthin-enriched *Haematococcus pluvialis* microalgae on the growth efficacy, immune response, antioxidant capacity, proinflammatory cytokines, and tissue histomorphology of hybrid red tilapia

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Abstract
Astaxanthin, a natural ketone carotenoid, is among the environmentally friendly antioxidants and immunopotentiators. A 60-day feeding experiment was proceeded to assess the feasibility of astaxanthin-rich *Haematococcus pluvialis* as a growth promoter, antioxidant, and immunostimulant in the hybrid red tilapia (*Oreochromis niloticus x O. mossambicus*). Experimental diets containing grade amounts of *Haematococcus pluvialis* powder (0, 0.5, 1.0, and 1.5 g kg^{-1} feed) were formulated to be fed to red tilapia (Initial weight 27 ± 0.5 g) and designated as control, $HP_{0.5}$, HP_1, and $HP_{1.5}$, respectively. The results indicated that the HP_1 and $HP_{1.5}$ promoted growth performance by decreasing FCR and increasing FBW, WG, WGR, and SGR confirmed by better intestinal morphology. Moreover, the HP_1 and $HP_{1.5}$ diets improved non-specific immunity via enhancing phagocytic activity, IgG and IgM contents, and nitric oxide, while decreasing MPO values compared to the control. Additionally, the *H. pluvialis* diets boosted antioxidant ability through elevating serum SOD and GSH activities, unlike the control group. The $HP_{0.5}$, HP_1, and $HP_{1.5}$ diets also exerted hepatoprotective effects via histological sections as well as, suppressing liver enzymes (ALT, AST, ALP, and GGT) and reducing serum TG and cholesterol contents confirmed our data. Besides, a notable decrease in the serum levels of IFN-γ and IL-4 along with hepatic mRNA levels of *TNF-α*, *IL-1β*, *IL-8*, and caspase-3 with the increasing doses of *H. pluvialis*. These results proposed that a diet supplemented with 1 and 1.5 g kg^{-1} *H. pluvialis* is exhorted to augment the growth performance, hepatoprotection, antioxidant capacity, immunity, and anti-inflammatory response of red tilapia.

Keywords Red tilapia · *Haematococcus pluvialis* · Growth · Immunity · Gene expression · Histomorphometry

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Highlights
- Dietary *H. pluvialis* significantly improved red tilapia's growth performance and feed utilization.
- Dietary *H. pluvialis* elevated the antioxidant properties and immune parameters.
- Dietary *H. pluvialis* suppressed the expression of pro-inflammatory cytokines and apoptotic genes.
- Dietary *H. pluvialis* increased intestinal villous width and thickness of absorptive epithelium.

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Introduction

The most global and valuable cultured fish species are tilapia species with a total yield of 6.3 million metric tons in 2021, estimated at over 12 billion US$ (FAO 2023). In general, the extensive expansion of tilapia farming is related to their swift growth and preferable resistance to environmental stressors and diseases (Eissa et al. 2023a, 2023b). Red hybrid tilapia is considered the most genetically enhanced tilapia (GIFT) by cross-breeding the Nile tilapia, *Oreochromis niloticus* with *Oreochromis mossambicus*. These species have gained rapid popularity among Egyptian consumers because of their morphological attractiveness, higher marketability, and their fast growth (Aboelward et al. 2020). The wide tolerance to salinity (1–25 ppt) and higher densities increase its value in the aquaculture (Rahmah et al. 2020). In addition, red tilapia provides a favorable taste with lower off-flavor along with an increase in the long polyunsaturated fatty acids contents in comparison with other tilapia species (Banuelos-Vargas et al. 2021). According to these previous characteristics, formulating red tilapia diets using immunostimulant and antioxidant supplements remains a major challenge for red tilapia production strategies and limitations of antibiotic resistance and other drugs in intensive culturing techniques and a variety of infections (Griesh et al. 2024).

The exploitation of appropriate algal feed additives facilitates the prospective expansion of the tilapia farming industry for use as an alternative to antibiotics. The unicellular green microalga, *Haematococcus pluvialis*, from the family Haematococcaceae, is a major exporter of natural astaxanthin (3,3′-dihydroxy-β-carotene-4,4′-dione), with a 1.5–6.0% content in dry powder (Yu et al. 2021). Therefore, *H. pluvialis* has commercial significance as it can accumulate remarkable astaxanthin content under stressful light, elevated temperature and salinity, and nitrogen scarcity (Ambati et al. 2014), as well as the constancy and bioavailability of natural astaxanthin are superior to synthetic astaxanthin (Ma et al. 2017). The in vitro studies have proved that the antioxidant feature of astaxanthin was higher by 100–500 fold than other natural antioxidants (Elbahnaswy and Elshopakey 2023). As well, astaxanthin as a feed additive gives noteworthy benefits to the physiological health and growth of aquatic animals (Cerezal-Mezquita et al. 2022; Gervasi et al. 2018). Besides, the naturalistic red pigmentation of salmon, trout, crabs, and shrimp species mainly originates from carotenoid astaxanthin produced from *H. pluvialis* (Cerezal-Mezquita et al. 2022; Lim et al. 2018). The valuable roles of *H. pluvialis* have been established in the fish culture industry, especially in improving the immunity and antioxidant capacity along with increasing the survival rate promoting against stressors and microbial diseases in different aquatic animals (Elbahnaswy and Elshopakey 2023; Long et al. 2023). The positive impact of *H. pluvialis* as a feed supplement was investigated on the growth, anti-inflammatory status, metabolic capacity, and intestinal histology of juvenile *Litopenaeus vannamei* shrimp (Fang et al. 2022). Dietary *H. pluvialis* could effectively enhance the antioxidant ability and innate immunity parameters of the Chinese mitten crab (Wu et al. 2017).

To date, data about the beneficial effects of *H. pluvialis* are little obtainable as a dietary supplement in the red tilapia reared in raceways. Therefore, in this work, 60 days feeding trial was carried out to explore the impact of *H. pluvialis* on the growth achievement, chemical carcass composition, antioxidant capability, physiological status, pro-inflammatory and anti-inflammatory gene expression, and hepatic and intestinal morphology of juvenile red tilapia.

Materials and methods

Fish management and experimental design

The experiment was carried out at a confidential fish farm in West El-kantra- Ismailia Governorate, where hybrid red tilapia (*O. niloticus*×*O. mossambicus*) fingerlings were cultured. Before the start of the feeding trial, six hundred red tilapia juveniles with estimated average size (initial body weight of 27 ± 0.5 g) were reared in 12 concrete tanks (3 × 8 × 1.2 m) with a water capacity of 24/m^3. Fish were randomly allotted in triplicates with a density of 50 fish per tank and supplemented the control diet for 2 weeks. During the acclimation period, red tilapias were assessed its healthy status through visual observation of clinical signs and some reflexes (ocular, defensive, and escape), along with microbial culturing to confirm fish was pathogen-free.

Over the 60-day feeding trial, water quality parameters were monitored twice a week. The maintained values were 26.5 ± 0.41 °C for water temperature; 2.5 ± 0.3 ppt for salinity; 6.7 ± 0.31 mg L^{-1} for D.O; 7.6 ± 0. 37 for pH; 0.4 ± 0.02 mg L^{-1} for ammonia nitrogen; 0.042 ± 0.01 mg L^{-1}. The tanks were provided with filters and continuous aeration via air blowers.

Experimental diets and feeding protocol

Four isonitrogenous dietary regimes containing almost 30% crude protein were prepared (Table 1) according to previous research (Bombardelli et al. 2017). The supplemental 2% red *H. pluvialis* powder (Bioalgo (WF), Co. Ltd., Astalgae ®, China, and 2% astaxanthin content) was used. The product was incorporated in the supplemented diets at four levels (0, 0.5, 1.0, and 1.5 g kg^{-1} feed), designated as control, $HP_{0.5}$, HP_1, and $HP_{1.5}$, respectively. The prepared diets were exposed to air at room temperature, mashed, and screened through 2–3 mm sieves, and kept in sterile plastic bags at 20 °C until used. During the trial, fish were supplied with test diets at 3% of the body weight containing different doses of *H. pluvialis* algae in the supplemented groups, twice a day (9:00 and 16:00 h) for 60 days. Uneaten feed and fecal wastes were removed via siphon feces were siphoned every morning, and about 25% of tank water was changed.

Sample collection

After 60 days of feeding, red tilapias in each tank were starved for 24 h. Then, all collected fish were prepared for the estimation of growth indices. Then, five fish from each group were randomly picked to be anesthetized with commercial clove oil solution (60 mg L^{-1}) to obtain the blood from the heart puncture using 3 mm sterile syringes. Blood samples were kept in un-heparinized Eppendorf tubes to coagulate at room temperature to collect serum by using a centrifuge at 4000 rpm for 15 min. Then, serum samples were reserved at – 20 °C for analysis of immunological and biochemical indices as well as antioxidant capacity. Liver samples were removed and put in RNA later® (Sigma, USA) for analysis of mRNA expression and then kept at – 20 °C. At the same time, liver and intestine sections were removed and fixed in 10% neutral formaldehyde for histological examination.

Table 1 Formulation and chemical analysis of the experimental diets (% dry weight)

Ingredients	Experimental diets (g kg^{-1})			
	Control	$HP_{0.5}$	HP_1	$HP_{1.5}$
Fish meal (60% CP)	100	100	100	100
Soybean meal (45% CP)	280	280	280	280
Yellow corn (8.5%)	130	129.950	129.850	129.700
Wheat bran (16.4%)	130	130	130	130
Rice bran (14.4%)	160	160	160	160
Haematococcus pluvialis	0	0.050	0.150	0.300
Linseed oil	20	20	20	20
Sunflower oil	20	20	20	20
Fish oil	20	20	20	20
Vitamins premix (1)	20	20	20	20
Minerals premix (2)	20	20	20	20
Proximate composition (%)				
Crude protein (CP)	30.14	30.02	30.20	30.19
Ether extract (EE)	2.73	2.18	2.07	2.06
Ash	5.63	4.55	4.43	4.43
Crude fiber (CF)	5.62	5.92	5.69	5.69
Nitrogen-free extract (NFE)[3]	55.88	57.33	57.61	57.63

(1)Vitamin premix (per kg of premix): thiamine, 2.5 g; riboflavin, 2.5 g; pyridoxine, 2.0 g; inositol, 100.0 g; biotin, 0.3 g; pantothenic acid, 100.0 g; folic acid, 0.75 g; para-aminobenzoic acid, 2.5 g; choline, 200.0 g; nicotinic acid, 10.0 g; cyanocobalamine, 0.005 g; a-tocopherol acetate, 20.1 g; menadione, 2.0 g; retinol palmitate, 100,000 IU; cholecalciferol, 500,000 IU

(2)Mineral premix (g kg^{-1} of premix): $CaHPO_4.2H_2O$, 727.2; $MgCO_4.7H_2O$, 127.5; KCl, 50.0; NaCl, 60.0; $FeC.6H_2O$, 7.3; $ZnCO_3$, 5.5; $MnCl_2.4H_2O$, 2.5; Cu (OAc).$2H_2O$, 0.785; $CoCl_3.6H_2O$, 0.477; $CaIO_3.6H_2O$, 0.295; $CrCl_3.6H_2O$, 0.128; $AlCl3.6H_2O$, 0.54; Na_2SeO_3, 0.03

[3]NFE = Nitrogen Free Extract (1000—{Moisture + Protein + Lipid + Ash + Fiber})

Growth indices

At the termination of the feeding trial, the growth indices and survival rates were recorded by counting and weighing the fish in every group. The calculations were proceeded as follows:

$$\text{Weight gain rate (WGR, \%)} = 100 \times (\text{mean final body weight} - \text{mean initial body weight}) / \text{mean initial body weight}$$

$$\text{Specific growth rate (SGR, \%/day)} = 100 \times (\text{In final body weight} - \text{In initial body weight}) / \text{number of days}$$

$$\text{FCR} = \text{feed intake(g)} / \text{weight gain (g)}$$

$$\text{Survival rate (\%)} = 100 \times (\text{final numbers of fish}) / (\text{initial numbers of fish})$$

Chemical analysis of diets

The chemical compositions of each diet were investigated according to the standard protocols of AOAC (Horwitz 2010). The analysis of moisture was done through heat drying in the ventilated oven at 105 °C until steady weight; crude lipid evaluation was followed by the Soxhlet ether-extraction method; crude protein was measured following the Kjeldahl method; ash was tested using a muffle furnace at 550 °C until constant weight. The nitrogen-free extract (NFE) was valued through the corresponding equation: NFE (g/kg) = 100 – (crude protein + crude lipids + ash + crude fiber).

Measurement of serum immunity indices, biochemical, and antioxidant capacity

The phagocytic activity was determined following the previous protocol (Kawahara et al. 1991). Phagocytic activity (PA) was assessed by dividing the number of phagocytic cells containing yeast by the total number of phagocytes multiplied by 100; meanwhile, the phagocytic index; phagocytic index (PI) = number of cells phagocytized/number of phagocytic cells (Dawood et al. 2020). As well, myeloperoxidase (MPO) activity was assessed using EnzChek assay Kits (Invitrogen™, Thermo Fisher Scientific, USA).

Following the manufacturer's procedures, the serum concentrations of immunoglobulin M (IgM) and immunoglobulin G (IgG) were estimated using Fish Immunoglobulins ELISA kits bought from MyBioSource Co. (San Diego, California, USA) (Eissa et al. 2023b; Elbahnaswy et al. 2023). Serum nitric oxide levels were measured depending on the stated approaches of commercial kits (Bio-diagnostics, Cairo, Egypt) (Bryan and Grisham 2007). Serum tumor necrosis factor-alpha (TNF-α), interleukin-4 (IL-4), and interferon-gamma (IFN-γ) values were measured by ELISA Kit supplied by Quantikine company corresponding to previous protocols (Juhász et al. 2013; Nicola 1994; Swanson et al. 2001).

The serum samples of red tilapia were used to estimate antioxidant enzymes, such as superoxide dismutase (SOD), reduced glutathione (GSH), and catalase (CAT), as well as, lipid peroxidation indicator malondialdehyde (MDA) following the previously published protocols (Benzie and Strain 1996; Ellman 1959; Nishikimi et al. 1972; Ohkawa et al. 1979). Serum lipids including total cholesterol, triglyceride (TG), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) were measured spectrophotometrically (Photometer BM Co., Germany, 5010) using commercial kits (Spinreact, Spain) according to the manufacturer's instructions (Bucolo and David 1973; Naito and Kaplan 1984).

MyBioSource Assay Kits (MyBioSource Co., California, USA) was used to examine serum creatinine, urea, total bilirubin, total protein, and albumin levels were investigated according to the standard methods using an automated spectrophotometer (Perkin Elmer Co., Waltham, USA) (Anavekar et al. 2004; Prætorius and Poulsen 1953; Wedemeyer and Yasutake 1977; Young 1997). The globulin level was also determined according to a previous method (Reitman and Frankel 1957). The efficacy of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT) was analyzed using an automated clinical spectrophotometer (Abbott Alcyon 300, USA) following the Pars-Azmon kit's instructions (Pars Azmon, Tehran, Iran).

RNA extraction, cDNA production, and quantitative PCR

Liver Sects. (100 mg) from red tilapia of each group (control, $HP_{0.5}$, HP_1, and $HP_{1.5}$) stored in RNA later solution, were subjected to total RNA extraction using Total RNA Extraction Kits (iNtRON Biotechnology, Inc., Gyeonggi-do, Korea) which stated the manufacturer's instructions. The purity of RNA samples was calculated by UV–Vis Nanodrop spectrophotometer (Quawell Technology, Inc., San Jose, CA, USA). The first strand of complementary DNA (cDNA) containing 1 µg of total RNA was formed using a cDNA synthesis kit (Enzynomics Co. Ltd., Daejeon, Korea). The primer sequences of proinflammatory (*IL-8*, *TNF-α*, and *IL-1β*), anti-inflammatory *IL-10*, and caspase-associated genes, were used (Table 2) (Elbahnaswy et al. 2021; Zahran et al. 2021). Housekeeping gene beta-actin (*β-actin*) equalized the mRNA expressions of these genes. qRT-PCR was done using SYBR Green PCR Master Mix (Enzynomics Co. Ltd., Daejeon, Korea) for quantifying red tilapia cDNAs (1 µl) according to the manufacturer's procedures via Quant studio 1 Real-Time PCR System (Applied Biosystems™, Thermo Fisher Scientific, Oslo, Norway). The thermocycling conditions were 95 °C for 15 min, followed by 45 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 20 s, followed by melt curve generation. The relative mRNA expression values of each target gene were normalized to *β-actin* mRNA transcripts using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008).

Examination of liver and intestinal histology

The excised liver and intestine samples were dried in ethanol, cleared in xylene, immersed in paraplast wax, and dissolved in 10% neutral formaldehyde (Zhao et al. 2020). Briefly, sections of 5–7 µm tissue thickness were then prepared using a rotary microtome, stained with hematoxylin and eosin, and investigated under a light microscope (Olympus CKX41, Tokyo, Japan). Also, the length and width of intestinal sections were statistically estimated.

Table 2 Forward and reverse primers applied for q-PCR analysis

Target gene	Primer sequence (5′-3′)	Amplicon size (bp)	Accession number
β-actin	F: CAGCAAGCAGGAGTACGATGAG R: TGTGTGGTGTGTGGTTGTTTTG	136	XM_003455949.2
Caspase-3	F: GGCTCTTCGTCTGCTTCTGT R: GGGAAATCGAGGCGGTATCT	80	GQ421464.1
TNF-α	F: CTGGGACACTAAGACCGCTG R: TGCAGTTTCACTGAGGCCAT	153	XM_003453563.5
IL-1β	F: AACAACTGCGAACACAGCAC R: CTTTTACTGAGTTAAGATCGGTTGA	139	XM_019365842.2
IL-8	F: GCACTGCCGCTGCATTAAG R: GCAGTGGGAGTTGGGAAGAA	180	XM_031747075.1
IL-10	F: AGCATTTCTGTGGACCAGCTA R: GAGCTTCTTGAGCCTGACGG	112	KP645180.1

Statistical analyses

GraphPad Prism version 8.0 (GraphPad Software, Inc., San Diego, California, USA) was implemented to perform statistics. Firstly, the normality verification using Shapiro–Wilk and Levene's tests was performed. The findings were indicated as mean ± standard deviation (SD). All data were statistically inspected by one-way analysis (ANOVA) with post-hoc Tukey's multiple range tests to detect whether *H. pluvialis* levels significantly affected the observed response. The notable difference was valued at $p < 0.05$.

Results

Growth and body chemical composition indexes

The results of the growth performance of red tilapia at the end of the 60-day feeding trial were summarized in Table 3. The final body weight, weight gain, weight gain rate, and specific growth ratio of fish were markedly increased with increasing dietary *H. pluvialis* levels up to $HP_{1.5}$ ($p < 0.05$) compared with the control group. In addition, a notable decrease was observed in FCR levels as dietary *H. pluvialis* levels increased from $HP_{0.5}$-$HP_{1.5}$ compared with the control ($p > 0.05$), and the lowest FCR was detected in HP_1 and $HP_{1.5}$ diets ($p < 0.05$). During the experiment, there was no marked variation in the survival rates among the four diet groups (Table 3).

The whole-body composition of red tilapia fed a variety of *H. pluvialis* diets is revealed in Table 4. A significant increase was noticed in the body protein and ash in the dietary *H. pluvialis* groups ($HP_{0.5}$, HP_1, and $HP_{1.5}$) when compared with the control ($p > 0.05$). A worthy decline in the lipids content of red tilapia fed by HP_1 and $HP_{1.5}$ diets, concerning the values shown in the control group ($p < 0.05$).

Table 3 Impact of dietary *H. pluvialis* on the growth efficacy of hybrid red tilapia

Parameters	Treatments			
	Control	$HP_{1.5}$	$HP_{0.5}$	HP_1
IBW (g)	27.1 ± 0.2	27 ± 0.2	26.9 ± 0.2	27.03 ± 0.15
FBW(g)	58.9 ± 1.5^{b}	64.3 ± 0.7^{a}	67.1 ± 1.2^{a}	67.7 ± 1.8^{a}
W.G. (g)	31.8 ± 1.3^{c}	37.3 ± 0.5^{b}	40.1 ± 1^{ab}	40.7 ± 1.6^{a}
WGR (%)	117.6 ± 4.1^{c}	138.3 ± 1.0^{b}	148.8 ± 2.7^{a}	150.5 ± 5.3^{a}
SGR (%/d)	1.3 ± 0.03^{c}	1.4 ± 0.00^{b}	1.5 ± 0.02^{a}	1.5 ± 0.03^{a}
FCR	1.5 ± 0.05^{c}	1.3 ± 0.00^{b}	1.2 ± 0.02^{ab}	1.2 ± 0.04^{a}
SR (%)	94.67 ± 3.0	96.67 ± 1.5	99.00 ± 1.7	99.00 ± 1.0

Values were expressed as means ± SD ($n = 3$). The different superscript letters in the same row significantly varied when $p < 0.05$. *IBW* initial body weight, *FBW* final body weight, *WG* weight gain, *WGR* weight gain rate, *SGR* specific growth rate, *FCR* feed conversion ratio, *SR* survival rate

Table 4 Effects of dietary *H. pluvialis* on whole body composition of red tilapia

Parameters	Control	$HP_{0.5}$	HP_1	$HP_{1.5}$
Moisture	78.3 ± 0.1	78.4 ± 0.05	78.5 ± 0.04	78.3 ± 0.1
Protein	13.3 ± 0.03^b	13.4 ± 0.03^b	13.6 ± 0.03^a	13.6 ± 0.04^a
Lipid	11.61 ± 0.02^a	11.55 ± 0.02^b	11.54 ± 0.02^b	11.53 ± 0.00^b
Ash	6.9 ± 0.01^a	7.0 ± 0.01^b	7.1 ± 0.03^b	7.1 ± 0.03^b

Values with different superscript letters in the same row significantly differed when $p < 0.05$

Serum immunological and pro-inflammatory cytokines indices and antioxidant capacity

The phagocytic activity and phagocytic index, as well as IgM content in $HP_{0.5}$, HP_1, and $HP_{1.5}$ groups notably increased when compared to the control group ($p < 0.05$) (Table 5). As well, IgG content significantly increased in HP_1 group ($p < 0.05$). Meanwhile, MPO levels markedly decreased with the higher levels of *H. pluvialis*, and the lowest increment was detected in $HP_{1.5}$, when compared with the control group ($p < 0.05$). Nitric oxide values markedly increased in the HP_1 group ($p < 0.05$). At the same time, IFN-γ and IL-4 levels were notably reduced in the serum of red tilapia-fed supplemented groups in comparison with the control one ($p < 0.05$). However, there was no disparity in TNF-α levels among all the groups (Table 5).

In comparison with control diet, the serum SOD and GSH activities manifested a notable increasing tendency in HP_1 and $HP_{1.5}$ supplemented groups ($p < 0.05$). Conversely, the serum levels of CAT showed no alterations in supplemented groups (HP_1 and $HP_{1.5}$) except for $HP_{0.5}$ group when compared with control ($p < 0.05$). MDA level revealed no change between the control and dietary groups (Fig. 1).

Table 5 Serum immunological parameters and proinflammatory cytokines (mean ± SD) of red tilapia fed on *H. pluvialis*–supplemented diets

Parameters	Control	$HP_{0.5}$	HP_1	$HP_{1.5}$
Phagocytic activity (μg ml^{-1})	1.6 ± 0.05^b	2.0 ± 0.01^a	2.0 ± 0.0^a	2.0 ± 0.01^a
Phagocytic index (%)	1.3 ± 0.06^c	1.8 ± 0.07^b	2.4 ± 0.07^a	2.5 ± 0.1^a
IgG (mg/dl)	33.5 ± 0.7^b	34.5 ± 0.7^b	40.5 ± 2.1^a	34 ± 1.4^b
IgM (mg/dl)	28.5 ± 2.1^b	65 ± 7^a	64.5 ± 14.9^a	86.5 ± 3.5^a
MPO (U/L)	29.5 ± 0.7^a	21.5 ± 2.1^b	13 ± 1.4^c	12.5 ± 0.7^c
Nitric oxide (Umol/L)	2.8 ± 0.3^b	2 ± 0.03^b	7 ± 1.3^a	4.6 ± 0.4^b
IFN-γ (pg/ml)	3.6 ± 0.07^a	1.5 ± 0.4^b	1.2 ± 0.2^b	1.4 ± 0.4^b
IL-4 (pg/ml)	6.9 ± 0.1^a	3.8 ± 0.3^b	2.9 ± 0.1^c	1.9 ± 0.0^d
TNF-α (pg/ml)	1.3 ± 0.06	1.1 ± 0.1	1.1 ± 0.06	1.6 ± 0.3

Values in the same row with different superscripts are significantly different ($p < 0.05$). *IgG* immunoglobulin G, *IgM* immunoglobulin M, *MPO* myeloperoxidase, *IFN-γ* interferon gamma, *IL-4* interleukin 4, *TNF-α* tumor necrosis factor alpha

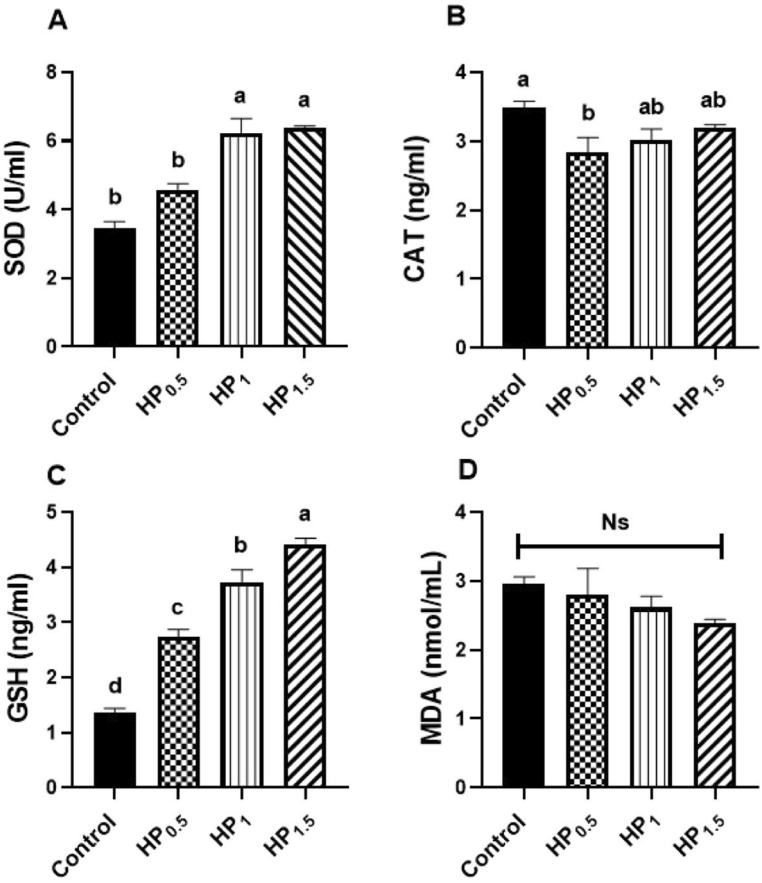

Fig. 1 Effects of *H. pluvialis* supplement (control group, $HP_{0.5}$, HP_1, and $HP_{1.5}$) on serum superoxide dismutase (SOD, **A**), catalase (**B**), reduced glutathione (GSH, **C**), and malondialdehyde (MDA, **D**) levels in red tilapia. Data were represented as Mean ± SD. Data in the same row assigned with the different superscripts are significantly different ($p < 0.05$) using ANOVA Post Hoc (Tukey test)

Serum biochemical parameters and hepatic function

Liver (ALT, AST, ALP, and GGT) biomarkers in the serum of hybrid red tilapia were greatly affected ($p < 0.05$) by supplementary *H. pluvialis* when compared to control (Table 6). These enzymes were notably diminished ($p < 0.05$) in all dietary *H. pluvialis* levels up to 1.5 g kg^{-1}. A similar trend has also been investigated regarding blood creatinine levels ($p < 0.05$); however, urea, total bilirubin, total protein, and albumin were not significantly affected by *H. pluvialis* supplementation. The highest globulin levels ($p < 0.05$) were acquired at 1 g kg^{-1} and 1.5 g kg^{-1} diet, respectively (Table 6).

Red tilapia fed the *H. pluvialis* diet at HP_1 and $HP_{1.5}$ had minimal levels of total cholesterol as well as elevated levels of TG than those of the control ($p < 0.05$). Meanwhile, the levels of TG were significantly decreased in the $HP_{0.5}$ dietary group ($p < 0.05$). However, no considerable amendment was distinguished in the levels of HDL and LDL among all dietary groups (Fig. 2).

Table 6 Effects of dietary *H. pluvialis* on serum biochemical parameters of red tilapia

Parameters	Control	$HP_{0.5}$	HP_1	$HP_{1.5}$
Creatinine (mg/dl)	1.37 ± 0.07^{a}	1.11 ± 0.05^{b}	1.12 ± 0.04^{b}	1.0 ± 0.02^{b}
Urea (mg/dl)	71.93 ± 0.78	61.16 ± 1.4	63.62 ± 2.1	65.26 ± 5.5
Total bilirubin (mg/dl)	0.52 ± 0.1980	0.31 ± 0.09	0.24 ± 0.03	0.35 ± 0.04
Total protein (mg/dl)	5.5 ± 0.18	6.1 ± 0.35	6.6 ± 0.5	6.9 ± 0.2
Albumin (mg/dl)	2.4 ± 0.23	2.7 ± 0.13	2.3 ± 0.06	2.6 ± 0.07
Globulin (mg/dl)	3.1 ± 0.05^{b}	3.4 ± 0.21^{ab}	4.4 ± 0.4^{a}	4.3 ± 0.1^{a}
ALT (U/ml)	12.2 ± 0.6^{a}	10.04 ± 0.2^{b}	8.5 ± 0.07^{c}	8.5 ± 0.07^{c}
AST (U/ml)	25.7 ± 0.7^{a}	20.2 ± 0.03^{b}	21.5 ± 0.3^{b}	21.5 ± 0.09^{b}
ALP (U/ml)	9.0 ± 0.2^{a}	7.9 ± 0.2^{b}	7.4 ± 0.07^{b}	7.4 ± 0.2^{b}
GGT (U/L)	121.0 ± 1.4^{a}	105.5 ± 4.9^{b}	86.5 ± 0.7^{c}	72.0 ± 1.4^{d}

Values in the same row with different superscripts are significantly different ($p < 0.05$). *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *ALP* alkaline phosphatase, *GGT* gamma-glutamyl transpeptidase

Gene expression

The hepatic expression of various genes (*TNF-α*, *IL-1β*, *IL-8*, *IL-10*, and *caspase-3*) in the red tilapia following dietary inoculation of the *H. pluvialis* was represented in Fig. 3. The significant downregulation of pro-inflammatory *TNF-α*, *IL-1β*, and *IL-8* genes was displayed ($p < 0.05$) in fish-fed *H. pluvialis* doses at 1 and 1.5 g kg^{-1} diet (HP_1 and $HP_{1.5}$) when compared with the control diet (Fig. 3A–C). A similar trend was also observed in group $HP_{0.5}$, except for *IL-1β* gene expression (Fig. 3B). On the other hand, the *IL-10* mRNA expression was notably upregulated in all dietary groups containing *H. pluvialis* ($p < 0.05$), in comparison with the control group (Fig. 3D). Nevertheless, *caspase-3* gene expression was markedly decreased in HP_1 and $HP_{1.5}$ supplemented diets ($p < 0.05$), with no change revealed in $\mathbf{HP_{0.5}}$ group (Fig. 3E).

Histological examination

As for the liver, there was an improvement in the hepatic tissue structures, an improvement in the endothelium lining of the hepato-central veins, and a decrease in the inflammatory cells detected in supplemented groups. The best improvement was observed in groups HP_1 (Fig. 4C), $HP_{1.5}$ (Fig. 4D), and $HP_{0.5}$ (Fig. 4B), in comparison with the control group (Fig. 4A). On the other hand, the third group (HP_1), improving the normal sizes, numbers of Kupffer cells, appearance of hepatocytes, and pancreatic acinar cells were observed as shown in Fig. 4C, and in the fourth group ($HP_{1.5}$), Kupffer cells were of medium size and number was shown in Fig. 4D, and in the second group ($HP_{0.5}$), numerous and swelling of Kupffer cells and inflammatory cells was demonstrated was shown in Fig. 4B compared to the control group was shown in Fig. 1A.

As for the intestine, there was an improvement in the intestinal mucosal layers, as well as other intestinal tissues in the HP_1 (Fig. 5C), and $HP_{1.5}$ groups (Fig. 5D), and a notable increase in the measurable length and width of the intestinal villus (Fig. 6) which a noticed boost in the higher level of the substance in the $\mathbf{HP_1}$ group caused of the improving effect of astaxanthin.

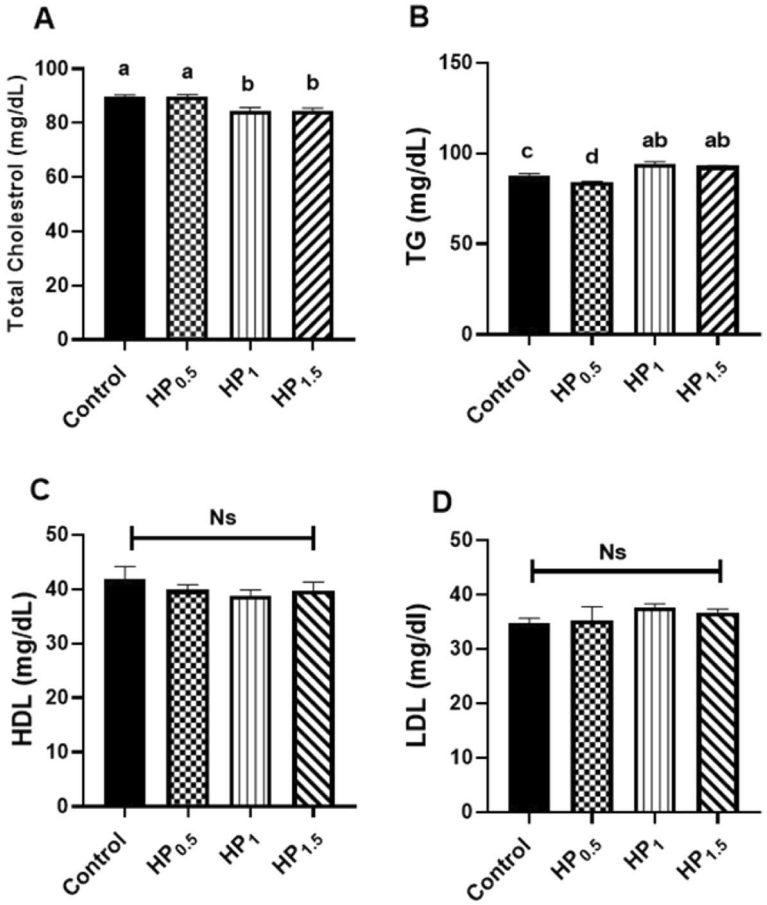

Fig. 2 Effects of *H. pluvialis* supplement (control group, $HP_{0.5}$, HP_1, and $HP_{1.5}$) on serum cholesterol (**A**), triglyceride (**B**), high-density lipoprotein, HDL (**C**), and low-density lipoprotein, LDL (**D**) levels of red tilapia. Data were represented as Mean ± SD. Data in the same row assigned with the different superscripts are significantly different ($p < 0.05$) using ANOVA Post Hoc (Tukey test)

Discussion

Haematococcus pluvialis is a significant microalgae species that produce astaxanthin, one of the most potent naturally occurring antioxidants (Shah et al. 2016). As far as the authors are aware, there is not enough data on the advantages of supplementing red tilapia raised in concrete tanks with *H. pluvialis*. A 60-day feeding trial was carried out in this study to check the effectiveness of *H. pluvialis* on the growth index, carcass chemical composition, antioxidant status, and anti-inflammatory potential of juvenile red tilapia.

In our research, red tilapia reared in raceways showed a significant increase in FBW, WG, WGR, and SGR of juvenile red tilapia as dietary *H. pluvialis* doses boosted from $HP_{0.5}$–$HP_{1.5}$ in comparison with the control group. These findings were similar to earlier research on tilapia (*Oreochromis mossambicus*) (Ju et al. 2017), large yellow croaker (*Pseudosciaena crocea*) (Li et al. 2014), and discus fish (*Symphysodon haraldi*) (Wang et al. 2016). The main explanation is due to the presence of a variety of important several

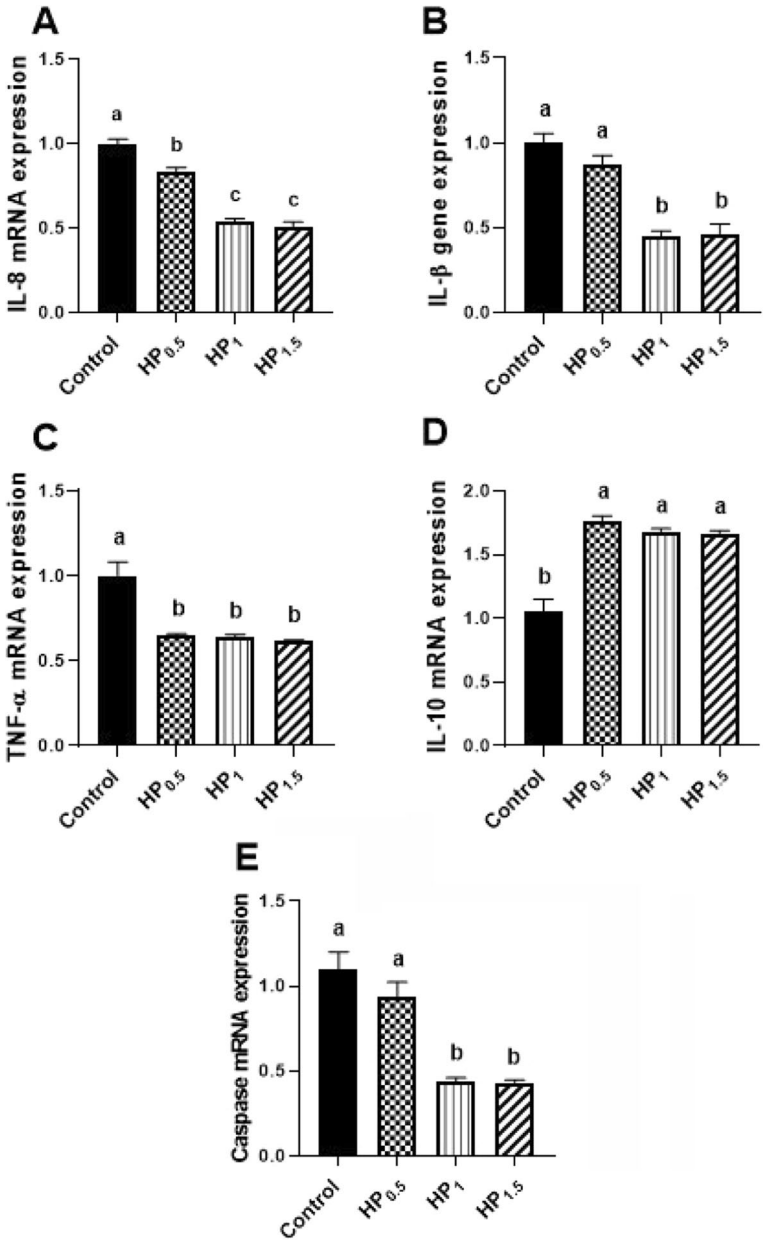

Fig. 3 Liver mRNA levels of proinflammatory (*IL-8*, *TNF-α*, and *IL-1β*), anti-inflammatory *IL-10*, and *caspase* genes of red tilapia-fed diet supplemented with *H. pluvialis* for 60 days. Four groups, the control group, $HP_{0.5}$, HP_1, and $HP_{1.5}$ were tested. Data were expressed as Means ± SD ($n = 3$) and were normalized to the *β-actin* mRNA levels. Data in the same row assigned with the different superscripts are significantly different ($p < 0.05$) using ANOVA Post Hoc (Tukey test)

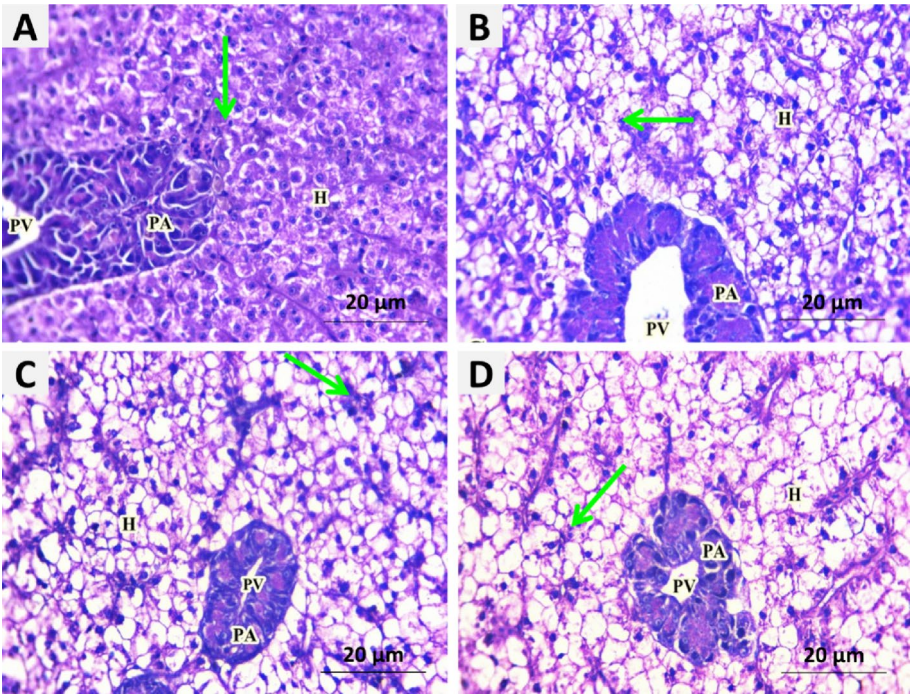

Fig. 4 Sections for hepatopancreatic micrograph of four red tilapia groups; **A** control group, **B** $HP_{0.5}$, **C** HP_1, and **D** $HP_{1.5}$, hepatocytes (H), pancreatic veins (PV), pancreatic acinar cells (PA), and Kupffer cells (green arrows), [H&E, bar = 20 µm]

nutrients in *H. pluvialis*, including minerals, vitamins, proteins (2–5%), fat (0.5%), carbohydrates (2–5%), and other bioactive substances (e.g., Carotenoids, Chlorophylls) that can promote the growth of fish (Yu et al. 2021). This might be also explained by the protein accumulation of hybrid tilapia that reflects the positive impact of the high protein content of *H. pluvialis*-supplemented diets; this could elucidate the improvement of fish growth. Similar effects were reported in hybrid red tilapia-fed *H. pluvialis* microalgae in the diet (Li et al. 2014), and in Nile tilapia-fed *Nannochloropsis oculate* at different levels (Zahran et al. 2023).

Additionally, this study exhibited a significant increase in the whole-body protein and ash along with a decrease in body lipids in the *H. pluvialis* groups ($HP_{0.5}$, HP_1, and $HP_{1.5}$) unlike the control one. It has been reported that spotted sea bass (*Lateolabrax maculatus*) fed diets containing *H. pluvialis* (2, 4, 6, 8, and 10 g/kg) exhibited considerably lower total body lipid levels than fish fed the control diet (Yu et al. 2021). This outcome was comparable to those who discovered that juvenile golden pompano-fed diets containing astaxanthin had lower total body lipid contents (Xie et al. 2017). Similarly, astaxanthin has been shown to improve lipid utilization and lower lipid levels in red porgy *Pagrus pagrus* (Kalinowski et al. 2011).

In both vertebrates and invertebrates, phagocytosis is the first cellular defense mechanism by which cells adhere to the surface of recognized particles such as bacteria, and other microorganisms, and then engulf these recognized particles (Carbone and Faggio 2016). Immunoglobulin (Ig) is a glycoprotein produced by the proliferation and differentiation of

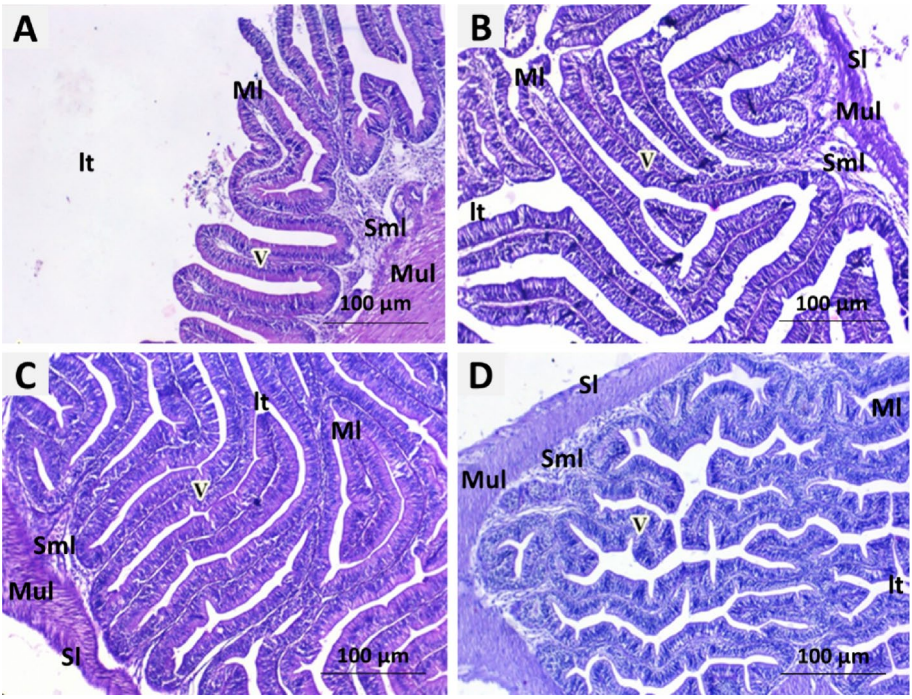

Fig. 5 Transversal sections for intestine tract micrograph of four red tilapia groups. Serosa layer (Sl), muscular layer (Mul), submucosa layer (Sml), mucosa layer (Ml), intestinal tract (It), inflammatory cell (asterisks), [H&E, bar = 50 µm]

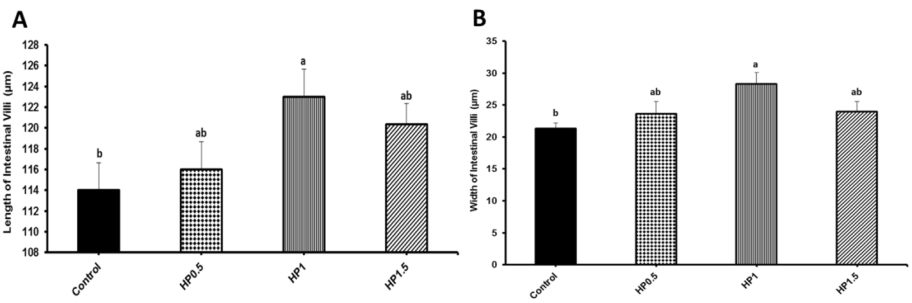

Fig. 6 Column bars for intestinal parameters of length and width from four red tilapia groups, $n = 3$ for each fish group. Mean ± SD, different letters as a, and b refer to different significantly comparing between fish groups

B lymphocytes into plasma cells upon antigen stimulation (Burgos-Aceves et al. 2021). Myeloperoxidases (MPO) are peroxidase enzymes that oxidize a variety of halides and pseudohalides to produce numerous hypohalous acids by utilizing H_2O_2. In the current results, the phagocytic activity as well as IgM, IgG, and NO content were significantly elevated in the HP_1 group ($p < 0.05$). Meanwhile, myeloperoxidase values markedly decreased with the gradual increase in *H. pluvialis* doses, and the lowest increment was revealed in $HP_{1.5}$. In the same line, after treating common carp, *Cyprinus carpio* with astaxanthin

supplemental diet at 50 and 100 mg kg^{-1} following *Aeromonas hydrophila* infection, a significant increase in serum phagocytic, respiratory burst, lysozyme, and bactericidal activities were noticed (Jagruthi et al. 2014). Moreover, rainbow trout that ingested astaxanthin had higher phagocytic indices and lysozyme activity (Amar et al. 2004). These results may help to explain why the fish fed on a diet high in astaxanthin may also enhance the bifacial microbiota in the digestive tract by enhancing the enzymes responsible for digesting fish, hence promoting the immune response (Elbahnaswy and Elshopakey 2023). As well, astaxanthin-treated human neutrophils elucidated a significant reduction in the generation of MPO and HClO in addition to a sharp decline in all reactive oxygen species (Guerra et al. 2012). Another result demonstrated the capacity of dietary astaxanthin from *Haematococcus pluvialis* (3 g/kg) to enhance the action of inducible nitric oxide synthase (i-NOS), a crucial indicator of oxidative stress which converts l-arginine to ultimately lead to increase production of NO in *L. vannamei* (Liu et al. 2022).

A previous study found that supplementing coral trout *Plectropomus leopardus* with 100–150 mg/kg of *H. pluvialis* astaxanthin could notably boost the IgM concentration in serum and liver, as well as the IgM mRNA expression in the liver both before and after challenge with *Vibrio harveyi* (Zhu et al. 2022). The inclusion of *H. pluvialis* astaxanthin in the meal considerably raised the total IgM content in the serum of Asian seabass, *Lates calcarifer* (Lim et al. 2019). The fact that supplemental astaxanthin was probably beneficial for fish Ig synthesis indicates that it may have an immunomodulatory effect on fish T lymphocyte activation, which is presumably the primary agent regulating the discrimination and proliferation of peripheral B cells that make Igs (Ashfaq et al. 2019).

The antioxidant enzymes demonstrated the functioning state of the body's antioxidant system, indicating the body's capacity to neutralize oxygen-free radicals and shield the fish's tissues from oxidative damage (Shan et al. 2019). Nonetheless, a previous study found a connection between fish responsiveness in aquaculture and antioxidant defense (Guerriero et al. 2002). In this experiment, when compared to the control diet, the antioxidant enzyme activities of red tilapia, such as SOD, and GSH were significantly elevated by nutritional supplementation of *H. pluvialis*, which is the primary source of esterified astaxanthin. These were consistent with other findings that recorded significant decrease in the levels of CAT, SOD, and GPx in the liver of *L. maculates* with an increase in dietary *H. pluvialis* in Chinese mitten crab *Eriocheir sinensis* (Wu et al. 2017) and large yellow croaker *Pseudosciaena crocea* (Li et al. 2014). Earlier research has also demonstrated that dietary supplements containing astaxanthin from *H. pluvialis* (Sheikhzadeh et al. 2012) may enhance the activities of antioxidative enzymes (T-AOC and SOD) in *O. mykiss*. According to another research, feeding *O. mykiss* a meal containing 30 mg/kg *H. pluvialis* astaxanthin for 4 months significantly raised the activities of T-AOC, T-SOD, and GPx in the blood and liver with decreased MDA levels (Long et al. 2023). A previous study on *Trachinotus ovatus* showed the body's antioxidant capacity may be enhanced by the addition of astaxanthin-rich *Oedocladium carolinianum* via the activation of the Nrf2-ARE signaling pathway (Zhao et al. 2022).

According to earlier research, serum AST and ALT activities are important indicators for evaluating liver function, and elevated levels of these enzymes in the serum may indicate hepatocyte dysfunction and liver injury (Ayyat et al. 2018). In this study, liver enzymes including ALT, AST, ALP, and GGT were notably decreased with increasing dietary *H. pluvialis* levels up to 1.0 g kg^{-1} diet and leveled off with a further increase to 1.5 g kg^{-1}, compared to the control group. These decreases in liver enzyme activities are usually related to astaxanthin hepatoprotective actions as hepatic tissue was in good condition, as seen by intact cellular structures and the staining characteristics of the hepatocytes,

and did not exhibit any congestion, inflammation, or vacuolization. Similarly, dietary *H. pluvialis* supplementation was observed to improve *T. ovatus* liver shape and decrease serum AST and ALT activity (Zhao et al. 2021). A minor elevation in blood creatinine level was also detected in this study; however, urea, total bilirubin, total protein, and albumin were not significantly affected by *H. pluvialis* supplementation. The primary cause of increased blood creatinine level is may be related to the negative effects of high astaxanthin or other elements in microalgae including heavy metals, toxins, and nucleic acids when high doses of algal were administrated (Spolaore et al. 2006).

Prior research has indicated that adding *H. pluvialis* or astaxanthin to the diet may improve the serum biochemical parameters of fish (Chimsung et al. 2013). The LDL-C/HDL-C ratio is assessed as an exponent of to transport capacity of cholesterol (Yun et al. 2011). In the current study, red tilapia fed the *H. pluvialis* diet at HP_1 and $HP_{1.5}$ had the lowest levels of cholesterol as well as the highest levels of TG than those of control. Meanwhile, the levels of TG were significantly decreased in the $HP_{0.5}$ group with no considerable alterations observed in the levels of HDL and LDL among all dietary groups. Our results corroborate another study that showed a significant decrease of TG and cholesterol on days 1 and 4 in fish (*Oncorhynchus mykiss*) given 3 g kg^{-1} of *H. pluvialis*; however, these metabolites were significantly increased when fish were supplied with 10 g kg^{-1} of algae (Sheikhzadeh et al. 2012). Conversely, dietary *H. pluvialis* might lower triglyceride and cholesterol levels in large yellow croaker *Pseudosciaena crocea* (Li et al. 2014). Serum biochemical parameter measurements in the current investigation revealed that fish fed the astaxanthin-rich *Oedocladium carolinianum* diet (5%) had greater levels of HDL with lower levels of TG, cholesterol, and LDL than fish fed control diet (Zhao et al. 2022). It is unclear why the highest treatment groups have greater triglyceride levels. However, it may be attributed to the high fat content of the diet used to mix the algae was to be the cause of this rise.

The inflammatory cytokines present in fish can be categorized into two groups: pro-inflammatory cytokines (*IL-8*, *TNF-α*, and *IL-1β*) and anti-inflammatory cytokines (*IL-10*). By upregulating the genes concerning anti-inflammatory cytokines after downregulating pro-inflammatory cytokines-related genes, the inflammation of fish can be decreased (Ottinger et al. 2016). The treatment of oral astaxanthin in this study resulted in a decrease in serum IFN-γ and IL-4 levels along with a drop in hepatic TNF-α, IL-1β, and IL-8 mRNA levels with increasing *H. pluvialis* levels up to 1.5 g kg^{-1} diet. Additionally, the expression of hepatic anti-inflammatory *IL-10* was statistically increased, suggesting that the inflammation in red tilapia can be lessened. A similar study also recorded that administration of 200, 400, and 800 mg/kg astaxanthin led to a decrease in *TNF-α*, *IL-1β*, and *IL-8* serum levels with a rise in *IL-10* levels in crucian carp (Wu and Xu 2021). Another finding clarified that astaxanthin could greatly suppress the expression of *TNF-α*, *IL-1β*, and *IL-8*, in the head kidney of snakeheads (Li et al. 2019). A recent report confirmed the anti-inflammatory effect of astaxanthin following exposure of *Channa argus* to LPS, demonstrated by a marked reduction in expression of *NF-κB* p65, *IL-1*, *IL-8*, and *TNF-α* in liver, kidney, spleen, and gut of fish (Zhu et al. 2022). The anti-inflammatory activity of astaxanthin is associated with its ability to block the *NF-κB* p65 pathway (Zhu et al. 2022).

The caspase family plays a crucial role in mediating apoptosis as well; caspase 3 serves as the essential executive molecule, commonly activated death protease, and is responsible for the precise cleavage of numerous important cellular proteins. Our findings showed that the caspase-3 gene was markedly decreased in HP_1 and HP1.5-supplemented diets. Adding *H. pluvialis* (3.3, 6.7, and 13.3 g kg^{-1}) to the diet also reduced the salinity stress response and apoptosis in post-larval white shrimp via controlling the caspase-3 mRNA

levels, suggesting that the dietary *H. pluvialis* suppressed the death of post-larval white shrimp (Xie et al. 2018). Furthermore, the intake of 150 mg kg^{-1} astaxanthin reduced the high expression of caspase-3, caspase-9, and BAD, suggesting that astaxanthin mitigated HFD-induced apoptosis in largemouth bass (Xie et al. 2020).

Generally, the intestinal histomorphology notably contributed to the growth performance of fish species that are represented by higher intestinal villi height (Eissa et al. 2023b). In the current study, significantly higher intestinal villi height and intestinal mucosal layer thickness were shown in the dietary *H. pluvialis* groups, referring that *H. pluvialis* enhances the growth performance of red tilapia via improving the digestibility and absorption capacity of nutrients; subsequently, greater contact surface area between the intestine and nutrients was revealed in the supplemented groups. Consistent with our findings, a remarkable increase in the length of intestinal villi and thickness of the intestinal mucosal layer was observed following dietary inoculation of *H. pluvialis* in *L. vannamei* shrimp (Fang et al. 2022). Besides, our histological data exerted a beneficial effect of *H. pluvialis* on the liver health of red tilapia that was strengthened by transcriptional levels of anti-inflammation and apoptosis-related genes. Nevertheless, dietary astaxanthin-rich microalgal *Oedocladium carolinianum* was found to have a hepatoprotective impact by prohibiting the apoptosis and inflammatory response of *trachinotus ovatus* fish (Zhao et al. 2022).

Conclusion

Dietary supplementation of *H. pluvialis* astaxanthin could improve the growth efficacy, antioxidant ability, and innate immunity of hybrid red tilapia after two months of feeding, also the hepatoprotective effects were exerted. Considering these positive benefits of *H. pluvialis*, the optimal supplementation of dietary *H. pluvialis* levels should be between 1 and 1.5%.

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Author contributions Elsayed A. A. Eldessouki and Sayed Hemdan Eissa: Methodology, Investigation, Validation, Formal analysis. Gehad E. Elshopakey: Methodology, Investigation, Formal analysis, Validation, Writing-original draft, Writing-review & editing. Samia Elbahnaswy: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing-original draft, Writing-review & editing and Follow-up publication. Medhat S. Shakweer, Abdelwahab A. Abdelwarith, Elsayed M. Younis, Simon J. Davies, Amira Mili, Sameh A. Abdelnour, Yasmin M. Abd El-Aziz: Methodology, Formal analysis, and Writing-original draft.

Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval Our trial has been accomplished with the approval of the Institutional Ethics Committee of the Faculty of Veterinary Medicine, Mansoura University, Egypt. It follows the general guidelines of the Canadian Council on Animal Care approved our experimental protocol (MU-ACUC (VM.R.24.04.162)).

Consent to participate All authors have participated in this work.

Consent for publication All authors have reviewed and approved the manuscript for publication.

Competing interests The authors declare no competing interests.

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