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Growth, health, and immune status of Nile tilapia *Oreochromis niloticus* cultured at different stocking rates and fed algal β-carotene

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A B S T R A C T

Bioactive substances such as β-carotene are regarded as an effective dietary aquafeed supplement to boost the immunity of fish cultured in high densities. The present study aimed to evaluate the influence of the dietary incorporation of β-carotene derived from *Spirulina platensis* (0, 0.5, and 1.0 g kg^{-1} diets) on growth performance, feed efficiency, hemato-biochemical indices, immunological responses, hepatic antioxidant enzymes, and associated immune-antioxidant genes expression in Nile tilapia, *Oreochromis niloticus* fingerlings (initial weight of 5.85 ± 0.06 g) reared at two stocking densities (SD_{100}, 100 fish m^{-3} and SD_{200}, 200 fish m^{-3}) for 70 days. Fish were randomly allocated into eighteen plastic tanks (0.5 m^3 each plastic tank) in triplicate and fed three times per day at 9:00 a.m., 11:00 a.m., and 3:00 p.m. with three isonitrogenous and isolipidic diets. The results showed that there was no difference in feed intake among the experimental diets ($P > 0.05$). The diet containing 0.5 g kg^{-1} β-carotene increased weight gain, final body weight, specific growth rate, and feed conversion ratio of fish reared at SD_{200}. Fish grown at SD_{100} or SD_{200} showed a significant increase in hematocrit and hemoglobin levels when fed a diet supplemented with 0.5 or 1.0 g kg^{-1} β-carotene compared to those on an unsupplemented diet ($P < 0.05$). Alanine amino transferase and aspartate amino transferase levels were lowest in fish group fed diet containing 1.0 g kg^{-1} β-carotene at SD_{100} as well as in fish fed diet supplemented with 0.5 and 1.0 g kg^{-1} β-carotene at SD_{200}. Fish raised at SD_{200} and supplemented with 1.0 g kg^{-1}β-carotene showed significantly improvements in total protein, albumin, and growth hormone levels compared to those fed on an unsupplemented diets ($P > 0.05$). Supplementing with 0.5 or 1.0 g kg^{-1} β-carotene diets significantly increased the concentrations of C3, C4, and IgM in fish reared at both SD_{100} and SD_{200} compared to those without supplementation ($P < 0.05$). The hepatic antioxidant activity of malondialdehyde was lowest in fish-fed diet supplemented with 1.0 g kg^{-1} β-carotene at SD_{100}. At SD_{200}, fish fed a diet supplemented with 1.0 g kg^{-1} β-carotene showed the highest levels of glutathione (GSH) and glutathione peroxidase (GPx) activity ($P < 0.05$). The highest activity of catalase (CAT) was in fish fed diet supplemented with 0.5 g kg^{-1} β-carotene at SD_{200} and SD_{100} whereas, the highest activity of superoxide dismutase (SOD) was in fish fed diet supplemented with 1.0 g kg^{-1} β-carotene at SD_{100}. The same trend was seen in the associated hepatic genes expression of superoxide dismutase 2, CAT, and GPx. When compared to other diets, fish raised at SD_{100} and fed a diet supplemented with 0.5 g kg^{-1} β-carotene showed the highest levels of gene expression for interleukin (IL)−1β and interleukin-8 (IL-8) ($P < 0.05$). But those reared at SD_{200} and fed a diet without β-carotene supplementation had the lowest transcript expressions of IL-1β and IL-8. In conclusion, adding β-carotene up to 1.0 g kg^{-1} to the basal diet improves and boosts growth performance, feed efficiency, immunological responses, hepatic antioxidant enzyme, and associated immune-antioxidant gene expression in Nile tilapia reared at SD_{200}.

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1. Introduction

To meet the increasing global demand for fish, aquaculture must be intensified (Montero et al., 1999; Turnbull et al., 2005; Lupatsch et al., 2010; Hassaan et al., 2015, 2018; Moawad et al., 2024; Abdo et al., 2024). Stocking density is a key factor to success in aquaculture management as it directly impacts fish survival, growth, behavior, health, production, feed consumption, and water quality (Abdel Fattah et al., 2020). The choice of stocking densities depends in part on economic factors and market demands (Rahman et al., 2016; Sachin et al., 2020). Increasing stocking density is essential for high productivity and can increase the potential economic revenue of fish farms by about 30% (Naderi et al., 2019; Jewel et al., 2023). However, high-stocking density may lead to an imbalance in the physiological processes of fish, affecting the growth performance and health status (Bayunova et al., 2002; Long et al., 2019; Sagar et al., 2019; Yadata et al., 2020). Excessive stress can suppress the immune system, leading to physiological problems and diseases that weaken the stamina of fish and cause mortality (Jeney et al., 1997; Hassaan et al., 2019; Bowyer et al., 2019; Hoseini et al., 2020; Mohammady et al., 2021; Omar et al., 2022; Ibrahim et al., 2023; Ali et al., 2023). Growth performance, survival, behavior, and immunological status of fish are important factors that researchers have focused on this area (El-Sayed, 2002; Huntingford et al., 2006; Ashley, 2007; Dawood et al., 2019; Karnatak et al., 2021; Chen et al., 2021; Ibrahim et al., 2021; El-Badawy et al., 2022; Mohammady et al., 2022).

Several studies shown that as stocking density increases, the growth efficiency of fish decreases, resulting in reduced final body weight (Rahman et al., 2016; Sachin et al., 2020). High stocking density has been found to negatively impact the immune system of farmed rainbow trout, *Oncorhynchus mykiss* (Ellis et al., 2002), European sea bass, *Dicentrarchus labrax* (Lupatsch et al., 2010), Amur sturgeon, *Acipenser schrenckii* (Ni et al., 2016), and juvenile Chinese sturgeon, *Acipenser sinensis* (Long et al., 2019), leading to decreased productivity, and feed conversion efficiency. Additionally, high stocking density can increase fish metabolic rate, leading to oxidative stress and damage to the integrity of the mitochondria and the cell membrane through lipid peroxidation (Evans and Cooke, 2004; Ahmad et al., 2000; Kucukbay et al., 2009). Thus, using natural immunostimulants to mitigate the adverse effects of crowding on fish is important for long-term sustainability. Previous studies have found that dietary supplements of antioxidants can improve the growth performance and health status of rainbow trout reared in high stocking density conditions (Kucukbay et al., 2009; Trenzado et al., 2008).

Natural pigments such as carotenoids, prodigiosin, and astaxanthin have been found in various plants and microalgae, particularly the cyanobacterium, *Spirulina platensis*. Studies have revealed that these pigments can improve the performance, physiological functions, immune response, and disease resistance in different fish species (Kim et al., 2010; Anbazahan et al., 2014; Jagruthi et al., 2014). Among these pigments, β-carotene is a significant source of vitamin A and has been reported to act as a natural immunostimulant, enhancing antioxidant and immunological responses in organisms (Babin et al., 2015). Several studies have been conducted on the role of carotenoids in enhancing growth, survival, and immunity as well as protecting against the harmful effects of lipid peroxidation in different aquatic species including rainbow trout (Amar et al., 2001), European bullhead, *Cottus gobio* (Dorts et al., 2012) and common carp, *Cyprinus carpio* (Anbazahan et al., 2014). Also, the addition of β-carotene to the diet has been found to enhance the ability of various aquatic species to cope with stressors such as infectious hematopoietic necrosis virus in rainbow trout (Amar et al., 2012), heavy metal toxicity in Nile tilapia, *Oreochromis niloticus* (Elseady and Zahran, 2013), cold shock in small-sclaed pacu, *Piaractus mesopotamicus* (Bacchetta et al., 2020), and high temperature in yellow catfish *Pelteobagrus fulvidraco* (Liu et al., 2019). However, the mechanism by which carotenoids enhance immunity and effectively scavenge free radicals in tilapia, requires further investigation (Sun et al., 2012; Anbazahan et al., 2014). Therefore, more research is needed to understand how carotenoids boost fish immunity. As a result, the current study aimed to assess the ameliorative effects of β-carotene extracted from *Spirulina platensis* on the growth, immunological responses, hepatic antioxidant enzymes, and associated immune-antioxidant genes expression of Nile tilapia farmed at different stocking densities.

2. Materials and methods

2.1. Experimental approach

A factorial experimental design (3×2) was carried out to examine the effects of various levels of β-carotene extracted from *S. platensis* (0, 0.5, and 1.0 g kg^{-1} β-carotene diets) on the performance of mono-sex Nile tilapia, *Oreochromis niloticus* fingerlings reared in two stocking densities SD_{100} (100 fish m^3) and SD_{200} (200 fish m^3).

2.2. β-carotene extraction

β-carotene was obtained and extracted from *S. platensis* according to Herrero-Martínez et al. (2021). Dried *S. platensis* were obtained from the National Research Center in Egypt. Approximately 20 g of the dried *S. platensis* were suspended in 50 mL of methylene chloride, refrigerated at −4°C overnight, and then dried at 40°C. The residue was dissolved in 10 mL petroleum ether. The wavelength of β-carotene absorbance present in the solution was read spectrophotometrically at 451 nm against the standard (tetrahydrofuran).

2.3. Preparation of experimental diets

A basal diet (310.9 g kg^{-1} crude protein and 18.93 MJ kg^{-1} gross energy) was formulated (Table 1) and supplemented with 0, 0.5, and 1.0 g kg^{-1} β-carotene diets. Each diet was tested at two different stocking densities: SD_{100} and SD_{200}. Using a pelleting hand noodle maker, all the components were thoroughly combined with the β-carotene before being formed into pellets with a diameter of 2 mm. These pellets were then allowed to dry overnight at room temperature and then kept at 4°C. Gross energy was determined as reported by Brett (1973) and is shown in Table 1 along with the proximate analysis of the ingredients and diets as analyzed by the AOAC (2012) method.

2.4. Fish rearing technique

Mono-sex Nile tilapia *O. niloticus* fingerlings (initial weight of 5.85 ± 0.06 g) were purchased from a private farm (El-Sahaba Hatchery, Egypt) and acclimated in 10 m^3 concrete pond (4×2×1.25 m) within a greenhouse for two weeks. Fish were fed a commercial feed purchased from Aller Aqua company, Egypt with 30% crude protein and 6% lipid at a rate of 3% of the total biomass throughout the acclimation period, supplied at equal portions at 9:00 a.m., 11:00 a.m., and 3:00 p.m. Following acclimation, healthy Nile tilapia were distributed at random in 18 plastic tanks (0.5 m^3/tank; filled with a 400 L water capacity) in triplicate for 70 days. The fish with an average initial body weight of 5.85±0.06 g were randomly stocked in the first nine tanks with 100 fish per m^3 (SD_{100}, 585 g/m^3). The other nine tanks were each allocated with 200 fish per m^3 (SD_{200}, 1170 g/m^3). Aeration, greenhouse housing, and automated heaters (150 watts) were all employed to keep the water temperature in the tanks between 26 and 28°C. Each day, the water in the tank was changed by about 30%. The amount of feed was calculated on the basis of 3% of total biomass and offered for experimental fish three times a day, at 9:00 a.m., 11:00 a.m., and 3:00 p.m. Fish were weighed every 15 days to adjust the amount of feed ration. Water quality parameters were monitored daily during the experiment. The temperature ranged from 26.2 to 28.2°C, dissolved oxygen was 6.0 ±0.03 mg L^{-1}, the total ammonia was 0.15±0.02 mg L^{-1}, nitrite (NO_2) was 0.02 ± 0.004 mg L^{-1}, nitrate (NO_3) was 0.74 ± 0.06 mg L^{-1}.

Table 1
Formulation and proximate composition of the experimental diets (g kg $^{-1}$ diet, dry matter).

Ingredients	g kg^{-1}
Fish meal	50
Soybean meal	450
Corn gluten	80
Yellow corn	280
Wheat bran	75
Fish oil	40
Premix[1]	25
Total	
Chemical composition (g kg^{-1})	
Protein	310.9
Lipid	61.75
Ash	45.32
Fiber	48.82
Nitrogen free extract[2]	533.2
Gross energy[3] (MJ kg^{-1})	18.93

[1]Vitamin and mineral mixture kg^{-1} of a mixture contains 4800 I.U. Vit A, 2400 IU cholecalciferol (Vit. D), 40 g Vit E, 8 g Vit K, 4.0 g Vit B12, 4.0 g Vit B2, 6 g Vit B6, 4.0 g, pantothenic acid, 8.0 g nicotinic acid, 400 mg folic acid, 20 mg biotin, 200 mg choline, 4 g copper, 0.4 g iodine, 12 g iron, 22 g manganese, 22 g zinc, 0.04 g selenium. Folic acid, 1.2 mg; niacin, 12 mg; d-calcium pantothenate, 26 mg; pyridoxine. HCl, 6 mg; riboflavin, 7.2 mg; thiamine. HCl, 1.2 mg; sodium chloride (NaCl, 39% Na, 61% Cl), 3077 mg; ferrous sulphate ($FeSO_4.7\ H_2O$, 20% Fe), 65 mg; manganese sulphate ($MnSO_4$, 36% Mn), 89 mg; zinc sulphate ($ZnSO_4.7\ H_2O$, 40% Zn), 150 mg; copper sulphate ($CuSO_4.5\ H_2O$, 25% Cu), 28 mg; potassium iodide (KI, 24% K, 76% I).
[2]NFE (Nitrogen free extract) = 100−(crude protein + lipid + ash + fibre content).
[3]Gross energy was calculated using gross calorific values of 23.63, 39.52, and 17.15 kj/g for protein, fat, and carbohydrate, respectively, according to Brett (1973).

Throughout the experiment, the water quality requirements were acceptable and appropriate for Nile tilapia culture (Boyed, 1990).

2.5. Growth and feed efficiency

Before the feeding trial and following it, each tank was counted and the number of fish was recorded. At the end of experiment (70 days), all the formulae employed to determine the growth parameters and feed utilization efficiency are listed as follow:

- Weight gain (WG) = final weight (g) - initial weight (g)
- Specific growth rate (SGR) = LnW2 - LnW1/t (days)*100, where Ln = the natural log; W1 = initial fish weight, W2 = final fish weight in grams, t = period in days (70 days)
- Feed conversion ratio (FCR) = feed intake (g)/weight gain (g)
- Protein efficiency ratio (PER) = weight gain (g) / protein ingested (g).

2.6. Hemato-biochemical indices

At the end of the experiment, five fish were used from each tank for blood collection from the caudal vein and were divided into two portions (1.5 mL of blood for two portions). The first portion of selected fish was collected utilising 10% EDTA to the estimate haematological parameters (Rawling et al., 2009). The Rosenfeld (1947) approach was used to calculate the differential counting of white blood cells (WBCs). The second portion of selected fish was taken without the use of an anticoagulant, left to coagulate at 4°C, then centrifuged at 3000 rpm for 10 minutes to obtain the serum. The serum was collected and stored −20°C until use for measuring the serum biochemical parameters. The procedure outlined by Reitman and Frankel (1957) was used to assess the levels serum enzymatic activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Total serum protein and albumin were determined according to Henry (1964) and Wotton and Freeman (1982), respectively. However, globulin was calculated by subtracting albumin from total protein according to Coles (1974). Following the manufacturer's instructions, a radioimmunoassay (RIA) kit was used to assess the serum growth hormones (GH) (Tianjin, China).

2.7. Immune responses

The level of serum total immunoglobulin M (IgM) was determined by an ELISA assay kit (Cusabio, Wuhan, Hubei, China). The test kits were purchased from Shenzhen Mindray Bio-medical Electronics Co., Ltd. The amounts of complement component 3 (C3) and complement component 4 (C4) were assessed using the immunoturbidimetric approach (Zhejiang Yilikang Biotech Co., Ltd).

2.8. Assessments of the liver's antioxidant activity

Hepatic samples (livers of three fish per replicate) were weighed, homogenized, and rinsed with ice-cold phosphate buffer (1:10; phosphate buffer: pH 7.4, 0.064 M), after anestheitzing the fish with 3-aminobenzoic acid ethyl ester (MS 222, 100 mg/L, Sigma, St. Louis, MO). Based on the Peskin and Winterbourn (2000) method, the homogenate was centrifuged for 10 min at 4°C and 4000 g, and the supernatant was used to assay the activity of superoxide dismutase (SOD). According to Dogru et al. (2008) the concentration of malondialdehyde (MDA) was assessed. A modified technique of Beers and Sizer (1952) was used to assess the catalase (CAT) activity. The activities of glutathione peroxidase (GPx) and glutathione (GSH) were assessed according to Moin (1986) and Giustarini et al. (2013), respectively.

2.9. Gene expressions

After anestheitzing fish by MS 222, 100 mg/L, liver samples from three fish from each treatment were taken and homogenized using a Tissue Lyser LT apparatus (QIAGEN; Cat No. /ID: 85600). The total ribonucleic acid (RNA) was extracted from these tissues using RNeasy® Mini kit (Qiagen, Cat No. 74104), based on the manufacturer's protocol. The reverse transcriptase reaction of RNA was conducted to synthesize complementary DNA (cDNA) synthesizing according to the protocol of the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). The cDNA was stored at −80°C for further molecular analyses. The primers used in this study (Table 2) were designed for the genes of CAT, SOD2, and GPx antioxidants, as well as the immune response genes; interleukin 1β (IL-1β) and interleukin 8 (IL-8). The primers were designed using the software GenScript Online PCR Primers Designs Tool. β-actin and 18 s rRNA were used as a housekeeping gene.

Quantitative PCR reaction contained 2.5 μL of 1 μg/μL cDNA, 12.5 μL SimplyGreen SYBR Green qPCR Master Mix, Low Rox (Cat SQ102–0100, GeneDireX, Inc), 0.3 μM of each of forward and reverse primers, 1 μL RNase inhibitor and RNase-Free water to a final volume of 25 μL. Reaction was run on an AriaMax Real Time PCR (Agilent Technologies, USA) using a two-step protocol: hot-start at 95℃ for 10 min, followed by 40 cycles of denaturation at 95℃ for 15 s and annealing/extension at 60℃ for 1 min, and ending with a melt curve from 65–95℃. The expression levels of the selected target genes were normalized to those of 18SrRNA gene. Changes in the expression levels of the target genes were presented as n-fold changes relative to the corresponding controls. Relative gene expression ratios (RQ) were estimated using the formula: RQ = $^{2-}$ΔΔCT Shved et al. (2011). According to Hassaan et al. (2021), RNA template negative and non-template controls were performed in triplicate along with each assessed sample.

Table 2
Functional group, genes name, oligonucleotide sequence and genebank accession number of primers used in quantitative real time polymerase chain reaction (qRT-PCR).

Functional group	Gene name	Primer Sequence (5′–3′)	GenBank No.
Immune response	IL-1β	F- 5′-TGCTGAGCACAGAATTCCAG-3′ R- 5′-GCTGTGGAGAAGAACCAAGC-3′	XM_019365842.2
	IL-8	F- 5′-GCACTGCCGCTGCATTAAG-3′ R- 5′-GCAGTGGGAGTTGGGAAGAA-3′	XM_019359413.2
Antioxidant activity	CAT	F- 5′-TCAGCACAGAAGACACAGACA-3′ R- 5′-GACCATTCCTCCACTCCAGAT-3′	NM_001279533.1
	GPx	F- 5′-ACAAGTGACATCGAGGCAGA-3′ R- 5′-CAAACCCAGGCCTGCTATAA-3′	NM_001279711.1
	SOD2	F- 5′-CTCCAGCCTGCCCTCAA-3′ R- 5′-TCCAGAAGATGGTGTGGTTAATGTG-3′	XM_003449940.3
reference genes	β-actin	F- 5′-CAGTGCCCATCTACGAGGGTTAT-3′ R- 5′-CGGCTGTGGTGGTGAAGGAGT-3′	AY116536.1
	18s rRNA	F- 5′-GTTGCAAAGCTGAAACTTAAAGG-3′ R- 5′-TTCCCGTGTTGAGTCAAATTAAGC-3′	AF497908.1

F: Forward primer; R: Reverse primer.

2.10. Data statistical analysis

Before analysis, the data were converted using arc-sin (Zar, 1984). Thereafter, the SAS ANOVA technique was used to analyze all the data (SAS, version 6.03, Soft Inc.,Tusla, OK, USA, SAS, 1993). To ascertain whether there was appreciable variation among the treatments, one-way ANOVA was utilized. When overall variations were detected, Duncan (1955) test was used to examine the variances between the means. Individual impacts of the two different densities, as well as different concentrations of β-carotene were examined using two-way ANOVA. Results are shown as mean values ± standard error of the means. The differences were deemed significant at $P < 0.05$.

3. Results

3.1. Growth and feed utilization efficiency

Growth indices and feed utilization parameters are presented in Table 3. Regardless of the effect of β-carotene, FBW, WG, SGR, and PER were significantly greater at SD_{100} than at SD_{200} ($P < 0.05$). The addition of β-carotene significantly influenced the SGR, WG, and PER of fish reared at both SD_{100} and SD_{200} ($P < 0.05$). The highest FBW, WG, SGR, and PER were observed in fish fed a diet with 0.5 g kg^{-1} β-carotene when stocked at densities of SD_{100} and SD_{200}, with no significant difference ($P < 0.05$). A lower FCR was recorded in the group reared at SD_{200} without β-carotene supplementation.The best FCR was recorded in fish fed a diet containing 0.5 g kg^{-1} β-carotene and stocked under SD_{100} and SD_{200} without any significant difference ($P < 0.05$). The FI values did not differ significantly among groups that were fed the experimental diets ($P > 0.05$).

3.2. Hemato-biochemical indices

Table 4 shows the hematological parameters values. Even without β-carotene supplementation, the levels of RBCs, WBCs, and monocytes were higher in SD_{200} compared to SD_{100}. Additionally, regardless of stocking density, adding 0.5 g kg^{-1} of β-carotene to the diet resulted in a significant increase in Hb, Hct, RBCs, WBCs, and their differential compared to 1.0 g kg^{-1} of β-carotene ($P < 0.05$). Fish fed a diet with 0.5 g kg^{-1} β-carotene at either SD_{100} or SD_{200} densities showed higher values of Hb, Hct, and RBCs with no statistically significant differences between them ($P > 0.05$).

Table 3
Growth performance and feed utilization of Nile tilapia *O. niloticus* fed diets containing β-carotene at different stocking densities for 70 days.

Treatment	β-carotene g kg^{-1} diet	SD*	Growth and feed utilization						
			IBW (g $fish^{-1}$)	FBW (g $fish^{-1}$)	WG (g $fish^{-1}$)	SGR (% day^{-1})	FI (g $fish^{-1}$)	FCR	PER (g)
Individual treatment means†									
T1	0.0	SD_{100}	6.00	15.5^{ab}	9.5^{ab}	1.58^{ab}	16.17	1.72^{b}	1.99^{a}
T2	0.5	SD_{100}	5.95	18.95^{a}	13^{a}	1.91^{a}	17.28	1.34^{b}	2.49^{a}
T3	1.0	SD_{100}	6.00	18.98^{a}	12.97^{a}	1.92^{a}	17.88	1.38^{b}	2.44^{a}
T4	0.0	SD_{200}	6.15	13.5^{b}	7.35^{b}	1.31^{b}	18.71	2.54^{a}	1.31^{b}
T5	0.5	SD_{200}	5.85	17.92^{a}	12.07^{a}	1.87^{a}	16.25	1.35^{b}	2.49^{a}
T6	1.0	SD_{200}	6.05	17.45^{ab}	11.4^{ab}	1.77^{a}	17.25	1.51^{b}	2.20^{a}
Pooled ±SE			0.066	0.650	0.662	0.063	0.913	0.078	0.106
Means of the main effect‡									
	0.0		6.07	14.50^{y}	8.42^{y}	1.44^{y}	17.44	2.13^{x}	1.65^{y}
	0.5		5.90	18.43^{x}	12.53^{x}	1.90^{x}	16.77	1.34^{y}	2.49^{x}
	1.0		6.02	18.21^{x}	12.18^{x}	1.84^{x}	17.57	1.44^{y}	2.31^{x}
		SD_{100}	17.95	53.43^{q}	35.47^{q}	5.41^{q}	51.33	4.44^{q}	6.92^{q}
		SD_{200}	18.05	48.87^{r}	30.82^{r}	4.95^{r}	52.21	5.40^{q}	6.00^{r}
ANOVA (P-value)									
SD			0.735	0.015	0.014	0.012	0.828	0.027	0.089
BC**			0.359	0.022	0.021	0.012	0.865	0.002	0.008
SD × BC			0.581	0.911	0.869	0.627	0.506	0.048	0.252

†Treatment means represent the average values of three tanks per treatment. SD*: Stocking density; BC**: β-carotene.
The Duncan multiple range test was conducted for individual means only if there was a significant interaction (ANOVA: $P < 0.05$). Means followed by the same letter in a column are not significantly different.
‡Means of the main effect followed by different letters in a column are significantly different at $P < 0.05$ by the Duncan multiple range test; p and q for SD levels and x, y and z for BC levels.

Table 4
Hematological parameters of Nile tilapia *O. niloticus* fed diets containing β-carotene at different stocking densities for 70 days.

Treatment	β-carotene g kg^{-1} diet	SD*	Hb (g/dl)	Hct (%)	RBCs ($\times 10^6$ cmm^{-1})	WBCs ($\times 10^3$ cmm^{-1})	Neutrophil (%)	Lymphocyte (%)	Monocyte (%)	Acidophil (%)
Individual treatment means[†]										
T1	0.0	SD_{100}	7.50^{b}	19.75^{c}	2.00^{b}	16.65^{f}	12.50^{c}	81.50^{ab}	4.00^{c}	2.00^{c}
T2	0.5	SD_{100}	9.55^{a}	29.40^{a}	3.25^{a}	59.50^{b}	18.50^{a}	74.50^{d}	4.50^{bc}	3.50^{ab}
T3	1.0	SD_{100}	9.50^{a}	25.35^{b}	3.00^{a}	45.50^{c}	11.00^{c}	78.50^{c}	4.00^{a}	4.50^{a}
T4	0.0	SD_{200}	7.50^{b}	19.00^{c}	3.30^{a}	63.75^{a}	15.50^{b}	80.50^{b}	4.00^{c}	1.00^{c}
T5	0.5	SD_{200}	8.40^{a}	25.45^{b}	3.05^{a}	36.40^{e}	10.50^{c}	82.50^{a}	5.00^{b}	3.25^{b}
T6	1.0	SD_{200}	8.25^{a}	24.50^{b}	3.00^{a}	40.45^{d}	17.00^{ab}	73.50^{d}	7.00^{a}	4.25^{ab}
Pooled ±SE			0.4639	0.3649	0.2114	0.311	0.408	0.288	0.1178	0.1863
Means of the main effect[‡]										
	0.0		7.50	19.73^{z}	2.65	40.20^{z}	14.00	81.00^{x}	4.00^{z}	1.50^{z}
	0.5		8.97	27.42^{x}	3.15	47.95^{x}	14.50	78.50^{y}	4.75^{y}	3.37^{y}
	1.0		8.87	24.95^{y}	3.00	42.97^{y}	14.00	76.00^{z}	5.50^{x}	4.37^{x}
		SD_{100}	8.85	24.83^{P}	2.75^{q}	40.55^{q}	14.00	78.16	4.16^{q}	3.33
		SD_{200}	8.05	22.98^{q}	3.11^{P}	46.86^{P}	14.33	78.83	5.33^{P}	2.83
ANOVA (P-value)										
SD			0.268	0.011	0.266	0.0001	0.584	0.1536	0.355	0.106
BC**			0.203	0.001	0.427	0.0001	0.729	0.0002	0.0001	0.0003
SD × BC			0.703	0.074	0.164	0.0001	0.0001	0.0001	0.421	0.455

[†]Treatment means represent the average values of three tanks per treatment. SD*: Stocking density; BC**: β-carotene.
The Duncan multiple range test was conducted for individual means only if there was a significant interaction (ANOVA: $P < 0.05$). Means followed by the same letter in a column are not significantly different.
[‡]Means of the main effect followed by different letters in a column are significantly different at $P < 0.05$ by the Duncan multiple range test; p and q for SD levels and x, y and z for BC levels.

Table 5 shows that ALT and AST values increased significantly at SD100, regardless of β-carotene supplementation ($P < 0.05$). However, supplementation of β-carotene significantly ($P < 0.05$) decreased ALT and AST values. The lowest ALT and AST levels were observed in fish fed a diet supplemented with 1.0 g kg^{-1} β-carotene at SD_{100}, followed by those fed a diet supplemented with 0.5 and 1 g kg^{-1} β-carotene at SD_{200}. Additionally, adding 0.5 g kg^{-1} β-carotene at SD_{200} had a significant impact on TP, albumin, and growth hormones ($P < 0.05$) (Table 5). The highest levels of TP, albumin, globulin, and growth hormones were found in fish kept at SD_{100} and fed diets enriched with 0.5 g kg^{-1} β-carotene, as well as those offered diets supplemented with 1 g kg^{-1} β-carotene at SD_{200}, but with no significant influence.

3.3. Immune response parameters

Table 6 shows the immune indices. The levels of C3, C4, and IgM were lower in fish under SD_{200} compared to fish under SD_{100}, regardless of β-carotene supplementation ($P < 0.05$). Fish fed a diet with 0.5 or 1.0 g kg^{-1} β-carotene diet had higher levels of C3, C4, and IgM in fish reared at either SD_{100} or SD_{200} compared to those without supplementation. Fish fed a diet with 0.5 g kg^{-1} β-carotene under SD_{100} and fish fed a diet supplemented with 1.0 g kg^{-1} β-carotene under SD_{200} had the highest levels of C3, C4, and IgM, with no significant differences between them ($P < 0.05$).

3.4. Hepatic antioxidant activities

Table 7 shows that the activities of CAT, GSH, GPx and SOD were significantly elevated in fish at SD_{200} regardless of β-carotene supplementation ($P < 0.05$). Additionally, the addition of β-carotene significantly enhanced the activities of CAT, GSH, GPx, and SOD ($P < 0.05$). The highest activity of CAT was observed in fish that were fed a diet

Table 5
Biochemical parameters of Nile tilapia *O. niloticus* fed diets containing β-carotene at different stocking densities for 70 days.

Treatment	β-carotene g kg^{-1} diet	SD*	ALT (U/L)	AST (U/L)	TP (g/dl)	Albumin (g/dl)	Globulin (g/dl)	GH (ng/dL)
Individual treatment means[†]								
T1	0.0	SD_{100}	121.50^{c}	34.90^{b}	2.85^{ab}	1.40^{ab}	1.45^{ab}	1.45^{d}
T2	0.5	SD_{100}	131.5^{b}	32.10^{bc}	3.25^{a}	1.65^{ab}	1.60^{a}	2.55^{b}
T3	1.0	SD_{100}	125.5^{bc}	26.30^{c}	2.15^{b}	0.70^{c}	1.45^{ab}	2.00^{c}
T4	0.0	SD_{200}	151.00^{a}	55.00^{a}	2.20^{b}	1.05^{bc}	1.15^{b}	1.10^{d}
T5	0.5	SD_{200}	126.00^{bc}	28.75^{bc}	2.80^{ab}	1.10^{bc}	1.70^{a}	2.15^{c}
T6	1.0	SD_{200}	124.00^{c}	31.25^{bc}	3.40^{a}	1.75^{a}	1.65^{a}	3.00^{a}
Pooled ±SE			1.099	1.181	0.1486	0.10206	0.0623	0.0656
Means of the main effect[‡]								
	0.0		136.25^{x}	44.95^{x}	2.52	1.22	1.30^{y}	1.27^{y}
	0.5		128.75^{y}	30.42^{y}	3.02	1.37	1.65^{x}	2.35^{x}
	1.0		124.75^{y}	28.77^{y}	2.77	1.22	1.55^{xy}	2.50^{x}
		SD_{100}	126.16^{q}	31.10^{q}	2.75	1.25	1.50	2.00
		SD_{200}	133.66^{P}	38.33^{P}	2.80	1.30	1.50	2.08
ANOVA (P-value)								
SD			0.002	0.004	0.8199	0.7409	1.0000	0.4038
BC**			0.002	0.0004	0.2314	0.6407	0.0429	0.0001
SD × BC			0.0002	0.003	0.0191	0.0077	0.1250	0.0013

[†]Treatment means represent the average values of three tanks per treatment. SD*: Stocking density; BC**: β-carotene.
The Duncan multiple range test was conducted for individual means only if there was a significant interaction (ANOVA: $P < 0.05$). Means followed by the same letter in a column are not significantly different.
[‡]Means of the main effect followed by different letters in a column are significantly different at $P < 0.05$ by the Duncan multiple range test; p and q for SD levels and x, y and z for BC levels.

Table 6
Immune response of Nile tilapia *O. niloticus* fed diets containing β-carotene at different stocking densities for 70 days.

Treatment	β-carotene g kg^{-1} diet	SD*	C3 (mg/dL)	C4 (mg/dL)	IgM (mg/dL)
Individual treatment means†					
T1	0.0	SD_{100}	7.50^{ab}	0.70^{b}	7.10^{b}
T2	0.5	SD_{100}	8.05^{a}	1.00^{a}	8.25^{a}
T3	1.0	SD_{100}	8.50^{a}	0.90^{a}	7.20^{b}
T4	0.0	SD_{200}	5.50^{b}	0.35^{c}	5.50^{c}
T5	0.5	SD_{200}	8.90^{a}	0.95^{a}	8.30^{a}
T6	1.0	SD_{200}	8.85^{a}	0.95^{a}	7.95^{ab}
Pooled ±SE					
Means of the main effect‡					
	0.0		6.50^{y}	0.52^{y}	6.30^{z}
	0.5		8.47^{x}	0.97^{x}	8.27^{x}
	1.0		8.67^{x}	0.92^{x}	7.57^{y}
		SD_{100}	8.01^{P}	0.86^{P}	7.51^{P}
		SD_{200}	7.75^{q}	0.75^{q}	7.25^{q}
ANOVA (P-value)					
SD			0.6697	0.1594	0.2554
BC**			0.0448	0.0044	0.0008
SD × BC			0.1941	0.1430	0.0103

†Treatment means represent the average values of three tanks per treatment. SD*: Stocking density; BC**: β-carotene.
The Duncan multiple range test was conducted for individual means only if there was a significant interaction (ANOVA: $P < 0.05$). Means followed by the same letter in a column are not significantly different.
‡Means of the main effect followed by different letters in a column are significantly different at $P < 0.05$ by the Duncan multiple range test; p and q for SD levels and x, y and z for BC levels.

supplemented with 0.5 g kg^{-1} β-carotene whether they were stocked at SD_{100} or SD_{200}, with no significant difference between them.The highest activities of GSH and GPx were recorded in fish fed a diet supplemented with 1.0 g kg^{-1} β-carotene at SD_{200}, while the highest activity of SOD was recorded in fish fed diet supplemented with 1.0 g kg^{-1} β-carotene at SD_{100}. Fish fed diets that included 0.5 g kg^{-1} β-carotene showed greater SOD activity, with negligible differences between them. On the other hand, MDA concentration was reduced with β-carotene supplementation at either SD_{100} or SD_{200} (Table 7). The lowest MDA concentration was recorded in fish fed diet supplemented with 1.0 g kg^{-1} β-carotene at either SD_{100} or SD_{200}.

3.5. Gene expressions

3.5.1. The relative expression level of antioxidant enzyme genes

The relative expressions of the antioxidant enzymes CAT, SOD2, and GPx genes were all significantly enhanced by β-carotene at either SD_{100} or SD_{200} ($P < 0.05$) (Fig. 1). The highest transcript expressions of CAT and SOD2 were observed in tilapia fed a diet enriched with 1.0 g kg^{-1} β-carotene at SD_{200} while, the highest transcript expressions of GPx was observed in tilapia fed a diet enriched with 0.5 g kg^{-1} β-carotene at SD_{200}.

3.5.2. The relative expression level of immune response genes

Fig. 2 shows the relative expression profiling of immune responses genes IL-8 and IL-1β in tilapia that were fed a diet supplemented with β-carotene and reared at SD_{100} and SD_{200}. Tilapia raised at either SD_{100} or SD_{200} and given a diet supplemented with 0.5 g kg^{-1} β-carotene showed significantly increased IL-1β and IL-8 expressions, with the highest values in those reared at SD_{100} ($P < 0.05$). However, those raised at either SD_{100} or SD_{200} and fed 1.0 g kg^{-1}β-carotene showed decreased transcript expressions.

4. Discussion

4.1. Growth indices and feed utilization

High stocking density reduces growth and feed intake, leading to lower fish production and meat quality and financial losses in aquaculture (Jewel et al., 2023). This also results decreased performance, productivity and fish health issues (Lovell, 1989; Trenzado et al., 2008; Kucukbay et al., 2009; Braun et al., 2010; Hoseini et al., 2020; Jewel et al., 2023). According to the current findings, high stocking density (SD_{200}) stress in Nile tilapia reduced feed intake, body weight gain, and feed efficiency. This decline in growth performance may be due to increased energy expenditure on swimming and competition for food and space (Ajani et al., 2015; Fauji et al., 2018). Also, fish reared in SD_{200} had higher FCR values, indicating that they were unable to use the supplied diets due to chronic stress induced by increased stocking density (Rahman et al., 2016; Sachin et al., 2020). The increased energy demand brought on by high-density stress may be responsible for this increase in FCR (Ellis et al., 2002). These results are consistent with previous research on various species, including tilapia (Silva et al., 2000;

Table 7
Hepatic antioxidant activities (U/g protein) of Nile tilapia *O. niloticus* fed diets containing β-carotene at different stocking densities for 70 days.

Treatment	β-carotene g kg^{-1} diet	SD*	MDA	CAT	GSH	GP_X	SOD
Individual treatment means†							
T1	0.0	SD_{100}	40.50^{a}	122.50^{c}	144.45^{f}	153.50^{e}	143.80^{d}
T2	0.5	SD_{100}	31.50^{b}	281.70^{a}	264.35^{d}	271.00^{d}	261.75^{c}
T3	1.0	SD_{100}	27.50^{d}	272.10^{b}	295.65^{c}	292.00^{c}	385.45^{a}
T4	0.0	SD_{200}	42.50^{a}	126.50^{c}	187.60^{e}	153.50^{e}	120.00^{e}
T5	0.5	SD_{200}	30.50^{bc}	281.00^{a}	352.50^{b}	358.00^{b}	359.95^{b}
T6	1.0	SD_{200}	28.50^{cd}	275.50^{ab}	365.40^{a}	382.50^{a}	376.60^{ab}
Pooled ±SE			0.4409	1.3302	1.818	1.711	3.078
Means of the main effect‡							
	0.0		41.50^{x}	124.50^{z}	166.02^{z}	153.50^{z}	131.90^{z}
	0.5		31.00^{y}	281.35^{x}	308.42^{y}	314.50^{y}	310.85^{y}
	1.0		28.00^{z}	273.80^{y}	330.52^{x}	337.25^{x}	381.02^{x}
		SD_{100}	33.16^{q}	225.43	234.81^{q}	238.83^{q}	263.66^{q}
		SD_{200}	33.83^{P}	227.66	301.83^{P}	298.00^{P}	285.51^{P}
ANOVA (P-value)							
SD			0.3262	0.2800	0.0001	0.0001	0.0024
BC**			0.0001	0.0001	0.0001	0.0001	0.0001
SD × BC			0.2160	0.5709	0.0011	0.0001	0.0001

†Treatment means represent the average values of three tanks per treatment. SD*: Stocking density; BC**: β-carotene
The Duncan multiple range test was conducted for individual means only if there was a significant interaction (ANOVA: $P < 0.05$). Means followed by the same letter in a column are not significantly different.
‡Means of the main effect followed by different letters in a column are significantly different at $P < 0.05$ by the Duncan multiple range test; p and q for SD levels and x, y and z for BC levels.

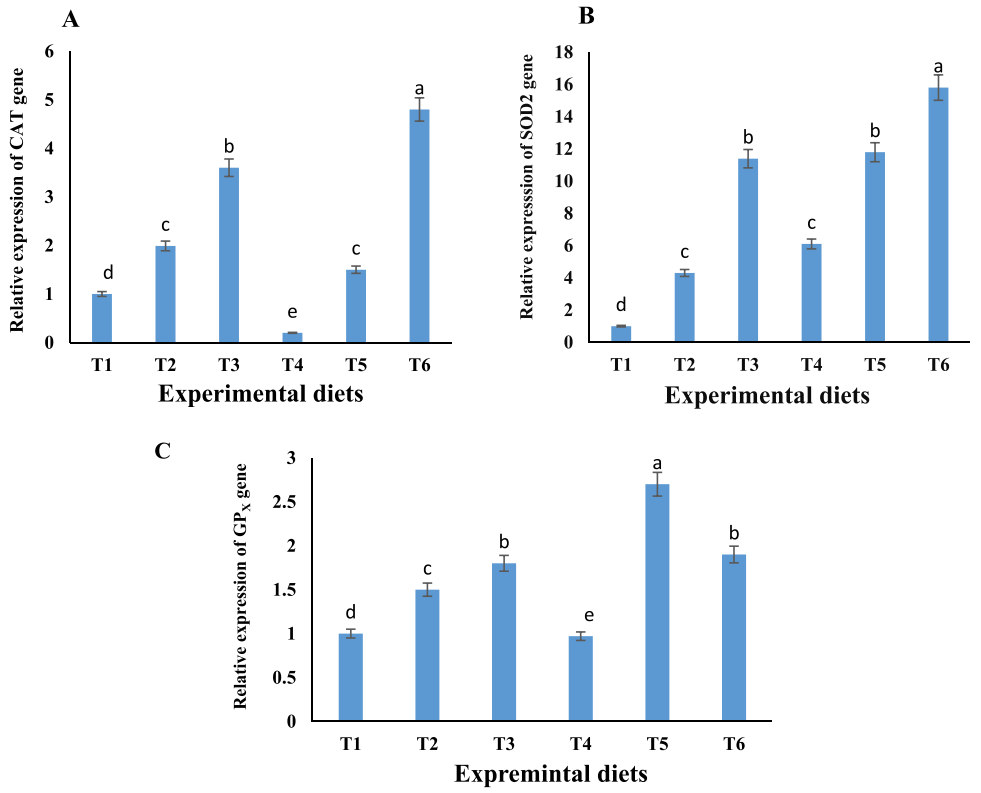

Fig. 1. Relative expressions profiling of antioxidant enzymes: (A) catalase (CAT), (B) superoxide dismutase 2 (SOD2), and (C) glutathione peroxidase (GP_X) genes in the liver of Nile tilapia *O. niloticus* fed diets containing β-carotene at different stocking densities SD_{100} and SD_{200} for 70 days. Data were normalized using β-actin as an internal reference gene. T1: SD_{100} with 0.0 g β-carotene kg^{-1} diet, T2: SD_{100} with 0.5 g β-carotene kg^{-1} diet, T3: SD_{100} with 1.0 g β-carotene kg^{-1} diet, T4: SD_{200} density with 0.0 g β-carotene kg^{-1} diet, T5: SD_{200} with 0.5 g β-carotene kg^{-1} diet, and T6: SD_{200} with 1.0 g β-carotene kg^{-1} diet.

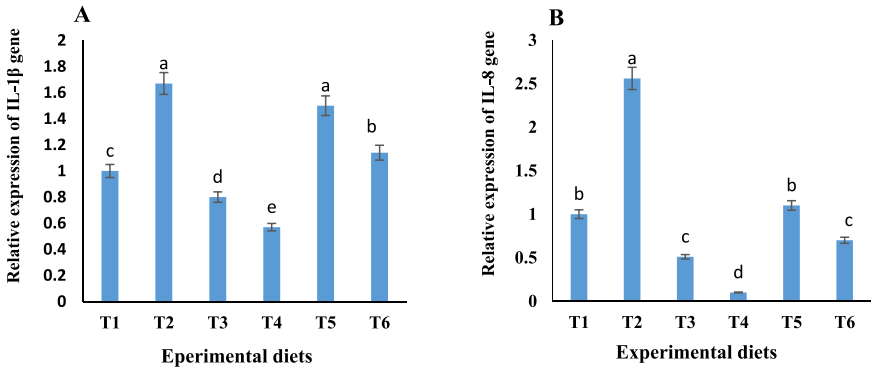

Fig. 2. Relative expressions profiling of immune responses: (A) Interleukin 1β (IL-1β) and (B) Interleukin-8 (IL-8) genes in the liver of Nile tilapia *O. niloticus* fed diets containing β-carotene at different stocking densities SD_{100} and SD_{200} for 70 days. Data were normalized relative to β-actin as an internal reference gene. T1: SD_{100} with 0.0 g β-carotene kg^{-1} diet, T2: SD_{100} with 0.5 g β-carotene kg^{-1} diet, T3: SD_{100} with 1.0 g β-carotene kg^{-1} diet, T4: SD_{200} density with 0.0 g β-carotene kg^{-1} diet, T5: SD_{200} with 0.5 g β-carotene kg^{-1} diet, and T6: SD_{200} with 1.0 g β-carotene kg^{-1} diet.

Ouattara et al., 2003; Tadesse, 2007; Gibtan et al., 2008), sturgeon, *Huso huso* (Rafatnezhad et al., 2008), Chinook salmon, *Oncorhynchus tshawytscha* (Martin and Wertheimer, 1989), African catfish (Haylor, 1991), Arctic charr, *Salvelinus alpinus* (Jørgensen et al., 1993), gilthead seabream, *Sparus aurata* (Canario et al., 1998), rainbow trout, *O. mykiss* (Ellis et al., 2002; Kucukbay et al., 2009), Atlantic cod, *Gadus morhua* (Lambert and Dutil, 2001), European seabass, *Dicentrarchus labrax* (Saillant et al., 2003), turbot, *Psetta maxima* (Aksungur et al., 2007), and juvenile Amur sturgeon, *Acipenser schrenckii* (Ni et al., 2014).

β-carotene is needed for essential functions, such as the improvement of growth efficiency, disease resistance, and immune response which have a good impact on tilapia health and performance (Anbazahan et al., 2014; Hassaan et al., 2021; Taalab et al., 2022). Thus, the present findings indicated that adding β-carotene to tilapia diets reared in high stocking density (SD_{200}) enhanced the growth and feed utilization, with no significant difference compared to tilapia reared in lower stocking density (SD_{100}). Likewise, our results showed that FCR improved in fish fed diet enriched with 0.5 or 1.0 g kg^{-1} β-carotene and reared in SD_{100} and SD_{200}, indicating the ability of fish to utilize the given feed due to the vital effects of β-carotene. Various scenarios may be connected to this improvement in growth indices and feed utilization: (1) the effectiveness of β-carotene in boosting stress tolerance in tilapia raised in high densities; (2) higher digestive efficiency, nutritional utilization, protein synthesis, and assimilation in fish as consequences of the

bioactive compounds in β-carotene that stimulate the creation of enzymes in fish metabolism as opposed to being stored (Gouveia et al., 1998; Tibaldi et al., 2015; Jagruthi et al., 2014); and (3) enhancement of the gut flora and its potential to break down the indigestible components of the feed (Eroglu et al., 2022). Similar positive effects of β-carotene supplementation have been observed in goldfish, Nile tilapia, and common carp (Aravindan et al., 2001; Hu et al., 2006; Jagruthi et al., 2014). Previous studies have shown that Nile tilapia diets containing *S. platensis* increased the growth and enhanced FCR of fish in comparison to the control group (Elsayed, 2012; Abu-Elala et al., 2016). Likewise, dietary astaxanthin increased body growth of common carp, *Cyprinus carpio* (Jagruthi, 2014). As far as we are aware, this is the first study to explore the potential benefits of supplementing β-carotene in the diets of the Nile tilapia to mitigate the negative effects of high stocking density on growth efficiency.

4.2. Hemato-biochemical indices

Recent studies have increasingly used hematological and biochemical parameters to measure fish stress levels (Barton and Iwama, 1991; Ajani, 2008; Kavitha et al., 2012; Fazio, 2019). The need to develop effective health management tools for the rapidly expanding aquaculture industry has renewed interest in understanding the fish body defense mechanisms and using immune system parameters to determine stress-related alterations (Mehdi et al., 2010). In the current study, RBC counts and WBC differentials of Nile tilapia increased with increasing stocking density (SD_{200}), irrespective of β-carotene supplementation. Regardless to β-carotene supplementation, there was a slight decrease in hematocrit in SD_{200} treatments consistent with previous studies on various fish species such as tilapia (Bejerano, 1984), rainbow trout (Kebus et al., 1992), Atlantic cod (Staurnes, 1994), European catfish, *Silurus glanis* (Docan et al., 2010), and common carp, *C. carpio* (Anbazahan et al., 2014). The supplementation of 0.5 g kg^{-1} β-carotene or 1.0 g kg^{-1} β-carotene diet elevated the values of hemoglobin and hematocrit in tilapia raised in SD_{100} and SD_{200} compared to those fed unsupplemented diets. Based on the current findings, it appears that the hematological parameters suggest that a diet supplemented with β-carotene at both levels can help Nile tilapia exposed to SD_{200} maintain their homeostasis by triggering an adaptive stress response. These results may be explained by the probable role of β-carotene in promoting fish health by strengthening the capacity of the immune system to combat stress and infection by increasing immunological parameters including WBCs (Nakono et al., 2003; 2016). Likewise, adding 10% *S. platensis* to a fish diet was reported to raise RBC and WBC counts (Abdel-Tawabe et al., 2008).

Serum ALT and AST are found in fish mitochondria and are important indicators of digestive function and liver damage (Abdel-Tawwab, 2016; 2023; Abdo et al., 2024). Fish exhibit higher ALT activity due to metabolic adjustments that counteract increases in energy demand caused by stress (Cho et al., 1994; Mirghaed et al., 2017). The activity of ALT and AST increased in fish reared in SD_{200} compared to those reared in SD_{100}, regardless of β-carotene ($P < 0.05$). Nile tilapia fed a diet with 0.5 or 1.0 g kg^{-1} β-carotene had significantly lower AST and ALT activity ($P > 0.05$). This could benefit the fish's overall health and nutritional state, possibly due to the production of cytokines that protect liver cells. Similar results were found for ALT activity in European seabass, *Dicentrarchus labrax* and characins, *Hyphessobrycon callistus*, when given diets with β-carotene supplements (Wang et al., 2006; Sallam et al., 2017).

The total protein content provides insight into the nutritional requirements and overall state of immunological health of the animal (Hwang and Lin, 2002). Serum albumin levels are essential for metabolism, transferring of both exogenous and endogenous metabolites, and other processes (Baker et al., 2002). Globulin plays a crucial role as carrier and transporter For living organisms (Preeti and Seema, 2014). In the present study, neither stocking density stress nor β-carotene supplementation had significant effects on TP, albumin, and growth hormones ($P > 0.05$). However, the addition of β-carotene at SD_{200} had a significant impact on these parameters,with the group fed a diet supplemented with 1.0 g kg^{-1} β-carotene at SD_{200} recording the highest values ($P < 0.05$). Similarly, Akbary et al. (2018) and Harikrishnan et al. (2021b) reported that dietary supplementation of *Ulva rigida* methanolic extracts (10 g/kg) to grey mullet, *Mugil cephalis* and rohu, *Labeo rohita* at varied amounts significantly enhanced globulin, albumin, and total serum protein.

4.3. Immune response parameters

Immunoglobulins such as IgM and complement play significant roles in both nonspecific and specific immunity of fish (Jang et al., 2004; Bag et al., 2009). The complement, which is the humoral component of the innate immune system is crucial for inflammatory responses, phagocytosis, microbes death, breakdown of immunological complexes, and antibody production (Harikrishnan et al., 2011; Devi et al., 2019). The primary components of the complement system are macrophages, hepatocytes, and serum complement produced by liver cells (Lambris, 1989; Sunyer et al., 1995; Rotllant et al., 1997). Stress reactions can lead to a decrease in complement contents (C3 and C4) (Lambris, 1989). In our data, the activities of C3, C4, and IgM were decreased ($P < 0.05$) in fish reared at SD_{200} conditions, regardless of β-carotene supplementation. Supplementing fish diets with 0.5 g kg^{-1} β-carotene diet significantly increased the activities of C3, C4, and IgM in fish raised at either SD_{100} or SD_{200} ($P < 0.05$). This improvement in non-specific immune response may be due to the complement, which helps maintain the fish body in a state of homeostasis (Anbazahan et al., 2014; Hassaan et al., 2021). Similar results were observed in other studies, where the immunological response of fish was enhanced by adding *Spirulina* was added to their diets, such as in Nile tilapia *O. niloticus* (Amer, 2016; Mahmoud et al., 2017; Yilmaz, 2019; Taalab et al., 2022) and coral trout *Plectropomus leopardus* (Yu et al., 2018). Other studies also showed increased serum C3, C4, and IgM in fish fed diets with carotenoid sources, such as *Dunaliella salina* and *Phaffia rhodozyma* for *O. mykiss* (Amar et al., 2004) and in yellow catfish *P. fulvidraco* fed carotenoid-containing diets after exposure to extreme temperature stress (Liu et al., 2019). Additionally, *O. niloticus* and *L. rohita* given polysaccharide ulvan extract from *Ulva clathrata* showed improved immune responses (del Rocío Quezada-Rodríguez and Fajer-Ávila, 2017; Harikrishnan et al., 2021a). Furthermore, dietary supplementation with carotenoid sources such as *Lentinula edodes* mushroom extract, black cumin seed oil (*Nigella sativa*), and peppermint (*Mentha piperita*) increased the level of immunoglobulins in *P. olivaceus*, and kutum *Rutilus frisii* (Pham et al., 2014; Adel et al., 2015). Additional research is needed to better understand how β-carotene boosts fish serum IgM, C3, and C4 levels.

4.4. Hepatic-antioxidant genes biomarker

The balance between the generation of ROS and the antioxidant systems can be damaged by stress, such as high stocking density in fish which causes ROS to be generated more rapidly (Halliwell and Gutteridge, 1989; Braun et al., 2010). This excessive production of ROS due to high stocking density can result in oxidative destruction, including oxidative damage and lipid peroxidation (Halliwell and Aruoma, 1991; Kucukbay et al., 2009). Several studies have shown that high stocking density causes oxidative injury in fish (Braun et al., 2010; Kucukbay et al., 2009; Trenzado et al., 2008). MDA is a by-product of lipid peroxidation and is recognized as an indication of lipid damage caused by ROS (Sumida et al., 1989). Therefore, the antioxidative state of fish can be reflected by TAC levels as well as CAT, SOD, MDA, and GSH levels. Adding β-carotene to the diet of high-density stressed fish resulted in a reduction of MDA levels and stronger activation of liver antioxidant enzymes (GPx, GSH, CAT, and SOD). To further understand how β-carotene enhances antioxidant responses, the expression of genes

associated with the antioxidant defense system was studied. Results revealed that under the stress of high stocking density, all examined genes (i.e., CAT, SOD2, and GPx) (Fig. 1) encoding antioxidant enzymes were upregulated in fish fed a 1.0 g kg^{-1} β-carotene diet. The upregulation of these genes may help boost the enzyme activity of these genes (Hou et al., 2015). Similarly, in studies by Tayag et al. (2010) and Lin et al. (2010), the concentration of *S. platensis* extract in white shrimp directly boosted SOD and GPx gene expressions and enzyme activities. Additionally, the results emphasize the importance of β-carotene in reducing oxidative stress and preventing lipid peroxidation in fish (Kiokias et al., 2018; Taalab et al., 2022; Anbazahan et al., 2014). Research has shown that β-carotene can help fish under high stocking density stress by scavenging free radicals (Edge and Truscott, 2018; Black et al., 2020).

Studies have also demonstrated the effectiveness of carotenoids in increasing liver antioxidant enzyme activity in various of fish species such as *H. callistus* in hypoxic and normoxic conditions, as well as *P. olivaceus*, *C. carpio*, *D. labrax*, and *P. fulvidraco* (Pan et al., 2010; Pham et al., 2014; Anbazahan et al., 2014; Sallam et al., 2017; Liu et al., 2019). Therefore, β-carotene may provide a comprehensive defense in terms of the antioxidant defense system. More research is needed to fully understand the molecular mechanism by which diets containing β-carotene regulate the expression of antioxidant enzyme genes.

4.5. Gene expression levels relating to immunological responses

Inflammation is a characteristic of immunological reactions often caused by cytokines released by immune cells (Zhao et al., 2013). For the current study, two genes from fish liver tissue, which is regarded as a crucial organ, were selected to examine the effects of β-carotene supplemented diets at different stocking densities (SD_{100} and SD_{200}) on the relative expression of immune response genes. IL-1β and IL-8 are pro-inflammatory cytokine genes crucial for controlling immunological and inflammation responses to infections or stresses by stimulating the release of more cytokines (lymphocyte activation) that activate macrophages, natural killer cells, and lymphocytes (Low et al., 2003; Secombes et al., 2001; Guzmán-Villanueva et al., 2014; Dawood et al., 2020; Anbazahan et al., 2014). Supplementing the diet with 0.5 g β-carotene kg^{-1} diet significantly increased the transcription of IL-1β and IL-8 in tilapia raised at SD_{100}. High transcription upregulation of IL-1β and IL-8 was found in fish raised at SD_{200} and fed 0.5 g β-carotene kg^{-1} diet. When carp *C.carpio*, tilapia, and yellow catfish *P. fulvidraco* were fed diets containing *A. platensis*, the IL-1β gene expressions also rose (Watanuki et al., 2006; Mahmoud et al., 2018; Liu etal., 2020). The improvement in beneficial gut microbiota, which produces peptide antimicrobials and manages the inflammatory response, is possibly connected to the immune-protective properties of bioactive compounds derived from *S. platensis*, such as β-carotene (Hassaan et al., 2021; Anbazahan et al., 2014). The effects of cytokines on fish are highly complicated and poorly understood. For this reason, additional research is required to better understand their associated mechanisms.

5. Conclusion

Adding β-carotene nutritional supplements to the diet of Nile tilapia *O. niloticus* can improve growth performance and regulate oxidative state when reared at high stocking density (SD_{200}). Economically, up to 1.0 g kg^{-1} of β-carotene can be added to Nile tilapia diets to enhance growth, feed efficiency, immune response, hemato-biochemical indices, hepatic antioxidant enzymes, and immune and antioxidative genes expression. Further research is needed to understand the biotransformation, absorption, and detection of carotenoid metabolites in the fish body.

Ethical approval

All experiments were approved by the authority of NIOF Committee for Institutional Care of Aquatic Organisms and Experimental Animals (NIOF-AQ4-F-23-R-017).

CRediT authorship contribution statement

Eman Y. Mohammady: Writing – review & editing, Writing – original draft. **Marwa M. Ali:** Data curation. **Mohamed R. Soaudy:** Data curation. **Janice A. Ragaza:** Writing – original draft, Funding acquisition. **Hoda S. El-Garhy:** Methodology. **Mohamed S. Hassaan:** Writing – review & editing, Data curation.

Declaration of Competing Interest

No conflict Interest

Data availability

Data will be made available on request.

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References

Abdel Fattah, A., Ahmed, F., Saleem, A.S., Mohammed, H., Youssef, M., Said, E., 2020. Effect of the different stocking density on behavior, performance and welfare of the Nile tilapia (*Oreochromis niloticus*). Egypt. J. Aquat. Biol. Fish. 24 (5), 539–560.

Abdel-Tawabe, M., Ahmad, M.H., Abdel-Hadi, Y.M., Seden, M.E.A., 2008. Use of *Spirulina* (*Arthrospir platensis*) as growth and immune promoter for Nile tilapia *Oreochromis niloticus* (L.) fry challenged with *Aeromonas hydrophila*. 8th Int. Symp. . tilapia Aquac. 2, 1015–1031.

Abdel-Tawwab, M., 2016. Effect of feed availability on susceptibility of Nile tilapia, *Oreochromis niloticus* (L.) to environmental zinc toxicity: Growth performance, biochemical response, and zinc bioaccumulation. Aquaculture 464, 309–315.

Abdo, H.S., Mohammady, E.Y., Tonsy, H.D., Ibrahim, A.-K., Hassaan, M.S., 2024. The potential synergistic action of quercetin and/or *Pediococcus acidilactici* on Nile tilapia, *Oreochromis niloticus* performance. Aquaculture 581, 740353.

Abu-Elala, N.M., Galal, M.K., Abd-Elsalam, R.M., Mohey-Elsaeed, O., Ragaa, N.M., 2016. Effects of dietary supplementation of *Spirulina platensis* and garlic on the growth performance and expression levels of immune-related genes in Nile tilapia (*Oreochromis niloticus*). J. Aquac. Res. Dev. 7 (7), 433–442.

Adel, M., Abedian, A.A., Zorriehzahra, J., Nematolahi, A., Esteban, M.Á., 2015. Effects of dietary peppermint (*Mentha piperita*) on growth performance, chemical body composition and hematological and immune parameters of fry Caspian white fish (*Rutilus frisii kutum*). Fish. Shellfish Immunol. 45, 841–847.

Ahmad, I., Hamid, T., Fatima, M., Chand, H.S., Jain, S.K., Athar, M., Raisuddin, S., 2000. Induction of hepatic anti oxidants in fresh water catfish (*Channa punctatus* Bloch) is a biomarker of paper mill effluent exposure. Biochim. Biophys. Acta 1523, 37–48.

Ajani, E.K., Setufe, S.B., Oyebola, O.O., 2015. Effects of stocking density on haematological functions of juvenile African catfish (*Clarias gariepinus*) fed varying crude protein levels. Afr. J. Food Sci. 9 (2), 65–69.

Ajani, F., 2008. Hormonal and heamatological responses of *Clarias gariepinus* (Burchell 1822) to ammonia toxicity. Afr. J. Biotechnol. 7 (19), 3466–3471.

Akbary, P., Molazaei, E., Aminikhoei, Z., 2018. Effect of dietary supplementation of *Ulva rigida* C. Agardh extract on several of physiological parameters of grey mullet, *Mugil cephalus* (Linnaeus). Iran. J. Aquat. Anim. Health 4 (1), 59–68.

Aksungur, N., Aksungur, M., Akbulut, B., Kutlu, İ., 2007. Effects of stocking density on growth performance, survival and food conversion ratio of Turbot (*Psetta maxima*) in the net cages on the southeastern coast of the Black Sea. Turkish. J. Fish. Aquat. Sci. 7 (2), 147–152.

Amar, E.C., Kiron, V., Satoh, S., Watanabe, T., 2001. Influence of various dietary synthetic carotenoids on bio-defence mechanisms in rainbow trout, Oncorhynchus mykiss (Walbaum). Aquac. Res. 32, 162–173.

Amar, E.C., Kiron, V., Satoh, S., Watanabe, T., 2004. Enhancement of innate immunity in rainbow trout (*Oncorhynchus mykiss* Walbaum) associated with dietary intake of carotenoids from natural products. Fish. Shellfish Immunol. 16, 527–537.

Amar, E.C., Kiron, V., Akutsu, T., Satoh, S., Watanabe, T., 2012. Resistance of rainbow trout *Oncorhynchus mykiss* to infectious hematopoietic necrosis virus (IHNV) experimental infection following ingestion of natural and synthetic carotenoids. Aquaculture 330, 148–155.

Amer, S.A., 2016. Effect of *Spirulina platensis* as feed supplement on growth performance, immune response and antioxidant status of mono-sex Nile Tilapia (*Oreochromis niloticus*). Benha Vet. Med. J. 30 (1), 1–10.
Ali, M.M., Elashry, M.A., Mohammady, E.Y., Soaudy, M.R., El-Garhy, H.S., El-Erian, M. A., Mustafa, Ahmed, Abouelsoud, Mohamed, Ragaza, Janice A., El-Haroun, Ehab R., Hassaan, M.S., 2023. Dietary Alpha-Monolaurin for Nile Tilapia (*Oreochromis niloticus*): Stimulatory Effects on Growth, Immunohematological Indices, and Immune-Related Gene Expressions. Aquac. Res. 2023.
Anbazahan, S.M., Mari, L.S.S., Yogeshwari, G., Jagruthi, C., Thirumurugan, R., Arockiaraj, J., Velanganni, A.A.J., Krishnamoorthy, P., Balasundaram, C., Harikrishnan, R., 2014. Immune response and disease resistance of carotenoids supplementation diet in *Cyprinus carpio* against *Aeromonas hydrophila*. Fish. Shellfish Immunol. 40, 9–13.
AOAC, 2012. Official Methods of Analysis, 19th ed. AOAC, Gaithersburg, p. 3000.
Aravindan, C.M., Preethi, S., Abraham, K.M., 2001. Effect of increased bio-availability of beta carotene on the pigmentation of gold fish *Carassius. auratus*. J. Inland Fish. Soc. India 33 (1), 49–53.
Ashley, P.J., 2007. Fish welfare: current issues in aquaculture. Appl. Anim. Behav. Sci. 104 (3–4), 199–235.
Babin, A., Saciat, C., Teixeira, M., Troussard, J.P., Motreuil, S., Moreau, J., Moret, Y., 2015. Limiting immunopathology: interaction between carotenoids and enzymatic antioxidant defences. Dev. Comp. Immunol. 49, 278–281.
Bacchetta, C., Ale, A., Rossi, A.S., Karakachoff, M., Cazenave, J., 2020. Effects of cold stress on juvenile *Piaractus mesopotamicus* and the mitigation by β-carotene. J. Therm. Biol. 88, 102497.
Bag, M.R., Makesh, M., Rajendran, K.V., Mukherjee, S.C., 2009. Characterization of IgM of Indian major carps and their cross-reactivity with anti-fish IgM antibodies. Fish. Shellfish Immunol. 26 (2), 275–278. https://doi.org/10.1016/j.fsi.2008.11.009.
Baker, R.T.M., Pfeiffer, A.M., Schöner, F.J., Smith-Lemmon, L., 2002. Pigmenting efficacy of astaxanthin and cantaxanthin in freshwater reared Atlantic salmon, *Salmo salar*. Anim. Feed Sci. Technol. 99, 97–106.
Barton, B.A., Iwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annu. Rev. Fish. Dis. 10, 3–26.
Bayunova, L., Barannikova, I., Semenkova, T., 2002. Sturgeon stress reactions in aquaculture. J. Appl. Ichthyol. 18 (4-6), 397–404.
Beers, R., Sizer, T., 1952. Spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195, 133–138.
Bejerano, I., 1984. Detection and control of stress conditions in warm water aquaculture. Res. on Aquacult. European Mariculture. Soc., Bredene, Belg. 56–68.
Black, H.S., Boehm, F., Edge, R., Truscott, T.G., 2020. The benefits and risks of certain dietary carotenoids that exhibit both anti-and pro-oxidative mechanisms—a comprehensive review. Antioxidants 9, 264.
Bowyer, P.H., El-Haroun, E.R., Hassaan, M., Salim, H., Davies, S.J., 2019. Dietary nucleotides enhance growth performance, feed efficiency and intestinal functional topography in European Seabass (*Dicentrarchus labrax*). Aquac. Res. 50 (7), 1921–1930.
Boyed, C.E., 1990. Water Quality in Ponds for Aquaculture. Birmingham Publishing Co.,, Birmingham, Alabama, USA.
Braun, N., de Lima, R.L., Baldisserotto, B., Dafre, A.L., de Oliveira Nuñer, A.P., 2010. Growth, biochemical and physiological responses of *Salminus brasiliensis* with different stocking densities and handling. Aquaculture 301 (1-4), 22–30.
Brett, J.R., 1973. Energy expenditure of sockeye salmon Oncorhynchus nerka, during sustained performance. J. Fish. Res. Board Can. 30, 1799–1809.
Canario, A.V., Condeca, J., Power, D.M., Ingleton, P.M., 1998. The effect of stocking density on growth in the gilthead sea-bream, *Sparus aurata* (L.). Aquac. Res. 29 (3), 177–181.
Chen, L., Yang, Y.O., Rizwan, M., Yue, D.D., Khan, I.M., Yao, F., Huang, S.J., 2021. Compensatory growth in gibel carp (*Carassius auratus* gibelio) after the stress of stocking density. Aquac. Res. 52 (4), 1697–1704.
Cho, Y.J., Kim, Y.Y., Lee, N.G., Choi, Y.J., 1994. Basic studies on developing equipment for waterless transportation of live fish. Bull. Korean Fish. Soc. 27, 501–508.
Chow, E.P.Y., Liong, H.K., Elke Schoeters, E., 2016. The Effect of Dietary Carotenoids of Different Forms: Microemulsified and Non-microemulsified on the Growth Performance, Pigmentation and Hematological Parameters in Hybrid Catfish (*Clarias macrocephalus* × *Clarias gariepinus*). J. Aquac. Res. Dev. 7, 437. https://doi.org/10.4172/2155-9546.1000437.
Coles, E.H., 1974. Plasma proteins. Veterinary Clinical Pathology, 2nd ed. W.B. Saunders Co,, Philadelphia, Pennsylvania, USA, pp. 558–560.
Dawood, M.A., Moustafa, E.M., Elbialy, Z.I., Farrag, F., Lolo, E.E., Abdel-Daim, H.A., et al., 2020. *Lactobacillus plantarum* L-137 and/or β-glucan impacted the histopathological, antioxidant, immune-related genes and resistance of Nile tilapia (*Oreochromis niloticus*) against *Aeromonas hydrophila*. Res. Vet. Sci. 130, 212–221.
Dawood, M.A., Koshio, S., Zaineldin, A.I., Van Doan, H., Moustafa, E.M., Abdel-Daim, M. M., Angeles Esteban, Hassaan, M.S., 2019. Dietary supplementation of selenium nanoparticles modulated systemic and mucosal immune status and stress resistance of red sea bream (*Pagrus major*). Fish. Physiol. Biochem. *45*, 219–230.
del Rocío Quezada-Rodríguez, P., Fajer-Ávila, E.J., 2017. The dietary effect of ulvan from *Ulva clathrata* on hematological-immunological parameters and growth of tilapia (*Oreochromis niloticus*). J. Appl. Phycol. 29, 423–431.
Docan, A., Cristea, V., Grecu, I., Dediu, L., 2010. Haematological response of the European catfish, Silurus glanis reared at different densities in "flow-through" production system. Prod. Syst. Arch. Zootech. 13 (2), 63–70.
Dogru, M.I., Dogru, A.K., Gul, M., Esrefoglu, M., Yurekli, M., Erdogan, S., Ates, B., 2008. The effect of adrenomedullin on rats exposed to lead. J. Appl. Toxicol. 28 (2), 140–146.
Dorts, J., Bauwin, A., Kestemont, P., Jolly, S., Sanchez, W., Silvestre, F., 2012. Proteasome and antioxidant responses in *Cottus gobio* during a combined exposure to heat stress and cadmium. Comp. Biochem. Phys. C. 155, 318–324.
Duncan, D.B., 1955. Multiple range and multiple F tests. Biometrics 11 (1), 1–42. https://doi.org/10.2307/3001478.
Edge, R., Truscott, T.G., 2018. Singlet oxygen and free radical reactions of retinoids and carotenoids—a review. Antioxidants 7, 5.
El-Badawy, A.S., Hassaan, M.S., Abdel-Hameid, N.A.H., El-Ezaby, M.M., El-Serafy, S., 2022. Synergistic effects between dietary zinc form supplementation and dietary protein levels on performance, intestinal functional topography, hemato-biochemical indices, immune, oxidative response, and associated gene expression of Nile tilapia Oreochromis niloticus. Biol. Trace Elem. Res. 1–17.
Ellis, T., North, B., Scott, A.P., Bromage, N.R., Porter, M., Gadd, D., 2002. The relationships between stocking density and welfare in farmed rainbow trout. J. Fish. Biol. 61 (3), 493–531.
Elsayed, B.B., Khalafalla, M.M.E., El-Hais, A.M.A., 2012. Use of spirulina (*Arthrospira fusiformis*) for promoting growth of Nile Tilapia fingerlings. Afr. J. Microbiol. Res. 6 (35), 6423–6431.
El-Sayed, A.F.M., 2002. Effects of stocking density and feeding levels on growth and feed efficiency of Nile tilapia (*Oreochromis niloticus* L.) fry. Aquac. Res. 33, 621–626.
Elseady, Y., Zahran, E., 2013. Ameliorating effect of β-carotene on antioxidant response and hematological parameters of mercuric chloride toxicity in Nile tilapia (*Oreochromis niloticus*). Fish. Physiol. Biochem. 39, 1031–1041.
Eroglu, A., Al'Abri, I., Kopec, R.E., Crook, N., Bohn, T. (2022). Carotenoids and their health benefits as derived via their interactions with gut microbiota. Advances in Nutrition.
Evans, M.D., Cooke, M.S., 2004. Factors contributing to the outcome of oxidative damage to nucleic acids. Bioessays 26, 533–542.
Fauji, H., Budiardi, T., Ekasari, J., 2018. Growth performance and robustness of African catfish *Clarias gariepinus* (Burchell) in biofloc-based nursery production with different stocking densities. Aquac. Res. 49 (3), 1339–1346.
Fazio, F., 2019. Fish hematology analysis as an important tool of aquaculture: a review. Aquaculture 500, 237–242.
Gibtan, A., Getahun, A., Mengistou, S., 2008. Effect of stocking density on the growth performance and yield of Nile tilapia (*Oreochromis niloticus* (L., 1758)] in a cage culture system in Lake Kuriftu, Ethiopia. Aquac. Res. 39 (13), 1450–1460.
Giustarini, D., Dalle-Donne, I., Milzani, A., Fanti, P., Rossi, R., 2013. Analysis of GSH and GSSG after derivatization with N-ethylmaleimide. Nat. Protoc. 8 (9), 1660–1669.
Gouveia, L., Choubert, G., Gomes, E., Rema, P., Empis, J., 1998. Use of *Chlorella vulgaris* as a carotenoid source for rainbow trout: effect of dietary lipid content on pigmentation, digestibility and retention in the muscle tissue. Aquac. Int. 6 (4), 269–279.
Guzmán-Villanueva, L.T., Tovar-Ramírez, D., Gisbert, E., Cordero, H., Guardiola, F.A., Cuesta, A., et al., 2014. Dietary administration of β-1, 3/1, 6-glucan and probiotic strain *Shewanella putrefaciens*, single or combined, on gilthead seabream growth, immune responses and gene expression. Fish. Shellfish Immunol. 39, 34–41.
Halliwell, B.E., Gutteridge, J.M.C., 1989. Lipid peroxidation: a radical chain reaction. Free Radicals in Biology and Medicine, 2nd ed. Oxford University Press,, New York, pp. 188–218.
Halliwell, B., Aruoma, O.I., 1991. DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. FEBS Lett. 281, 9–19.
Hassaan, M.S., Soltan, M.A., Abdel-Moez, A.M., 2015. Nutritive value of soybean meal after solid state fermentation with Saccharomyces cerevisiae for Nile tilapia, Oreochromis niloticus. Anim. Feed Sci. Technol. *201*, 89–98.
Hassaan, M.S., Soltan, M.A., Mohammady, E.Y., Elashry, M.A., El-Haroun, E.R., Davies, S.J., 2018. Growth and physiological responses of Nile tilapia, Oreochromis niloticus fed dietary fermented sunflower meal inoculated with Saccharomyces cerevisiae and Bacillus subtilis. Aquaculture 495, 592–601.
Hassaan, M.S., El-Sayed, A.I.M., Soltan, M.A., Iraqi, M.M., Goda, A.M., Davies, S.J., El-Haroun, E.R., Ramadan, H.A., 2019. Partial dietary fish meal replacement with cotton seed meal and supplementation with exogenous protease alters growth, feed performance, hematological indices and associated gene expression markers (GH, IGF-I) for Nile tilapia, Oreochromis niloticus. Aquaculture 503, 282–292.
Hassaan, M.S., Mohammady, E.Y., Soaudy, M.R., Palma, J., Shawer, E.E., El-Haroun, E., 2020. The effect of dietary sericite on growth performance, digestive enzymes activity, gut microbiota and haematological parameters of Nile tilapia, *Oreochromis niloticus* (L.) fingerlings. Anim. Feed Sci. Technol. 262, 114400.
Harikrishnan, R., Kim, M.C., Kim, J.S., Han, Y.J., Jang, I.S., Balasundaram, C., Heo, M.S., 2011. Immunomodulatory effect of sodium alginate enriched diet in kelp grouper Epinephelus brneus against *Streptococcus iniae*. Fish. Shellfish Immunol. 30 (2), 543–549.
Harikrishnan, R., Devi, G., Van Doan, H., Balasundaram, C., Arockiaraj, J., Jagruthi, C., 2021a. Efficacy of ulvan on immune response and immuno-antioxidant gene modulation in Labeo rohita against columnaris disease. Fish. Shellfish Immunol. 117, 262–273.
Harikrishnan, R., Devi, G., Van Doan, H., Balamurugan, P., Arockiaraj, J., Balasundaram, C., 2021b. Hepatic antioxidant activity, immunomodulation, and pro-anti-inflammatory cytokines manipulation of κ-carrageenan (κ-CGN) in cobia, *Rachycentron canadum* against *Lactococcus garvieae*. Fish. Shellfish Immunol. 119, 128–144.
Hassaan, M.S., Mohammady, E.Y., Soaudy, M.R., Sabae, S.A., Mahmoud, A.M., El-Haroun, E.R., 2021. Comparative study on the effect of dietary β-carotene and phycocyanin extracted from *Spirulina platensis* on immune-oxidative stress biomarkers, genes expression and intestinal enzymes, serum biochemical in Nile tilapia, *Oreochromis niloticus*. Fish. Shellfish Immunol. 108, 63–72.

Haylor, G., 1991. Controlled hatchery production of *Clarias gariepinus* (Burchell, 1922): growth and survival of fry at high stocking density. Aquac. Fish. Manag. 22, 405–422.
Henry, R.J., 1964. Colorimetric determination of total protein. Clinical Chemistry. Harper and Row Publ,, New York.
Hoseini, S.M., Mirghaed, A.T., Ghelichpour, M., Pagheh, E., Iri, Y., Kor, A., 2020. Effects of dietary tryptophan supplementation and stocking density on growth performance and stress responses in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 519, 734908.
Hou, J., et al., 2015. Hepatic positive and negative antioxidant responses in zebrafish after intraperitoneal administration of toxic microcystin-LR. Chemosphere 120, 729–736.
Hu, C.J., Chen, S.M., Pan, C.H., Huang, C.H., 2006. Effects of dietary vitamin A or β-carotene concentrations on growth of juvenile hybrid tilapia, *Oreochromis niloticus*× *O. aureus*. Aquaculture 253 (1-4), 602–607.
Huntingford, F.A., Adams, C., Braithwaite, V., Kadri, S., Pottinger, T., Sandøe, P., Turnbull, J., 2006. Current issues in fish welfare. J. Fish. Biol. 68, 332–372.
Hwang, D., Lin, T., 2002. Effect of temperature on dietary vitamin C requirement and lipid in common carp. Comp. Biochem. Physiol. Bbiochem. Mol. Biol. 131, 1–7.
Ibrahim, M.S., El-gendy, G.M., Ahmed, A.I., Elharoun, E.R., Hassaan, M.S., 2021. Nanoselenium versus bulk selenium as a dietary supplement: Effects on growth, feed efficiency, intestinal histology, haemato-biochemical and oxidative stress biomarkers in Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) fingerlings. Aquac. Res. 52 (11), 5642–5655.
Ibrahim, M.S., Mohammady, E.Y., EL-Erian, M.A., Ragaza, J.A., El-Haroun, E.R., Hassaan, M.S., 2023. Dietary Zinc Oxide for Growth and Immune Stimulation of Aquatic Animals Species: A Review. In Proceedings of the Zoological Society. Springer India,, New Delhi, pp. 1–14.
Jang, H.N., Woo, J.K., Cho, Y.H., Kyong, S.B., Choi, S.H., 2004. Characterization of monoclonal antibodies against heavy and light chains of flounder (*Paralichthys olivaceus*) immunoglobulin. BMB Rep. 37 (3), 314–319.
Jagruthi, C., Yogeshwari, G., Anbazahan, S.M.M., Mari, L.S.S., Arockiaraj, J., Mariappan, P., Sudhakar, G.R.L., Balasundaram, C., Harikrishnan, R., 2014. Effect of dietary astaxanthin against *Aeromonas hydrophila* infection in common carp, *Cyprinus carpio*. Fish. Shellfish Immunol. 41 (2), 674–680.
Jeney, G., Galeotti, M., Volpatti, D., Jeney, Z., Anderson, D.P., 1997. Prevention of stress in rainbow trout (*Oncorhynchus mykiss*) fed diets containing different doses of glucan. Aquaculture 154, 1–15.
Jewel, M.A.S., Ara, J., Haque, M.A., Hossain, M.A., Md Noor, N., Simon Kumar Das, S.K., 2023. Effect of stocking density on the growth, body composition, and blood parameters of cage-reared Gangetic mystus catfish (*Mystus cavasius*). Aquac. Rep. 28, 101428 https://doi.org/10.1016/j.aqrep.2022.101428.
Jørgensen, E., Christiansen, J., Jobling, M., 1993. Effects of stocking density on food intake, growth performance and oxygen consumption in Arctic charr (*Salvelinusalpinus*). Aquaculture 110, 191–204.
Karnatak, G., Das, B.K., Mishal, P., Tayung, T., Kumari, S., Sarkar, U.K., Ali, Y., 2021. Impact of stocking density on growth, feed utilization and survival of cage reared minor carp, *Labeo bata* (Hamilton, 1822) in Maithon reservoir, India. Aquaculture 532, 736078.
Kavitha, C., Ramesh, M., Kumaran, S.S., Lakshmi, S.A., 2012. Toxicity of Moringa oleifera seed extract on some hematological and biochemical profiles in a freshwater fish, *Cyprinus carpio*. Exp. Toxicol. Pathol. 64 (7-8), 681–687.
Kebus, M.J., Collins, M.T., Brownfield, M.S., Amundson, C.H., Kayes, T.B., Malison, J.A., 1992. Effect of rearing density on the stress response and growth of rainbow trout. J. Aquat. Anim. Health 4, 1–6.
Kim, J.S., Harikrishnan, R., Kim, M.C., Balasundaram, C., Heo, M.S., 2010. Dietary administration of Zooshikella sp. enhance the innate immune response and disease resistance of *Paralichthys olivaceus* against Sreptococcus iniae. Fish. Shellfish Immunol. 29 (1), 104–110.
Kiokias, S., Proestos, C., Oreopoulou, V., 2018. Effect of natural food antioxidants against LDL and DNA oxidative changes. Antioxidants 7, 133.
Kucukbay, F.Z., Yazlak, H., Karaca, I., Sahin, N., Tuzcu, M., Cakmak, M.N., Sahin, K., 2009. The effects of dietary organic or inorganic selenium in rainbow trout (*Oncorhynchus mykiss*) under crowding conditions. Aquac. Nutr. 15, 569–576.
Lambert, Y., Dutil, J.D., 2001. Food intake and growth of adult Atlantic cod (*Gadus morhua* L.) reared under different conditions of stocking density, feeding frequency and size-grading. Aquaculture 192 (2-4), 233–247.
Lambris, J.D., 1989. The third component of the complement system. Chemistry and Biology. Curr. Top. Microbiol. Immunol. 153, 45–73.
Lin, Y.C., Tayag, C.M., Huang, C.L., Tsui, W.C., Chen, J.C., 2010. White shrimp *Litopenaeus vannamei* that had received the hotwater extract of *Spirulina platensis* showed earlier recovery in immunity and up-regulation of gene expressions after pH stress. Fish. Shellfish Immunol. 29 (6), 1092–1098.
Liu, F., Qu, Y.K., Wang, A.M., Yu, Y.B., Yang, W.P., Lv, F., Nie, Q., 2019. Effects of carotenoids on the growth performance, biochemical parameters, immune responses and disease resistance of yellow catfish (*Pelteobagrus fulvidraco*) under high-temperature stress. Aquaculture 503, 293–303.
Liu, C., Liu, H., Han, D., Xie, S., Jin, J., Yang, Y., Xiaoming Zhu, X., 2020. Effects of dietary *Arthrospira platensis* supplementation on the growth performance, antioxidation and immune related-gene expression in yellow catfish (*Pelteobagrus fulvidraco*). Aquac. Rep. 17, 100297.
Long, L., Zhang, H., Ni, Q., Liu, H., Wu, F., Wang, X., 2019. Effects of stocking density on growth, stress, and immune responses of juvenile Chinese sturgeon (*Acipenser sinensis*) in a recirculating aquaculture system. Comp. Biochem. Physiol. Part - C: Toxicol. Pharmacol. 219, 25–34.
Lovell, T., 1989. Nutrition and Feeding of Fish. An AVI Book-Van Nostrand Reinhold,, New York, NY, USA, 260pp.
Low, C., Wadsworth, S., Burrells, C., Secombes, C.J., 2003. Expression of immune genes in turbot (*Scophthalmus maximus*) fed a nucleotide-supplemented diet. Aquaculture 221, 23–40.
Lupatsch, I., Santos, G.A., Schrama, J.W., Verreth, J.A.J., 2010. Effect of stocking density and feeding level on energy expenditure and stress responsiveness in European sea bass *Dicentrarchus labrax*. Aquaculture 298 (3-4), 245–250.
Mahmoud, H.K., Al-Sagheer, A.A., Reda, F.M., 2017. Dietary curcumin supplement influence on growth, immunity, antioxidant status, and resistance to Aeromonas hydrophila in *Oreochromis niloticus*. Aquaculture 475, 16–23. ⟨https://doi. org/10.1016/j.aquaculture.2017.03.043⟩.
Mahmoud, M.M.A., et al., 2018. Spirulina (*Arthrospira platensis*) supplementation improves growth performance, feed utilization, immune response, and relieves oxidative stress in Nile tilapia (*Oreochromis niloticus*) challenged with *Pseudomonas fluorescens*. Fish. Shellfish Immunol. 72, 291–300.
Martin, R., Wertheimer, A., 1989. Adult production of chinook salmon reared at different densities and released as two small sizes. Progress. Fish. Cult. 51, 194–200.
Mehdi, N., Masood, A., Behzan, N.H., Ali, A., Mosa, Z.D., Hamid, F.L., 2010. Physiological stress responses in Kutum *Rutilus frisii kutum* subjected to captivity. Int. Aquat. Res. 2, 55–60.
Mirghaed, A.T., Ghelichpour, M., Hoseini, S.M., Amini, K., 2017. Hemolysis interference in measuring fish plasma biochemical indicators. Fish. Physiol. Biochem. 43 (4), 1143–1151.
Moawad, D.A., El-Garhy, H.A., Refaat, M.H., Hassaan, M.S., 2024. Protecting deleterious effects of high stocking density of Nile tilapia, Oreochromis niloticus using dietary microalgae *Golenkinia longispicula*: Growth, immune-oxidative markers and associated gene expression. Aquaculture 581, 740430.
Mohammady, E.Y., Soaudy, M.R., Abdel-Rahman, A., Abdel-Tawwab, M., Hassaan, M.S., 2021. Comparative effects of dietary zinc forms on performance, immunity, and oxidative stress-related gene expression in Nile tilapia, Oreochromis niloticus. Aquaculture 532, 736006.
Mohammady, E.Y., Soaudy, M.R., Mohamed, A.E., EL-Erian, M.M.A., Farag, A., Badr, A. M., Bassuony, Nemat I., Ragaza, Janice A., El-Haroun, Ehab R., Hassaan, M.S., 2022. Can dietary phytogenic mixture improve performance for growth, digestive enzyme activity, blood parameters, and antioxidant and related gene expressions of Nile tilapia, Oreochromis niloticus? Anim. Feed Sci. Technol. 290, 115369.
Mohammady, E.Y., Aboseif, A.M., Soaudy, M.R., Ramadan, E.A., Hassaan, M.S., 2023. Appraisal of fermented wheat bran by Saccharomyces cerevisiae on growth, feed utilization, blood indices, intestinal and liver histology of Nile tilapia, *Oreochromis niloticus*. Aquaculture, 739755.
Moin, V.M., 1986. A simple and specific method for determining glutathione peroxidase activity in erythrocytes. Lab Delo 12, 724–727.
Montero, D., Izquierdo, M.S., Tort, L., Robaina, L., Vergara, J.M., 1999. High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. Fish. Physiol. Biochem. 20 (1), 53–60.
Naderi, M., Keyvanshokooh, S., Ghaedi, A., Salati, A.P., 2019. Interactive effects of dietary Nano selenium and vitamin E on growth, haematology, innate immune responses, antioxidant status and muscle composition of rainbow trout under high rearing density. Aquac. Nutr. 25, 1156–1168.
Nakono, T., Yamaguchi, T., Sato, M., Iwama, G.K., 2003. Biological Effects of Carotenoids in Fish, Effective Utilization of Marine Food Resource, Songkhla, Thailand.
Ni, M., Wen, H., Li, J., Chi, M., Bu, Y., Ren, Y., Ding, H., 2014. The physiological performance and immune responses of juvenile Amur sturgeon (*Acipenser schrenckii*) to stocking density and hypoxia stress. Fish. Shellfish Immunol. 36 (2), 325–335.
Ni, M., Wen, H., Li, J., Chi, M., Bu, Y., Ren, Y., Ding, H., 2016. Effects of stocking density on mortality, growth and physiology of juvenile Amur sturgeon (*Acipenser schrenckii*). Aquac. Res. 47 (5), 1596–1604.
Omar, S.S., Anwar, A.Y., Hassaan, M.S., El-Haroun, E., Davies, S.J., 2022. Preliminary assessment of a novel fermented wheat protein concentrate from a bio-distillation source as a dietary ingredient contribution for tilapia (Oreochromis niloticus). Aquac. Rep. *24*, 101101.
Ouattara, N.I., Teugels, G.G., N'Douba, V., Philippart, J.C., 2003. Aquaculture potential of the black-chinned tilapia, *Sarotherodon melanotheron* (*Cichlidae*). Comparative study of the effect of stocking density on growth performance of landlocked and natural populations under cage culture conditions in Lake Ayame (Côte d'Ivoire). Aquac. Res. 34, 1223–1229.
Pan, C.H., Chien, Y.H., Wang, Y.J., 2010. The antioxidant capacity response to hypoxia stress during transportation of characins (*Hyphessobrycon callistus* Boulenger) fed diets supplemented with carotenoids. Aquac. Res. 41, 973–981.
Peskin, A.V., Winterbourn, C.C., 2000. A microtiter plate assay for superoxide dismutase using a water-soluble tetrazolium salt (WST-1). Clin. Chim. Acta 293 (1–2), 157–166.
Pham, M.A., Byun, H.G., Kim, K.D., Lee, S.M., 2014. Effects of dietary carotenoid source and level on growth, skin pigmentation, antioxidant activity and chemical composition of juvenile olive flounder *Paralichthys olivaceus*. Aquaculture 431, 65–72.
Preeti, M., Seema, G., 2014. Modulatory effect of eclipta alba on biochemical parameters of catfish, *Clarias. batrachus*. World J. Pharm. Res. 3 (4), 1223–1233.
Rafatnezhad, S., Falahatkar, B., Gilani, M.H.T., 2008. Effects of stocking density on haematological parameters, growth and fin erosion of great sturgeon (*Huso huso*) juveniles. Aquac. Res. 39 (14), 1506–1513.
Rahman, M.M., Mondal, D.K., Amin, M.R., Muktadir, M.G., 2016. Impact of stocking density on growth and production performance of monosex Tilapia (*Oreochromis*

niloticus) in Ponds. Asian J. Med. Biol. Res. 2, 471–476. https://doi.org/10.3329/ajmbr.v2i3.30120.
Rawling, M.D., Merrifield, D.L., Davies, S.J., 2009. Preliminary assessment of dietary supplementation of Sangrovit® on red tilapia (*Oreochromis niloticus*) growth performance and health. Aquaculture 294, 118–122.
Reitman, A., Frankel, S., 1957. Determination of aspartate glutamic aminotransferase and alanine aminotransferase. Am. J. Clin. Pathol. 28, 56.
Rosenfeld, G., 1947. Dye pancrômico for hematology and clinical cytology. New combinaçço components of the May-Grünwald and Giemsa in one quick job coranate. Mem. Inst. Butan 20, 329–334.
Rotllant, J., Pavlidis, M., Kentouri, M., Abad, M.E., Tort, L., 1997. Non-specific immune responses in the red porgy *Pagrus pagrus* after crowding stress. Aquaculture 156, 279–290.
Sachin, O.K., Shanthanagouda, A.H., Vijay, K.R.S., 2020. Influence of Stocking Density on Growth and Physiological Responses of Nile tilapia (GIFT Strain) in Cages. J. Exp. Zool. 23, 731–735.
Sagar, V., Sahu, N.P., Pal, A.K., Hassaan, M., Jain, K.K., Salim, H.S., Kumar, Vikas, El-Haroun, E.R., 2019. Effect of different stock types and dietary protein levels on key enzyme activities of glycolysis-gluconeogenesis, the pentose phosphate pathway and amino acid metabolism in Macrobrachium rosenbergii. J. Appl. Ichthyol. *35* (4), 1016–1024.
Saillant, E., Fostier, A., Haffray, P., Menu, B., Laureau, S., Thimonier, J., Chatain, B., 2003. Effects of rearing density, size grading and parental factors on sex ratios of the sea bass (*Dicentrarchus labrax* L.) in intensive aquaculture. Aquaculture 221 (1-4), 183–206.
Sallam, A.E., Mansour, A.T., Srour, T.M., Goda, A.M.A., 2017. Effects of different carotenoid supplementation sources with or without sodium taurocholate on growth, feed utilization, carotenoid content and antioxidant status in fry of the European seabass, *Dicentrarchus labrax*. Aquac. Res. 48, 3848–3858.
Shved, N., Berishvili, G., Mazel, P., Baroiller, J.-F., Eppler, E., 2011. Growth hormone (GH) treatment acts on the endocrine and autocrine/paracrine GH/IGF-axis and on TNF-α expression in bony fish pituitary and immune organs. Fish. Shellfish Immunol. 31 (6), 944–952.
Silva P., Souza V., Padua D., Dalacorte P., Goncalves D., 2000. Effects of stocking density on growth and fillet composition of tetra hybrid red tilapia, Israeli strain. In: Tilapia Aquaculture in the 21st Century: Proceedings From 5th International Symposium on Tilapia Aquaculture (ed. by K. Fitzsimmons & C. Filho), pp. 341– 345. The world aquaculture society, FL, USA.
Secombes, C., Wang, T., Hong, S., Peddie, S., Crampe, M., Laing, K., et al., 2001. Cytokines and innate immunity of fish. Dev. Comp. Immunol. 25, 713–723.
Staurnes, M., Sigholta, T., Pedersenb, P.H., Rustad, T., 1994. Physiological effects of simulated high-density transport of Atlantic cod (*Gadus morhua*). Aquaculture 119 (4), 381–391.
Sumida, S., Tanaka, K., Kitao, H., Nakadomo, F., 1989. Exercise-induced lipid peroxidation and leakage of enzymes before and after vitamin E supplementation. Int. J. Biochem. 21, 835–838.
Sun, Y.J., Qiao, L.P., Zhong, L.Z., Ye, X.Q., 2012. Recent advances of carotenoids bioactivities. J. Chin. Inst. Food Sci. Technol. 12, 160–166.
Sunyer, J.O., Gómez, E., Tort, L., Navarro, V., Quesada, J., 1995. Physiological responses and depression of humoral components of the immune system in gilthead sea bream (*Sparus aurata*) following daily acute stress. Can. J. Fish. Aquat. Sci. 52, 2339–2346.
Taalab, H.A., Mohammady, E.Y., Hassan, T.M., Abdella, M.M., Hassaan, M.S., 2022. β-Carotene of *Arthrospira platensis* versus vitamin C and vitamin E as a feed supplement: Effects on growth, haemato-biochemical, immune-oxidative stress and related gene expression of Nile tilapia fingerlings. Aquac. Res. 53 (13), 4832–4846.
Tadesse, A., 2007. The effect of stocking density and supplementary feeding on growth performance of Nile tilapia [*Oreochromis niloticus* (L., 1758)] in cage culture system in Lake Elen, Ethiopia (Doctoral dissertation, MSc Thesis, Addis Ababa University, Ethiopia).
Tayag, C.M., Lin, Y.-C., Li, C.-C., Liou, C.-H., Chen, J.-C., 2010. Administration of the hot-water extract of *Spirulina platensis* enhanced the immune response of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus*. Fish. Shellfish Immunol. 28 (5), 764–773.
Tibaldi, E., Zittelli, G.C., Parisi, G., Bruno, M., Giorgi, G., Tulli, F., Poli, B.M., 2015. Growth performance and quality traits of European sea bass (*D. labrax*) fed diets including increasing levels of freeze-dried Isochrysis sp.(T-ISO) biomass as a source of protein and n-3 long chain PUFA in partial substitution of fish derivatives. Aquaculture 440, 60–68.
Trenzado, C.E., Morales, A.E., De-La Higuera, M., 2008. Physiological changes in rainbow trout held under crowded conditions and fed diets with different levels of vitamins E and C and highly unsaturated fatty acids (HUFA). Aquaculture 277, 293–302.
Turnbull, J., Bell, A., Adams, C., Bron, J., Huntingford, F., 2005. Stocking density and welfare of cage farmed Atlantic salmon: application of a multivariate analysis. Aquaculture 243 (1-4), 121–132.
Wang, Y.J., Chien, Y.H., Pan, C.H., 2006. Effects of dietary supplementation of carotenoids on survival, growth, pigmentation, and antioxidant capacity of characins, *Hyphessobrycon callistus*. Aquaculture 261, 641–648.
Watanuki, H., et al., 2006. Immunostimulant effects of dietary *Spirulina platensis* on carp, *Cyprinus carpio*. Aquaculture 258 (1–4), 157–163.
Wotton, I.D., Freeman, H., 1982. Micro analysis in Medical Biochemistry. Churchill,, New York, USA.
Yadata, G.W., Ji, K., Liang, H., Ren, M., Ge, X., Yang, Q., 2020. Effects of dietary protein levels with various stocking density on growth performance, whole body composition, plasma parameters, nitrogen emission and gene expression related to TOR signaling of juvenile blunt snout bream (*Megalobrama ambylcephala*). Aquaculture 519, 734730.
Yilmaz, S., 2019. Effects of dietary blackberry syrup supplement on growth performance, antioxidant, and immunological responses, and resistance of Nile tilapia, *Oreochromis niloticus* to *Plesiomonas shigelloides*. Fish. Shellfish Immunol. 84, 1125–1133.
Yu, W., Wen, G., Lin, H., Yang, Y., Huang, X., Zhou, C., Li, T., 2018. Effects of dietary *Spirulina platensis* on growth performance, hematological and serum biochemical parameters, hepatic antioxidant status, immune responses and disease resistance of Coral trout *Plectropomus leopardus* (Lacepede, 1802). Fish. Shellfish Immunol. 74, 649–655.
Zar, J.H., 1984. Biostatistical Analysis. Prentice-Hall, Englewood Cliff,, NJ, USA.

Further reading

Goswami, U.C., 1993. Metabolism of carotenoids in freshwater fish:(i) biogenesis of 3-4 dehydroretinol,(ii) supplementation of carotenoids with fish food for better survival and growth. 10th Int. Symp. . Carotenoids, Trondheim 20–25.
Herrero-Martínez, J.M., Eeltink, S., Schoenmakers, P.J., Kok, W.T., Ramis-Ramos, G., 2006. Determination of major carotenoids in vegetables by capillary electrochromatography. J. Sep. Sci. 29 (5), 660–665.
Liu, B., Xu, P., Xie, J., Ge, X., Xia, S., Song, C., Zhou, Q., Miao, L., Ren, M., Pan, L., Chen, R., 2014. Effects of emodin and vitamin E on the growth and crowding stress of Wuchang bream (*Megalobrama amblycephala*). Fish. Shellfish Immunol. 40, 595–602.
Rao, A.V., Agarwal, S., 1999. Role of lycopene as antioxidant carotenoid in the prevention of chronic diseases: a review. Nutr. Res. 19, 305–323.
Statistical, S.A.S., 1993. Analysis system. SAS/STAT User Guide Release 6, 03 edition. SAS Institute Inc,, Cary, North Carolina, USA.