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Separate and combined effects of *Spirulina platensis* and Pot Marigold (*Calendula officinalis*) powder on the growth performance, carcass composition, intestinal enzyme activities, immune response, and antioxidant defense in Macro fish (*Labidochromis caeruleus*)

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Abstract

Salary, O. B., Mohammadizadeh, F., Bahri, A., Salarzadeh, A. & Yahyavi, M. (2026). Separate and combined effects of *Spirulina platensis* and Pot Marigold (*Calendula officinalis*) powder on the growth performance, carcass composition, intestinal enzyme activities, immune response, and antioxidant defense in Macro fish (*Labidochromis caeruleus*). *Bulg. J. Agric. Sci., 32*(5), 1203–1214

This study investigated the impact of *Spirulina platensis* and pot marigold (*Calendula officinalis*) powder both individually and in combination, on various aspects of growth performance, carcass composition, immune response, intestinal enzymes, antioxidant defence, and astaxanthin levels in Macro fish (*Labidochromis caeruleus*) tissue. 240 Macro fish, with an average weight of 3.68±0.1 g, were randomly assigned to 12 aquariums, which were then divided into four treatment groups, as follows: control (standard feed without additives), SP (25 g/kg *Spirulina* powder added to the basal diet), MG (25 g/kg pot marigold powder added to the basal diet), and SP+MG (25 g of *C. officinalis* powder and 25 g of Spirulina per kg of basal diet). Within each group, three replications of 60 fish were maintained. The combination of SP and MG showed superior results in terms of specific growth rate (SGR), final weight, weight gain, and astaxanthin content. Furthermore, the SP+MG treatment led to improvements in hematological parameters (lymphocyte percentage, red blood cells (RBC) count, and hematocrit level) and immune factors (IgM concentration, total protein level, and lysozyme activity). It also increased crude protein levels in the carcass and antioxidant enzyme activities (catalase, superoxide dismutase, and glutathione peroxidase). Notably, the digestive enzyme activity in the SP+MG treatment differed significantly from the other groups. These findings suggest that the combined use of *S. platensis* and Pot Marigold powder could be a beneficial feed supplement in the diets of *L. caeruleus*.

Keywords: antioxidant; *Calendula officinalis*; immunity; *Labidochromis caeruleus*; pigmentation; *Spirulina platensis*

Introduction

In recent decades, breeding ornamental fish has experienced significant growth due to advancements in aquarium equipment and increased interest in keeping them as pets for their beauty in shape and color. Ornamental fish production is an essential part of the aquaculture industry, with over 7 000 species being bred and supplied to the market. It is estimated that of the total number of species, approximately 5 000 are freshwater, and 1 800 are marine (Hoseinifar et

al., 2023). The largest producers of ornamental fish in Iran are the provinces of Tehran, Alborz, Ardabil, Qazvin, and Markazi (Hoseinifar et al., 2023). This thriving industry also faces challenges, as reported mortality rates in the trade of ornamental fish vary significantly, ranging from 2 to 73 percent (Stevens et al., 2017). This potentially indicates a significant economic cost burden on this industry.

As with all other animals held in captivity, ornamental fish are exposed to stress factors that can affect their immune system, nutrition, growth, coloration, and survival. However, limited information on the nutritional needs of ornamental fish has led to the use of data from non-ornamental fish species, resulting in poor performance, coloration, and survival rates in the ornamental fish industry (Hoseinifar et al., 2023). Therefore, the use of nutritional supplements that can improve growth performance, health, coloration, and resistance to environmental stressors in ornamental fish would be beneficial (Hoseinifar et al., 2023).

Skin color in fish is primarily due to the presence of specialized cells in the skin called chromatophores. In ornamental fish, color serves as a tool for attracting mates and other social behaviors. Additionally, the pigment pattern is a key indicator of the health, quality, and marketability of ornamental fish (Hoseinifar et al., 2023). To enhance skin coloration, a wide range of artificial and natural carotenoids are used as dietary supplements in aquaculture (Bisht et al., 2023). Astaxanthin is the most important carotenoid pigment in aquatic organisms, controlling various biological functions such as preventing oxidation of essential unsaturated fatty acids, protection against the negative effects of ultraviolet radiation, immune indicators, and reproduction control (Hamrang Omshi et al., 2019). The cost of astaxanthin has led to the use of other natural substitutes that can be utilized as a cost-effective source of astaxanthin in feed.

In the category of compounds that contain these carotenoids, Spirulina and marigold represent notable examples (Hamrang Omshi et al., 2019). Spirulina is a strain of cyanobacteria that can be used in aquaculture due to its nutritional benefits such as high levels of protein, vitamins, essential amino acids, minerals, essential fatty acids, and antioxidant pigments like carotenoids (beta-carotene, astaxanthin, lutein, cryptoxanthin, and zeaxanthin) (Adel et al., 2016). It has been shown to have positive effects on the growth and health of fish species, particularly ornamental fish like zebrafish (Lo Cascio et al., 2017) and gourami (*Trichogaster lalius*) (Biabani Asrami et al., 2020). Additionally, Spirulina is known to have antioxidant properties, which can help reduce oxidative stress and improve overall health in fish species. By incorporating Spirulina into their diets, fish can experience improved digestion and nutrient absorption, leading to better overall health and performance.

Marigold (*Calendula officinalis*) has been shown to possess a variety of activities such as antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, pain relief, liver and heart protection, wound healing, among other effects (Manzoor et al., 2022). Marigold is a flower that is commonly used in fish diets to improve skin and scale pigmentation. Marigold contains a compound called lutein, which is a natural pigment that can enhance the coloration of fish, particularly in species like goldfish (Villar-Martínez et al., 2013) and blue gourami (*Trichogaster trichopterus*) (Jorjani et al., 2019). In addition to enhancing color, marigold also contains antioxidant properties that can help boost immune function and protect fish from oxidative damage (Ghafarifarsani et al., 2023). The incorporation of marigold within the diet of fish species has enhanced their aesthetic, characterized by a more vibrant colouration, also experience improved overall health and resilience to diseases (Ghafarifarsani et al., 2023). Investigating the synergistic effects of these natural additives could improve the overall well-being and aesthetic appeal of macro fish in aquaculture. By determining how Spirulina and marigold complement each other, we can develop more effective feeding plans for this fish species. The objective of this study was to evaluate the effects of *S. platensis* and pot marigold (*C. officinalis*) powder, both individually and in combination, on the growth performance, carcass composition, immune response, intestinal enzyme activity, antioxidant defense mechanisms, and astaxanthin levels in the tissues of Macro fish.

Materials and Methods

Fish and breeding conditions

A total of 240 healthy Macro fish, averaging 3.68 ± 0.1 g in weight and measuring 4.5 ± 0.2 cm in length, were procured from a local ornamental fish farm in Bandar Abbas. They were transported to the Marine and Fisheries Technologies Research Center (Bandar Abbas branch, Islamic Azad University, Bandar Abbas, Iran), in a thick plastic bag containing two-thirds water and one-third air. The fish were acclimated to the laboratory conditions for 14 days and divided into 12 aquariums (60×40×30 cm). Fish were fed with the basal diet (Table 1), which contained 58% protein, 15% fat, 0.5% fiber, 2.5% ash, and 9% moisture. Aeration was provided equally to all aquariums through a central pump and stones. The water parameters during the experiment were as follows: temperature 28 ± 1.0°C, pH 7.2 ± 0.5, water hardness 104 mg/l of calcium carbonate, and dissolved oxygen level 7.8 ± 0.1 mg/l. Throughout the experiment, daily water changes were conducted at a rate of 30% of the tank water.

Table 1. Dietary formulation and proximate composition of basal diet

Ingredients	Composition (% dry matter Basis)
Fish meal	40
Wheat flour	18
Gluten	5.5
Soy bean meal	13.5
Soy bean oil	5.8
Fish oil	6.0
Mineral premix [a]	3
Vitamin premix [a]	2
Binder [b]	2
Antifungi [c]	0.5
Antioxidant [d]	0.5

[a]Premix detailed by Dernekbasi et al. (2010); [b]Ametbinder™, MehrTaban-e-Yazd, Iran; [c]ToxiBan antifungal (Vet-A-Mix, Shenan- doah, IA); [d]Butylated hydroxytoluene (BHT) (Merck, Germany).
Source: Authors' own elaboration

Animal experimental protocol and institutional approvals

Approval was granted for this experimental work from the Marine and Fisheries Technologies Research Center (Bandar Abbas Branch, Islamic Azad University) Ethical Review Board, under internal documentation codes of practice. They were modelled on the European, UK and Canadian legislation Acts. At all stages experimental stock were monitored for any behavioral changes. If potentially moribund fish were observed and reached a threshold of excessive abnormal swimming and evidence of respiratory stress, they were euthanized with anaesthetic overdose of clove oil (200 mg/L) until sessile, and subsequently a sharp blow was applied to the dorsal region of the head (cranial percussion), followed by brain destruction with insertion of a seeker to destroy neural mass. This was a Schedule 1 method similar to guidelines of humane killing of fish under the UK Animal Scientific Procedures Act of 1986.

Experimental diet

The *Spirulina platensis* powder was obtained from the department of fisheries at the Caspian Sea Ecology Research center (Sari, Iran), while the pot marigold (*Calendula officinalis*) flower powder was purchased from Giah Kala Company (Tehran, Iran). Prior to use, both powders were sieved through a 100-micron sieve.

The standard diet was combined with *S. platensis* and/or pot marigold flower at suitable concentrations to create four distinct experimental diets: a control diet (C) (without any additives), a diet with Spirulina (SP) (25 g/kg of Spirulina powder mixed into the basal diet), a diet with pot marigold flower (MF) (25 g/kg of Calendula flower powder added to the basal diet), and a combined diet (SPMF) (which included both 25 g of Calendula flower powder and 25 g of Spirulina per kg of basal diet). *S. platensis* and/or pot marigold flowers were weighed aseptically and mixed with the required amount of sterile water while stirring continuously until fully dissolved. This mixture was then evenly sprayed onto the basal diet. In the control treatment, the same volume of water was also added. The enriched diets were subsequently dried in an oven at 55°C, then stored in plastic containers at 4°C in the refrigerator until needed.

Measurement of growth indices and survival rate

In order to determine growth rates and survival rates, all fish were subjected to a 24-h fasting period before being weighed and sampled. The following parameters were assessed at the end of the eight-week feeding trial:

Weight gain: W2 (final weight in g) – W1 (initial weight in g);

Specific growth rate (SGR%/day) = [Ln (final body weight, g) − Ln (initial body weight, g)] × 100/T, where T represents the number of days in the feeding period;

Feed conversion ratio (FCR): feed intake (g)/weight gain (g);

Condition factor (CF): weight of the fish (g)/fish length^3 (cm^3);

Survival rate (%SR): (final number of fish/initial number of fish) ×100.

Measurement of astaxanthin in the fish tissues

Measurement of astaxanthin in the fish tissues was conducted using an assay described by Jorjani et al. (2019). To examine astaxanthin, a solution was prepared by combining methanol, tetrahydrofuran, stannous chloride, distilled water, and hexane. Standard solutions (Sigma Aldrich in St. Louis, MO, and Fluka in Shanghai, China) were then created by diluting this initial standard to achieve varying concentrations ranging from 2.5 to 540 µg/ml. All sample preparations were carried out in sanitized containers and under controlled low-light conditions. For sample preparation, 50 mg of the test material was mixed with 3 ml of chilled stannous chloride, followed by brief vortexing and centrifugation at 1500 rpm for 5 min. Subsequently, 2.5 ml of distilled water and 2 ml of hexane were added to 0.5 ml of the upper layer with additional mixing and centrifugation steps. The upper layer (hexane) was then transferred to a clean tube, into which 250 µg of methanol was introduced. After thorough mixing, 70 µg of the resulting mixture was injected into the HPLC system (Young Lin Acme 9000, China) equipped with an Autochro 2000 injector, UV detector (730D), and

SP930D pump. The mobile phase A consisted of a mixture of methanol and water, while mobile phase B was a blend of methanol, tetrahydrofuran, and water. The HPLC separation conditions included a flow rate of 1.4 ml/min, a detection wavelength of 474 nm, a temperature of 25°C, and a C18 column (Inertsil ODS-3 V250 × 4.6 mm).

Chemical composition of the carcass

At the end of the period, 36 pieces of fish (3 fish from each replicate) were randomly selected and frozen at -20°C to determine the approximate chemical composition of the carcass. The determination of fish body composition in the laboratory was done using the standard AOAC (2005) method. Crude protein was measured using a Kjeldahl apparatus, crude fat by the Soxhlet method, moisture by oven drying at 105 °C for 24 h, and ash content using a muffle furnace at 550 °C for 4 h.

Blood sampling and hematological analysis

After completing the experimental trial, 9 fish were selected from each tank and anesthetized with clove oil (100 mg/l). Blood samples (1 ml) were taken by ablating the caudal fin of the fish with hematocrit tube and divided into two halves. One half was placed in a tube with an anticoagulant (heparin) for hematological analysis, while the other half of serum was put in non-heparinized tubes for assessing antioxidant and immune parameters. Serum samples were obtained by centrifuging the blood at 3000 x g for 10 min and stored at -80 °C (2 h) until needed. Total red blood cells (RBC: 10^6 mm^{-3}) and white blood cells (WBC: 10^3 mm^{-3}) were counted using an improved Neubauer hemocytometer with Hayem and Turck diluting fluids. Hematocrit (Ht %) was determined using the standard microhematocrit method and expressed as a percentage. Hemoglobin (Hb, g dl^{-1}) levels were measured using the cyanomethemoglobin procedure. Additionally, differential leukocyte counts were obtained by preparing Giemsa-stained smears, which were then examined under a light microscope for blood cell counts.

Antioxidant enzyme parameters

The enzyme activity of superoxide dismutase (SOD) was measured using Pajouhan Medical Kits according to the following procedure. 10 µL of serum sample, 250 µL of Solution 1R, 10 µL of Solution 2R, 10 µL of distilled water, and 20 µL of Chromogen were added to the reaction mixture. A blank sample was prepared with distilled water instead of Chromogen. Serum and blank samples were read at 0 and 2 minutes at a wavelength of 420 nm using an analyzer (Heikkila and Cabbat, 1976).

Glutathione peroxidase (GPx) activity was measured according to the method of Lawrence and Burk (1976). In summary, a reaction solution containing potassium phosphate buffer (pH 7), EDTA, sodium azide, NADPH, glutathione reductase, and reduced glutathione was incubated at 25 °C for 5 min. Then, H_2O_2 and the serum sample were added to the assay solution, and the absorbance at 340 nm was read for 1 min. The decrease in absorbance at 340 nm was monitored using a spectrophotometer to calculate GPx activity.

The activity of catalase was assessed using the method described by Goth (1991). In summary, a serum sample was combined with a reaction mixture containing potassium phosphate buffer (pH = 7) and H_2O_2, and then incubated at 37°C. The reaction was halted by adding an ammonium molybdate solution, and the absorbance at 405 nm was measured using a spectrophotometer and compared to a blank (distilled water). Catalase activity was quantified in units, where one unit was defined as the enzyme amount needed to break down 1 µmol of H_2O_2 per min.

Immunological parameters

Serum IgM levels were measured utilizing the microprotein quantification method (C-690; Sigma). To determine IgM levels, precipitation was carried out with a 12% polyethylene glycol solution, and the change in protein content pre- and post-precipitation was used as an indicator of IgM levels.

The assessment of serum lysozyme activity was conducted following the procedure outlined by Ellis (1990), with certain adjustments. In summary, 25 µl of serum was combined with 1 ml of a *Micrococcus lysodeikticus* suspension (0.2 mg/ml) in a 0.05 M sodium phosphate buffer (pH 6.2), and the absorbance was recorded at 670 nm after 30s and 180s using a spectrophotometer (Biophotometer Eppendorf). The estimation of total protein (TP) in fish sera was conducted using commercial kits from Pars Azmoon Company in Tehran, Iran, and a biochemical autoanalyzer from Eurolyser in Belgium.

Digestive enzyme activity

At the end of the period, 3 pieces of fish from each replicate were sampled to investigate the activity of digestive enzymes. The digestive system of each fish was homogenized in Tris-HCl buffer using a homogenizer at 4°C. The homogenate was centrifuged and the supernatant was transferred to a -20°C freezer. Enzyme activities of trypsin, pepsin, amylase, and lipase were measured using specific methods. Protease activity was measured according to the method described by Liu et al. (2008), amylase activity was determined according to Biabani Asrami et al. (2020), and lipase activity was evaluated by the assay recommended by Metin and Akpinar (2000).

Statistical analysis

Data analysis was conducted using SPSS version 26.0. The Kolmogorov-Smirnov test was used to check the normality of the data, and the Levene test was employed to assess the homogeneity of variances. One-way analysis of variance (ANOVA) was used to evaluate the differences between treatments. The Dunnett's post-hoc test with a 95% confidence level was utilized to determine the differences between treatments. A *P*-value of < 0.05 was considered significant. All tests were performed in triplicate.

Results and Discussion

Growth performance and astaxanthin content

The results of the effects of *S. platensis* and pot marigold powder on growth parameters (as shown in Table 2) indicated that the highest levels of final weight, total length, specific growth rate, and weight gain were observed in the SP+MG combined treatment, which had a significant difference compared to the control treatment ($P < 0.05$). Additionally, the highest feed conversion ratio (FCR) was observed in the control treatment, while the lowest was in the SP+MG treatment, although no significant differences were found among the other treatments ($P > 0.05$). The highest increase in the condition factor was associated with the control treatment, with no significant differences observed among the other treatments ($P > 0.05$). Recent findings indicate the non-significant impact of *S. platensis* and pot marigold powder supplementation on the survival rates of macro fish in the studied treatments.

After feeding the experimental diets, the highest level of astaxanthin in the tissues of macro fish fed with MG and the SP+MG treatment was observed ($P < 0.05$, as shown in Fig. 1). Additionally, the astaxanthin content in the tissues of fish fed with Spirulina was significantly higher compared to the control treatments ($P < 0.05$).

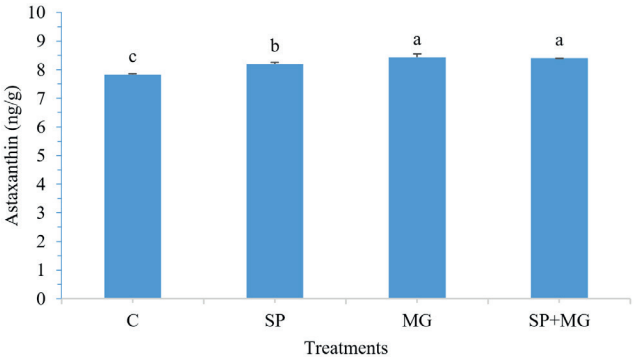


Fig. 1. Changes in the amount of astaxanthin in Macro fish (*Labidochromis caeruleus*) tissue fed with diets supplemented with different levels of Spirulina and/or Marigold powder for 8 weeks

Values are presented as mean ± S.D. Bars with different superscripts indicate significant differences ($P < 0.05$)

Source: Authors' own elaboration

Hematological indices

According to the data presented in Table 3, the highest level of red blood cells was seen in the SP+MG treatment with values of 1.66±0.01 (10^6 mm^{-3}), which significantly differed from the control group and other experimental treatments ($P < 0.05$). Nevertheless, no significant differences were found in the levels of RBC, Hb, MCV, MCH, and MCHC among the experimental treatments ($P > 0.05$). The highest lymphocyte count and hematocrit level were observed in the SP+MG treatment, which differed significantly from the control treatment ($P < 0.05$). However, no significant differences were observed in the differential counting of white blood cells among neutrophils, monocytes, and eosinophil percentages.

Carcass composition

The results of the carcass composition analysis showed that the highest percentage of crude protein at a value of 60.5

Table 2. Growth performance of Macro fish (*Labidochromis caeruleus*) fed with diets supplemented with different levels of Spirulina and/or Marigold powder for 8 weeks

Index	Control	SP	MG	SP+MG
Final weight (g)	5.27 ±0.15 ^{b}	6.43 ±0.3^{a}	6.67±0.15^{a}	2.83 ±0.20^{a}
Final length (mm)	7 .7 ±0.09^{b}	9.00 ±0.3^{a}	8.93 ±0.07^{a}	9.37 ±0.07^{a}
WG (g)	1.59 ±0.15^{b}	2.75 ±0.3^{a}	2.99 ±0.15^{a}	3.15 ±0.2^{a}
SGR (%/day)	0.4 ±0.03^{b}	0.62 ±0.05^{a}	0.66 ±0.02^{a}	0.69 ±0.03^{a}
FCR	1.91 ±0.18^{a}	1.11 ±0.11^{b}	1.00 ±0.05^{b}	0.95 ±0.06^{b}
CF	1.13 ±0.04^{a}	0.89 ±0.05^{b}	0.94 ±0.03^{b}	0.83 ±0.01^{b}
SR (%)	93.3 ±3.3^{a}	96.6 ±3.3^{a}	96.6 ±3.3^{a}	96.6 ±3.3^{a}

Data are presented as mean ± S.D. Data in the same row without superscript are not significantly different ($P > 0.05$). WG, weight gain; SGR, specific growth rate; FCR, fed conversion ratio; CF, condition factor; SR, Survival rate.

Source: Authors' own elaboration

Table 3. Hematological indices of Macro fish (*Labidochromis caeruleus*) fed diets enriched with different levels of Spirulina and/or Marigold powder for 8 weeks

Parameter	Control	SP	MG	SP+MG
RBC (10^6 mm^3)	1.6 ±0.03^a	1.61 ±0.01^a	1.60 ±0.01^a	1.66 ±0.01^a
WBC (10^3/ mm^3)	5.23 ±0.01^b	5.6 ±0.1^b	5.5 ±0.1^b	6.06 ±0.1^a
HCT (%)	27.0 ±0.03^b	27.6 ±0.3^{ab}	27.6 ±0.3^a	28.3 ±0.3^a
Hb (g/dl)	6.03±0.02^a	6.10 ±0.01^a	6.11 ±0.02^a	6.23 ±0.13^a
MCV (fl)	169.5 ±2.5^a	171.0 ±1.5^a	170.3 ±1.8^a	170.7 ±2.3^a
MCH (g/dl)	37.7 ±0.3^a	37.8 ±0.4^a	38.0 ±0.5^a	37.5 ±0.3^a
MCHC (g/dl)	32.3 ±0.5^a	22.0 ±0.6^a	22.0 ±0.3^a	21.9 ±0.2^a
Lymphocytes (%)	82.3 ±0.3^b	83.7 ±0.3^a	83.6 ±0.4^a	84.3 ±0.3^a
Neutrophils (%)	14.0 ±0.7^a	13.3 ±0.5^a	13.5 ±1.0^a	12.4±0.7^b
Monocyte (%)	4.6 ±0.3^a	4.7 ±0.4^a	4.67 ±0.3^a	5.33 ±0.3^a
Eosinophils (%)	0.00 ±0.00^a	0.00 ±0.00^a	0.00 ±0.00^a	0.67 ±0.3^a

Data are presented as mean ± S.D. Data in the same row with different superscript are significantly different ($P < 0.05$). RBC, red blood cells; WBC, white blood cells; HCT, hematocrit; Hb, haemoglobin concentration.
Source: Authors' own elaboration

Table 4. Carcass composition of Macro fish (*Labidochromis caeruleus*) after feeding with different levels of Spirulina and/or Marigold powder incorporated diet

Parameter (%)	Control	SP	MG	SP+MG
Crude protein	58.16 ± 0.62^c	59.58 ± 0.46^b	59.1 ± 0.32^b	60.5 ± 0.16^a
Crude lipid	10.4 ± 0.15^a	10.3 ± 0.18^a	10.5 ± 0.12^{ab}	10.2 ± 0.15^b
Ash	2.25 ± 0.04^a	2.21 ± 0.05^a	2.18 ± 0.03^a	2.24 ± 0.04^a
Moisture	70.3 ± 0.18^a	70.1 ± 0.23^a	70.2 ± 0.16^a	70.0 ± 0.12^a

Data are presented as mean ± S.D. Data in the same row with different superscript are significantly different ($P < 0.05$).
Source: Authors' own elaboration

± 0.16% was observed in the combined treatment (SP+MG), which had a significant difference compared to the other treatments ($P < 0.05$) (Table 4). Additionally, the percentage of crude fat in this treatment was lower compared to spirulina-fed and control treatments ($P < 0.05$). However, there was no significant difference in moisture and ash content percentages among the studied treatments ($P > 0.05$).

Serum immune parameters

The results demonstrated that the highest increase in IgM level value at 53.3±1.2 mg g^{-1} was observed in the MG treatment compared to the other treatments (Fig. 2) ($P < 0.05$). Following that, the SP+MG treatment showed a significant increase, although no significant differences were observed between Spirulina-fed and control treatments ($P > 0.05$).

Macro fish fed diets containing *S. platensis* and pot marigold powder had the highest total protein content (Fig. 3) and serum lysozyme activity (Fig. 4), which significantly differed from the other treatments ($P < 0.05$).

Antioxidant enzyme activities

The highest activity levels of the catalase, superoxide dismutase, and glutathione peroxidase were observed in the SP+MG treatment (Table 5), which had a significant difference compared to other treatments, especially the control treatment ($P < 0.05$). The level of the enzyme GPx in the control treatment was measured at the lowest level with val-

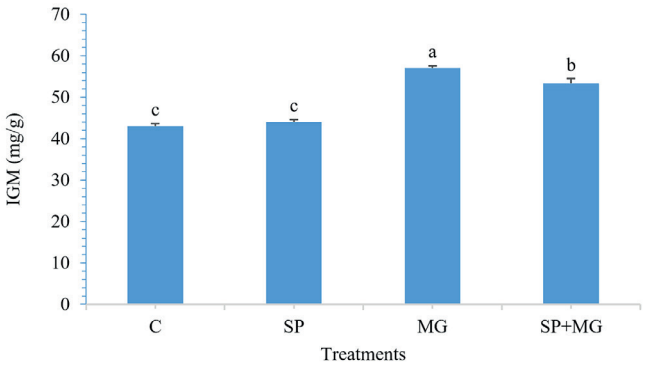


Fig. 2. Immunoglobulin M (IgM) level in Macro fish (*Labidochromis caeruleus*) fed with diets supplemented with different levels of Spirulina and/or Marigold powder for 8 weeks
Values are presented as mean ± S.D. Bars with different superscripts indicate significant differences ($P < 0.05$)
Source: Authors' own elaboration

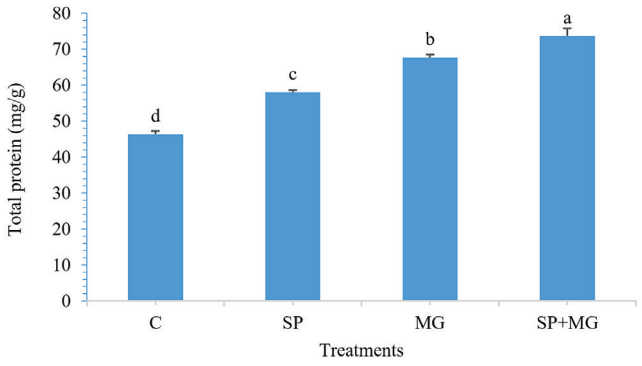


Fig. 3. Total protein level in Macro fish (*Labidochromis caeruleus*) fed with diets supplemented with different levels of Spirulina and/or Marigold powder for 8 weeks

Values are presented as mean ± S.D. Bars with different superscripts indicate significant differences ($P < 0.05$)

Source: Authors' own elaboration

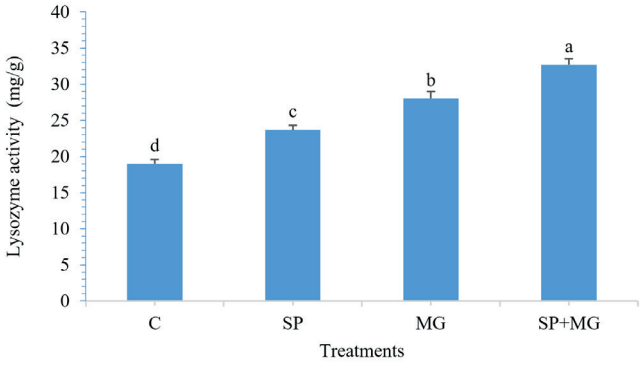


Fig. 4. Lysozyme activity in Macro fish (*Labidochromis caeruleus*) fed with diets supplemented with different levels of Spirulina and/or Marigold powder for 8 weeks

Values are presented as mean ± S.D. Bars with different superscripts indicate significant differences ($P < 0.05$)

Source: Authors' own elaboration

ues of 45.2±1.2 (U mg protein^{-1}), while in the SP+MG treatment, it was at the highest value at 61.3 ±1.7 U mg protein^{-1} ($P < 0.05$).

Digestive enzyme activities

The analysis of digestive enzyme activity in macro fish fed with individual and combined treatments of Spirulina and pot marigold powder indicated (Table 6) that the lipase activity level in the SP+MG combined treatment differed significantly from the other treatments ($P < 0.05$). Furthermore, the protease enzyme level in the SP+MG and SP treatments exhibited a notable increase compared to fish fed with Spirulina and the basal diet ($P < 0.05$). Although there was a relative rise in the amylase enzyme level in the SP+MG treatment, it did not demonstrate a significant variance compared to the other treatments ($P > 0.05$).

The outcomes of the impact of *S. platensis* and pot marigold powder on growth parameters of macro fish indicated that the SP+MG combined treatment exhibited the highest level of final weight, total length, specific growth rate, and weight gain ($P < 0.05$). Furthermore, the control treatment had the highest feed conversion ratio, while the SP+MG treatment had the lowest. In the research of Ghafarifarsani et al. (2023), the growth performance of rainbow trout was remarkably affected after being fed pot marigold-supplemented food. Based on their results, the inclusion of pot marigold in the diet significantly decreased the feed conversion ratio (FCR), particularly in fish that were fed the 1.5% MP diet. In a study by Swian et al. (2014), marigold was utilized as a natural source of carotenoids in the diet of koi carp, *Cyprinus carpio* L., resulting in positive effects on fish weight gain (WG) and feed conversion ratio (FCR). Similarly, anthocyanin extracted from Roselle Calyx, *Hibiscus sabdariffa*, another natural carotenoid source, enhanced the growth rate, specific growth rate (SGR), weight gain (WG), and feed conversion ratio (FCR) of goldfish, *Carassius auratus* (Pérez-Escalante et al., 2012). Similar results of the positive effect of Spirulina on growth have been reported in guppy fish, yellowtail carp, Oscar fish, Nile tilapia, and coral trout (Li et al., 2022).

Researchers have stated that the growth enhancement resulting from compounds such as Spirulina and pot marigold may be related to their high protein content and the presence of bioactive compounds such as carotenoid pigments and an-

Table 5. Antioxidant enzyme parameters in serum of Macro fish (*Labidochromis caeruleus*) fed diets enriched with different levels of Spirulina and/or Marigold powder for 8 weeks

Parameter	Control	SP	MG	SP+MG
Catalase (IU/min/mg protein)	62.4 ±1.2^{c}	68.9 ±1.4^{b}	68.3 ±1.1^{b}	72.6±1.5^{a}
SOD (U/ mg protein)	73.7 ±1.0^{c}	78.6 ±1.4^{b}	79.1 ±1.5^{b}	83.3 ±1.0^{a}
GPx (U/ mg protein)	45.2±1.2^{c}	58.2±1.4^{b}	57.8±1.6^{b}	61.3 ±1.7^{a}

Data are presented as mean ± S.D. Values in each row with different superscripts shows significant difference ($P < 0.05$). SOD, superoxide dismutase; MDA, malondialdehyde; GPx, glutathione peroxidase.

Source: Authors' own elaboration

Table 6. Digestive enzyme activities of Macro fish (*Labidochromis caeruleus*) fed diets enriched with different levels of Spirulina and/or Marigold powder for 8 weeks

Parameter	Control	SP	MG	SP+MG
Lipase (µg per mg soluble protein)	3.87 ±0.02^{c}	4.12 ±0.04^{b}	4.08 ±0.04^{b}	4.24±0.05^{a}
Amylase (µg per mg soluble protein)	3.44 ±00.05^{b}	3.64 ±0.08a	3.6 ±0.02^{a}	3.7 ±0.5^{a}
Protease activity (µg per mg soluble protein)	241.4 ±1^{c}	252.8 ±3^{b}	250.1 ±5^{a}	264.3 ±4^{a}

Data are presented as mean ± S.D. Values in each row with different superscripts shows significant difference ($P < 0.05$).
Source: Authors' own elaboration

tioxidant compounds (Patil et al., 2022). The antioxidant effect of these compounds increases immunity and ultimately leads to further growth (Li et al., 2022). Additionally, the high levels of amino acids and essential fatty acids in Spirulina also contribute to these effects (Lo Cascio et al., 2017). Recent findings, akin to those of Cavdar et al. (2020), indicate the non-negative impact of MG (*Tagetes erecta*) supplementation on the survival rates of *Labidochromis caeruleus* and pindani (*Pseudotropheus socolofi*) fry fish. Astaxanthin is the most important carotenoid pigment present in aquatic organisms, and it controls various biological functions such as preventing the oxidation of essential polyunsaturated fatty acids, protection against the negative effects of ultraviolet radiation, immune indicators, and reproduction (Hamrang Omshi et al., 2019). After feeding the experimental diets, the highest level of astaxanthin was recorded in the tissues of macro fish fed with MG and the SP+MG treatment.

Additionally, the astaxanthin content in the tissues of fish fed with Spirulina was significantly higher compared to the control treatment. In a similar study, Jorjani et al. (2019) indicated that feeding blue gourami (*Trichogaster trichopterus*) with marigold powder alters their skin pigmentation. Specifically, a dietary marigold level of 2.5% has been shown to enhance the total carotenoid content in the skin of blue gourami. Additionally, Sarma and Jha (2010) established a direct correlation between the addition of carotenoids to the diet and the growth of the fish. Spirulina, Göçer et al. (2006) found that incorporating red pepper and marigold flower meal as sources of natural carotenoids in the diet of *Penaeus semisulcatus* effectively enhanced pigmentation in the species. Spirulina and marigold contain pigments such as chlorophyll, carotenoids, and xanthophylls, which can enhance the coloration of fish skin (Villar-Martínez et al., 2013; Li et al., 2022). These pigments are deposited in the skin cells and can lead to brighter, more vibrant colors in fish species. Additionally, the antioxidants present in Spirulina and marigold can help protect skin cells from damage and promote healthy skin (Villar-Martínez et al., 2013; Li et al., 2022).

The percentage and amount of daily feed intake and the dietary composition are among the factors influencing the levels of carcass chemical compounds (Adel et al., 2016). The findings from the analysis of the chemical composition of macro fish carcasses revealed that the SP+MG treatment exhibited the highest level of crude protein in the carcass. Furthermore, the percentage of crude fat in this treatment was lower in comparison to the Spirulina-fed and control treatments. In a similar study, Nath Jha et al. (2012) demonstrated that the crude protein content of snow trout (*Schizothorax richardsonii*) increased with the inclusion of marigold flower and beetroot meal in the fish diet. In contrast, there were no notable variations in the body composition of blue gourami that were given varying levels of *C. officinalis* powder over a period of 70 days (Jorjani et al., 2019). Inconsistencies in results may be due to differences in the composition of ingredients and the utilization of compounds in the diet, the fish species studied, the duration of compound use in the diet, and varying experimental conditions (Jorjani et al., 2019).

Hematological indices are valuable biological indicators that reflect responses to physiological stresses and overall health status. Levels of red blood cells, hemoglobin, and hematocrit percentage are indicators of fish health and are recognized as evidence in determining the health of fish under abnormal conditions (M Al-Zayat, 2019). The SP+MG treatment exhibited the highest red blood cell levels, lymphocyte count, and hematocrit level, which significantly differed from the control treatment. The increase in the percentage of lymphocytes in fish blood is indicative of a positive response in the immune system, suggesting enhanced immune function and potential resistance to pathogens. The outcomes concerning blood cell levels may also be linked to the observed enhancement of growth observed in the present study, as well as to other functions associated with immunity. The findings of Yeganeh et al. (2015) indicated a notable increase in the levels of RBC, WBC, hemoglobin, total protein, and albumin in rainbow trout when supplemented with 10% *S. platensis* in their basic diet. Additionally, Youssef et al. (2023) suggested adding S. platensis to the diets of Nile tilapia (Oreochromis niloticus), as this led to a notable increase in hemoglobin levels, packed cell volume (PCV), red blood cell (RBC) count, and white blood cell (WBC) count. Aligning with our results, there was a dose-dependent increase in RBC count, hemoglobin concentrations, and hema-

tocrit values in fry that were given supplemented diets, with the most pronounced enhancements seen in great sturgeon (Huso huso) fed 5–10% *S. platensis* over an eight-week experimental period (Adel et al., 2016). The observed improvements in blood cell parameters suggest that the addition of S. platensis plays a crucial role in boosting the overall health and defense mechanisms of the fish studied. Specifically, the nutritional elements of S. platensis have a beneficial effect on both hematopoiesis (the production of blood cells) and immune response (Biabani Asrami et al., 2020).

The innate or non-specific immune system of fish is considered a fundamental defense mechanism against pathogens, and enhancing this system is highly valuable for cultured fish as they are vulnerable to various bacterial agents (Adel et al., 2016). Lysozyme is one of the key components of fish's non-specific immune system, causing bacterial cell wall destruction, complement activation, and enhancing phagocytic activity in fish. Increasing the lysozyme activity level indicates an improvement in the innate immune status of fish, aiding in better coping with infectious agents and stressors. An increase in lysozyme activity has been observed following the administration of certain immune stimulants, vaccines, and some probiotics in fish (Ghafarifarsani et al., 2023).

Macro fish fed diets containing SP+MG powder had the highest total protein content and serum lysozyme activity, which significantly differed from the other treatments. Plasma proteins include albumin and globulin. Elevating serum protein levels serves as a suitable indicator for assessing the immune defense status of fish. It is thought that the increase in albumin, globulin, and total serum protein levels is related to the stimulation of the host's non-specific immune system (Rauta et al., 2012). In line with the results obtained, Sadeghi Goghari et al. (2021) investigated the effect of Lactoferrin and Spirulina algae powder on the immune system of female kutum fish. In their study, the amount of lysozyme in the Spirulina treatment increased compared to the control. Also, studies by Mohammadiazarm et al. (2021) on Oscar fish, M Al-Zayat (2019) on Nile tilapia fish, Yu et al. (2018) on coral humpback fish and Adel et al. (2016) on great sturgeon indicated an increase in protein levels, and lysozyme activity due to the addition of Spirulina supplement to the diet. In the research conducted by Ghafarifarsani et al. (2023), an increase in the levels of lysozyme and complement components (C3 and C4), particularly in the 1.5% group, among *O. mykiss* that were given MG-enriched diets. The authors suggested that this enhancement might be attributed to the presence of high levels of antioxidants and polyphenols in MP, which could stimulate the immune system mechanisms. The bioactive compounds in both Spirulina and Calendula, such as antioxidants, may boost the immune system, leading to higher lysozyme activity, which is a marker of immune function in aquatic species.

The production of immunoglobulins in fish involves a series of interactions between antigen-presenting cells, activated helper T cells, and interleukins which stimulate B lymphocytes (Rauta et al., 2012). Immunoglobulins are important proteins that play a crucial role in the humoral immune response. Based on the current results, the upregulation of IgM in serum is similar to lysozyme enzyme. The study results indicate that the MG treatment exhibited the greatest elevation in IgM levels compared to the other treatment groups ($P < 0.05$). There is very little research on the effect of MG flower on immunoglobulins. In a study by Salary et al. (2024), it was found that incorporating SP+MG and MG into the diets of zebrafish (*Danio rerio*) led to a notable increase in total protein, lysozyme, and astaxanthin content in fish tissue compared to the control group. Additionally, the MG treatment showed the highest amount of IgM among the treatments. Regarding the effect of Spirulina, Yu et al. (2018) on Coral trout fish, Sabetmand et al. (2021) on common carp indicated an increase in immunoglobulins levels in Spirulina-fed treatments. Wagner et al. (1985)'s study has demonstrated that the polysaccharide (PS) components of *C. officinalis* extract possess significant immune-boosting properties.

Antioxidant enzymes are the first line of defense for organisms, including fish, under stress conditions, and evaluating them can be used to assess the status of their immune system (Yu et al., 2018). The most important antioxidant defense enzymes in fish include glutathione S-transferase, glutathione reductase, and glutathione peroxidase (Yu et al., 2019). Assessment of antioxidant markers in serum revealed that the SP+MG treatment exhibited the highest levels of activity for the enzymes catalase, superoxide dismutase, and glutathione peroxidase. According to the findings of Ghafarifarsani et al. (2023), the serum oxidative stress biomarkers showed enhancement in the rainbow trout groups that received MG, with notable improvements observed in fish that were fed the 1.5% MG diet. Their results indicate that diets supplemented with MG boosted the activities of SOD and CAT enzymes. The entire calendula plant has strong antioxidant properties, with the flowers being the most abundant source of flavonoids. These include both aglycones and glycosides of flavonols such as isorhamnetin and quercetin, as well as saponins, lipids (including sterols and carotenoids), organic acids, and sugars (Verma et al., 2018). According to Nath Jha et al. (2012), the study demonstrated that snow trout fed diets enriched with marigold flower or beetroot exhibited significantly elevated antioxidant enzyme levels compared to the control group after a 60-day period. Spirulina and

marigold are both rich in antioxidants such as carotenoids, flavonoids, and vitamin C, which help to neutralize free radicals and reduce oxidative stress in the body (Li et al., 2022; Patil et al., 2022). These antioxidants can protect cells and tissues from damage, leading to an overall improvement in health and growth parameters (Ghafarifarsani et al., 2023).

The activity of digestive enzymes serves as a digestive indicator and nutritional status in fish, and any manipulation in fish diets leads to significant changes in the activity of digestive enzymes (Hoseinifar et al., 2023). The analysis of digestive enzyme activity in macro fish fed with separate and combined treatments of Spirulina and pot marigold powder revealed that the lipase enzyme activity in the SP+MG treatment significantly differed from the other treatments. Furthermore, the protease enzyme activity in the SP+MG and SP treatments exhibited a significant increase compared to fish receiving Spirulina and a basal diet. These results are similar to those found by Biabani Asrami et al. (2020) using Spirulina in *dwarf gourami* enhanced digestive enzyme activities (lipase, amylase, and protease), with 3% considered the optimal substitution level with fishmeal. Additionally, according to Ghafarifarsani et al. (2023), diets containing pot marigold powder have positive effects on the intestinal protease and lipase levels in rainbow trout. Spirulina and marigold contain bioactive compounds that can boost the activity of intestinal enzymes involved in digestion and nutrient absorption, ultimately enhancing nutrient utilization efficiency in fish, leading to improved growth parameters and overall health. Plant compounds such as Spirulina and marigold stimulate digestive activity through four possible mechanisms: firstly, by stimulating bile secretion rich in bile acids that play a vital role in the digestion and absorption of fats; secondly, by stimulating enzymes responsible for digesting nutrients; thirdly, by controlling the microbial environment in the intestine to promote the growth of beneficial gut bacteria and increase the secretion of extracellular enzymes; and fourthly, by improving the structure and morphology of the host intestine to enhance absorption surface (Effendi et al., 2022).

Conclusions

In conclusion, incorporating Spirulina and marigold into the diets of macro fish offers numerous benefits, including enhanced growth, improved immune function, better intestinal health, and increased antioxidant activity. These natural supplements serve as cost-effective alternatives to synthetic compounds, significantly promoting the overall health and growth of fish in aquaculture settings. Additionally, they may improve fish behavior, leading to increased activity levels and reduced stress, which are essential for maintaining optimal health, especially in environments with high stocking densities and variable water quality.

Further research is necessary to explore the specific benefits of these dietary supplements across different ornamental fish species, as their responses may vary. Tailored approaches to supplement formulation and feeding strategies could foster broader acceptance of natural additives in the aquaculture community, contributing to more environmentally friendly practices. By aligning fish production with sustainability goals and reducing reliance on synthetic additives, the integration of Spirulina and marigold could pave the way for more resilient and sustainable aquaculture, ultimately enhancing the quality of seafood available to meet the demands of a growing population.

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Ethical approval

This article does not contain any studies with human participants by any of the authors. All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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