



EFFECTS OF FISHMEAL REPLACEMENT WITH SPIRULINA (*ARTHROSPIRA PLATENSIS*) AND *SARGASSUM ILICIFOLIUM* MEAL ON GROWTH AND HEALTH INDICES OF ASIAN SEABASS (*LATES CALCARIFER*) JUVENILES

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Abstract

The influence of dietary fishmeal (FM) replacement with a mixture (1:1) of spirulina (*Arthrospira platensis*) and a brown seaweed (*Sargassum ilicifolium*) in Asian seabass (*Lates calcarifer*, 45.0±0.2) performance was evaluated. Dietary FM was replaced with the algal mixture at 5 (MIX5%) and 10% (MIX10%) and a diet without algal mixture served as control group. One hundred and thirty five *L. calcarifer* juveniles were stocked into nine 300-L circular containers (15 fish/tank) filled with seawater (salinity = 46.0±0.2 g L⁻¹; temperature = 31.5±2.5°C) with 80% daily water exchange rate. Each dietary treatment was conducted in triplicate. The experimental feeds were offered to fish thrice daily up to satiation for 60 days. Fish in MIX5% group had the highest weight gain (approximately 12%) that was associated with higher feed intake compare to the control. Viscerosomatic index was reduced in fish fed MIX10% diet compared to control group (8.6 vs. 7.6%). Amylase, alkaline phosphatase, total protease and trypsin activities significantly increased in fish fed MIX5% group (P<0.05). Liver superoxide dismutase activity (36.1 U/mg protein) and glutathione (33.3 µmol/g tissue) level increased in fish fed MIX5% diet. MIX5% group had higher white blood cell counts than other groups. Plasma cholesterol decreased (approximately 20 mg/dL) but plasma calcium content increased in fish fed algal mix-supplemented diets (P<0.05). Plasma triglycerides, total protein, globulin, and alkaline phosphatase decreased in fish fed MIX10% diet. Based on the above-mentioned findings, dietary FM replacement with micro- and macroalgae mixture at 5% is recommended for growth and improving health status in *L. calcarifer* juveniles.

Key words: algae mixture, Asian seabass, antioxidant capacity, digestive enzymes, hypocholesterolemic effect, superoxide dismutase

Fishmeal (FM) and fish oil (FO) are two main marine-derived ingredients in aquafeeds, particularly for carnivorous fish species. Due to the fast expanding rate of aquaculture industry, demands increased for FM and FO that enhanced their prices in the market (Kok et al., 2020; Naylor et al., 2021; Macusi et al., 2023). Furthermore, overfishing of pelagic fish stocks (e.g. sardines, herrings and anchovies) as the main resources for FM and FO should be eliminated due to drastic ecological impacts on marine wildlife (Kok et al., 2020; Naylor et al., 2021). In addition, more than 90% of these resources have food grade quality and can be consumed directly by human or for producing pharmaceuticals, nutraceuticals, and also feeds for other livestock. Thus, finding alternative resources can reduce aquafeed production expenditures and supports the aquaculture profitability and sustainability (Turchini et al., 2019; Rombenso et al., 2022).

Among various novel alternatives for FM, microalgae and macroalgae are considered as promising resources because of their high biomass productivity, low carbon and water footprints, and wide range of species that could

be grown even in unconventional waste resources such as agricultural wastewater (Wells et al., 2017; Øverland et al., 2019; Wan et al., 2019; Nagarajan et al., 2021; Saadaoui et al., 2021; Valente et al., 2021; Moreira et al., 2022; Ma and Hu, 2023; Siddik et al., 2024; Vijayaram et al., 2024). Microalgae contain high amount of protein containing all essential amino acids (EAA), long chain polyunsaturated fatty acids (LC-PUFA), sterols, essential vitamins and minerals, bioactive compounds and pigments (Saadaoui et al., 2021; Valente et al., 2021; Ma and Hu, 2023; Siddik et al., 2024). For instance, spirulina (*Arthrospira platensis*) (SP), is considered as promising protein source due to its high protein level (50–70%), balanced EAA, high amounts of vitamins (e.g. E, B₁, B₃ and B₆) especially B₁₂, polysaccharides, minerals (e.g., Zn, Mn, Cu, Se), and many biologically active pigments (e.g., chlorophyll, β-carotene and zeaxanthin) (Zhang et al., 2020; Alagawany et al., 2021; Li et al., 2022). It contains a wide range of bioactive compounds, including carotenoids, phenolic substances, phycobiliproteins (e.g. phycocyanin), and γ-linolenic acid with antioxidant and

immunostimulatory characteristics (Zhang et al., 2020; Alagawany et al., 2021; Li et al., 2022).

On the contrary, macroalgae species generally contain low amount of protein (5%–30% dry weight) and their amino acids profile is less balanced, but they are a good source of PUFA, soluble fibers, pigments, minerals, and vitamins (Wells et al., 2017; Øverland et al., 2019; Wan et al., 2019). Among different seaweeds, *Sargassum* sp. contains sulfated polysaccharides and numerous bioactive compounds such as steroids, glycerides, alkaloids, polysaccharides, β -glucans, polyphenols, saponins, terpenoids, sargaquinoic acids, sargachromenol, plastoquinones, and flavonoids with immunostimulatory, antioxidant, antibacterial and antiviral properties (Immanuel et al., 2012; Norambueña et al., 2015; Wilke et al., 2015; Prabu et al., 2016; Schleder et al., 2017; Mohan et al., 2019; Ferreira et al., 2021). However, the application of microalgae biomass and bio-products as aquafeed for aquaculture is challenged by some problems, such as bioaccumulation of heavy metals, poor digestibility of algal biomass, anti-nutritional factors, and high cost of biomass utilization (Chen et al., 2021). Regarding macroalgae, high levels of fiber, ash and low digestibility, protein and lipid contents are the main challenges as fishmeal replacer in aquaculture (Wan et al., 2019).

Although algae lack lignin and have low amount of hemicellulose, the presence of complex structural polysaccharides may adversely affect their digestibility (Nicolai et al., 2019; Annamalai et al., 2021; Nagappan et al., 2021). For instance, some nutrients such as phosphorus and lysine are low in SP meal (Macias-Sancho et al., 2014). In this context, it has been proved that total replacement of FM with SP meal showed some EAA deficiencies, mainly in the proportions of lysine, methionine, histidine, arginine and threonine (Rosas et al., 2019 a, b). In this regard, it is suggested that the combination of micro- and macroalgae species could improve their nutritional values of diets and can exert beneficial synergistic effects (Amaro et al., 2019; Batista et al., 2020 a, b). The simultaneous inclusion of two or more micro- and macroalgae can provide more balanced and superior nutritional mixture, by combining macroalgae that are poor in lipids, with microalgae that are rich in n-3 LC-PUFA fatty acids. Furthermore, a mixture may allow synergistic antibacterial and antioxidant activities derived from the distinct micro- and macroalgae species (Amaro et al., 2019; Batista et al., 2020 a, b; Ferreira et al., 2021). Asian seabass (*Lates calcarifer*) is one of the most promising carnivorous fish to expanding marine cage culture activity in the south of Iran, mainly in the Persian Gulf and Oman Sea (Mozanzadeh et al., 2021). According to the annual statistical report of the Iran Fisheries Organization, the total production of Asian seabass was 6400 tons in Iran; meanwhile, its annual production was 120,000 tons in 2020 and it will increase by 5% yearly until 2035 (Future Market Insight, 2021). Partial replacement of dietary FM with various macroalgae species such as *Sar-*

gassum ilicifolium (Zeynali et al., 2020; Morshedi et al., 2024), *Gracilaria pulvinata* (Morshedi et al., 2018) or *Padina australis* (Morshedi et al., 2024) up to 6% or SP meal up to 10% (Yong et al., 2017; Siddik et al., 2022) for *L. calcarifer* is possible without compromising its performance. Thus, the present research aimed to assess the effects of replacing dietary FM with a mixture of SP and *Sargassum* sp. meals on performance of *L. calcarifer* reared in sea water.

Material and methods

Ethical statement

The husbandry and treatment of the experimental fish complied with the guidelines of ARRIVE (<https://arrive-guidelines.org/>) and the ethical recommendations guide for using laboratory animals.

Experimental diets

Brown macroalgae, *Sargassum ilicifolium* (6.5% crude protein, 1.0% crude lipid, 31% ash content) used in the current study were obtained from the coast of the Persian Gulf in southern Iran. Macroalgae were washed, then dried (60°C, 48 h) and powdered with an electric mill. Spirulina meal (65% crude protein, 3.5% crude lipid, 10.1% crude ash) was purchased (Algotab, Markazi, Delijan Iran). Micro- and macroalgae were equally mixed together (1:1) and then replaced with fishmeal (61.8% crude protein) at 5 (MIX5%) and 10 (MIX10%) and a control diet did not contain algal meal (Table 1). In summary, dry ingredients were blended (20 min), then a mixture of soy lecithin and oils was added and mixed (10 min), and finally gelatin was dissolved in warm water and added to prepare a dough. The prepared dough was pelleted with a meat grinder (3 mm). The pellets were dried (25°C, 48 h), then packed and stored (−20°C) until use. The proximate composition of all the protein sources and the experimental diets were analyzed based on the standard methods (AOAC, 2000).

Husbandry

The present study was carried out in Imam Khomeini Marine Fish Research Station, Sarbandar, Khuzestan, Iran. One hundred and thirty five *L. calcarifer* juveniles were randomly distributed into nine 300-L circular polyethylene tanks (15 fish/tank) containing 200 L sand filtered and disinfected (10 ppm calcium chloride, neutralized with 5 ppm sodium thiosulphate) seawater with 80% daily water exchange rate. Each dietary treatment was replicated in triplicate. Feeding was thrice daily (08:00, 12:00 and 16:00) up to satiation, ensuring no pellet was left uneaten for 60 days. Temperature (31.5±2.5°C), salinity (46.0±0.2 g L⁻¹), pH (8.2±0.2) and dissolved oxygen (5.5±0.5 mg L⁻¹) were monitored twice a week. The photoperiod was artificially set at 12 h light and 12 h dark.

Table 1. Ingredients and chemical composition (% of dry matter) of experimental diets containing *Spirulina*

Experimental diets	Control	MIX5%	MIX10%
Ingredients (g/kg diet) ^a			
Fishmeal	460	410	360
Wheat gluten meal	121	121	121
Corn gluten meal	121	121	121
Soybean meal ^b	50	46	42
Beef gelatin	20	20	20
<i>Spirulina</i> meal	-	25	50
<i>Sargassum</i> meal	-	25	50
Wheat middling	135	109	83
Fish oil	20	20	20
Canola oil ^c	20	20	20
Soy lecithin ^c	20	20	20
DL-methionine	1	1	1
L-lysine	2	2	2
Vitamin premix ^d	10	10	10
Mineral premix ^e	10	10	10
Butyric acid ^f	2.5	2.5	2.5
Sodium diformate ^g	2.5	2.5	2.5
Vitamin C ^h	5	5	5
Total	1000	1000	1000
Chemical composition			
dry matter	90.5	90.9	90.7
crude protein	46.3	45.8	46.7
crude lipid	16.3	15.1	16.5
ash	9.1	9.5	9.8

^aComposition of ingredients as % dry-weight basis [fishmeal (60.5% crude protein, 18.0% crude lipid); corn gluten (71.4% crude protein, 4.1% crude lipid); wheat gluten (53.3% crude protein, 2.8% crude lipid); soybean meal (41.0% crude protein, 4.2% crude lipid); gelatin (85.0% crude protein, 4.2% crude lipid); *Gracilaria* powder (11.9% crude protein; 1.4% crude lipid); *Sargassum* powder (9.8% crude protein; 1.4% crude lipid); wheat middling (12.0% crude protein, 3.0% crude lipid)].

^bProduct of Kesht Va Sanat Shomal Vegetable Oil Factories Complex (Neca, Iran).

^cBehpak industrial company, Behshahr, Mazandaran, Iran.

^dVitamin premix (IU/kg of premix): ascorbic acid, 350,000; retinol, 1,000,000,000; cholecalciferol, 500,000,000; tocopherols, 500,000; vitamin K₃, 960,000; thiamine, 980,000; riboflavin, 800,000; pyridoxine, 990,000; folic acid, 950,000; cobalamin, 10,000; biotin, 20,000; niacin, 995,000; pantothenic acid, 980,000.

^eMineral premix (mg/kg of premix): magnesium, 6,400; copper, 2000; ferrous, 11,000; zinc, 7,000; selenium, 100; iodine, 300; cobalt, 50; sodium, 5,000. ATA Company, Tabriz, Iran.

^fMerck, Germany.

^gConjugated salt of formic acid, HCOOH·HCOO-Na, contained 195 g/kg sodium + 390 g/kg formic acid + 381 g/kg formate + 34 g/kg silicate and water; Formi® NaDF, Addcon Nordic AS.

^hRooyan Darou, Semnan, Iran.

Sampling

Before sampling, fish were unfed for a day. Biometry of fish was individually carried out for weight and length. Six fish of each tank were anesthetized (2-phenoxyethanol, 300 μ L L⁻¹, Mohtashempour et al., 2024) and blood was caught from the caudal vein with heparinized (heparin sodium 5000 IU) syringes. To measure complete blood count, an aliquot of the blood (500

μ L) was transferred into 1 mL micro-tube and kept in a refrigerator (4°C). To measure plasma biochemical parameters, another aliquot (1000 μ L) of blood was centrifuged (5000 g, 4°C for 10 min) and the plasma was kept in a freezer (-20°C). The gut and liver of the same anaesthetized fish were dissected on a piece of ice and transferred into 2 mL microtubes, then kept in a freezer (-80°C).

Digestive enzymes and antioxidant factors

The gut samples were homogenized in ice-cold mannitol buffer (50 mM mannitol + 2 mM tris-HCl, pH 7) (30:1; v/w) for 60 s (Castro-Ruiz et al., 2019). The homogenate was centrifuged (9000 g, 10 min, 4°C), then the supernatant was separated. For separating the gut's brush border, the supernatant was centrifuged (34,000 g, 30 min, 4°C) and the precipitate was dissolved in one mL of buffer (0.1 M KCl, 5 mM Tris-Hepes, 1 mM DL-dithiothreitol (Sigma-0632); pH 7.5) to evaluate alkaline phosphatase (ALP) (Crane et al., 1979; Gisbert et al., 2019). Soluble protein content (Bradford, 1976), trypsin (Bergmeyer, 1974), chymotrypsin (Hummel, 1959), protease (Folin and Ciocalteu, 1929), ALP (Bessey, 1946), α -amylase (Bernfeld, 1955), and lipase (Tietz and Fiereck, 1966) activities were measured based on standard methods.

The liver samples were homogenized (1: 9, w/v) in cold potassium phosphate buffer (0.1 M, pH = 7.4, 4°C, 10000 g) for 60 s. The homogenate was centrifuged (9,000 g, 30 min, 4°C); the supernatant was separated to evaluate antioxidant parameters. Catalase (CAT) activity was measured by determining the reduction in absorbance at 240 nm in the presence of 13.2 mM H₂O₂ in 50 mM phosphate buffer (pH 7.0) and 100 μ L of serum (Aebi, 1984). Superoxide dismutase (SOD) activity was measured by using a buffer containing 1200 μ L sodium pyrophosphate buffer + 100 μ L phenazine methosulfate + 300 μ L nitroblue tetrazolium (McCord and Fridovich, 1969). Two hundred μ L of the plasma was added to the assay mixture, and the total volume was increased to 2.8 mL with distilled water. Then 200 μ L of NADH was added to the previous mixture to initiate the reaction at 30°C for 90 s. Then the reaction was stopped by adding 1.0 mL of glacial acetic acid. Then 4.0 mL of n-butanol was added to the mixture, shaken and stood for 10 min, and was centrifuged (5000 g, 10 min). The inhibition activity of SOD was measured spectrophotometrically at 560 nm in the butanol layer for 60 s. Glutathione level (GSH) was measured by reaction with dithionitrobenzoic acid and recording absorbance at 412 nm (Beutler et al., 1963).

Hematological and plasma biochemical indices

Complete blood count parameters including red blood cell (RBC), white blood cell (WBC), hemoglobin (HB), and hematocrit (Hct) were assessed, as described by Blaxhall and Daisley (1973). Blood indices, including the mean cell hemoglobin (MCH), the mean cell vol-

ume (MCV) and the mean cell hemoglobin concentration (MCHC), were calculated according to Dacie and Lewis (1990). Respiratory burst activity (RBA) was examined by measuring the reactive oxygen species production of leucocytes against nitro blue tetrazolium (NBT) as a target (Siwicki et al., 1994). Lysozyme activity (Ellis, 1990) of plasma was determined by using lyophilized *Micrococcus lysodeikticus* (Sigma, St Louis, MO, USA) as the target to determine lysozyme activity in plasma. Plasma biochemical parameters, including glucose (GLU), cholesterol (CHOL), triglycerides (TRIG), total protein (TP), albumin (ALB), calcium (Ca), alkaline phosphatase (ALP), and aspartate aminotransferase (AST) were measured spectrophotometrically by applying diagnostic kits (Pars Azmoon Co., Tehran, Iran) based on the following equation:

(Absorption of sample / Absorption of standard) × concentration of the standard (Ghanei-Motlagh et al., 2020).

The amount of globulin (GLOB) was calculated after subtracting albumin from total protein content.

Statistics

Data were analyzed by a SPSS statistics software (Ver 23.0, Chicago, IL, USA) and presented as means ± standard error (n = 3). First, data normality of distribution was evaluated by Kolmogorov-Smirnov test, then Levene's test was used to evaluate data homogeneity of variances. A one-way ANOVA followed by Tukey's post test were used to evaluate significant differences at $P < 0.05$.

Results

Survival rate was 100% in all groups (Table 2). Fish in MIX5% group had the highest final weight, specific growth rate and weight gain. Feed intake in fish fed MIX5% and MIX10% diets was higher than the control. Feed conversion ratio (FCR), Fulton's condition factor (K) and hepatosomatic index (HSI) were not affected by the experimental diets. Viscerosomatic index in fish fed MIX10% group was significantly lower than control group and fish in MIX5% had intermediate value.

Fish fed MIX5% diet had higher amylase, alkaline phosphatase, and trypsin activities than the other treatments (Table 3). Total protease activity in fish fed MIX5% and MIX10% was higher than the control group. Lipase and chymotrypsin activities were not affected by the experimental diets ($P > 0.05$). Catalase activity in the liver of fish fed MIX5% diet was lower than the other groups. Superoxide dismutase activity and glutathione level in the liver of fish fed MIX5% diet were higher than the other treatments.

White blood cell count increased in fish fed MIX5% diet. Control group had higher HCT percentage compared to the other groups. Other hemato-immunolog-

ical parameters were not affected by the experimental diets.

Table 2. Growth performance of *L. calcarifer* juveniles fed different experimental diets for 63 days. Data are presented as the mean ± pooled SE of three replicates

Treatments	Control	MIX5%	MIX10%
IBW (g)	45.4±0.2	45.4 ±0.2	44.8±0.0
FBW (g)	116.3±0.2 b	122.2±0.9 a	115.3±0.2 b
FBL (cm)	22.3±0.4	22.5±0.3	22.3±0.4
SGR (%/day)	1.5±0.1 b	1.6±0.0 a	1.5±0.0 b
WG (%)	156.4±7.5 b	169.2±2.0 a	157.8±0.5 b
FI (g/fish)	75.6±2.0 b	82.6±0.8 a	86.4±0.9 a
FCR	1.1±0.1	1.1±0.0	1.2±0.0
K (%)	1.1±0.0	1.1±0.0	1.1±0.0
HSI (%)	1.9±0.1	1.8±0.1	1.8±0.1
VSI (%)	8.6±0.2 a	8.2±0.2 ab	7.6±0.2 b
Survival (%)	100±0.0	100±0.0	100±0.0

IBW: initial body weight.

FBW: final body weight.

FBL: initial body length.

SGR: specific growth rate = ((Ln (FBW)-Ln (IBW)) / feeding period (63 days)) × 100.

WG: weight gain = (FBW (g) - IBW (g)) / IBW (g) × 100.

FI: feed intake = total feed intake (g) / number of fish.

FCR: feed conversion ratio = FI (g) / WG (g).

K: Fulton's condition factor = FBW (g) / $L^3 \times 100$.

HSI: hepatosomatic index = liver weight (g) / body weight (g) × 100.

VSI: viscerosomatic index = visceral weight (g) / body weight (g) × 100.

Survival = final number of fish / initial number of fish × 100.

Table 3. Digestive and liver antioxidant enzymes activity of *L. calcarifer* juveniles fed different experimental diets for 63 days. Data are presented as the mean ± pooled SE of three replicates

Treatments	Control	MIX5%	MIX10%
Gut digestive enzymes			
Amylase (U/mg protein)	0.3±0.1 b	2.1±0.3 a	0.5±0.1 b
Lipase (U/mg protein)	402.2±46.4	338.0±21.8	390.6±55.6
Alkaline phosphatase (U/mg protein)	6.3±0.7 b	8.9±1.1 a	6.5±1.0 b
Total protease (U/mg protein)	1158.3±120.6 b	2747.4±331.1 a	1979.2±308.2 a
Trypsin (U/mg protein)	14.3±0.6 b	18.4±1.3 a	15.2±2.2 b
Chymotrypsin (U/mg protein)	0.2±0.0	0.2±0.0	0.2±0.0
Liver antioxidant parameters			
Catalase (U/mg protein)	227.1±23.5 a	139.9±18.8 b	232.3 ±12.7 a
Superoxide dismutase (U/mg protein)	22.2±2.1 b	36.1±3.3 a	19.2±2.8 b
Glutathione (μmol/g tissue)	26.7±2.0 b	33.3±4.2 a	28.6±2.9 b

Table 4. Hemato-immunological parameters of *L. calcarifer* juveniles fed different experimental diets for 63 days. Data are presented as the mean $\pm$ pooled SE of three replicates

Treatments	Control	MIX5%	MIX10%
RBC ($\times 10^6/\mu\text{L}$)	2.1 $\pm$ 0.4	2.0 $\pm$ 0.3	1.9 $\pm$ 0.1
WBC ($\times 10^3/\mu\text{L}$)	3.3 $\pm$ 0.6 b	6.6 $\pm$ 0.5 a	3.5 $\pm$ 0.3 b
HCT (%)	44.0 $\pm$ 3.0 a	34.2 $\pm$ 2.2 b	35.7 $\pm$ 1.9 b
HB (g/dL)	6.4 $\pm$ 0.5	6.0 $\pm$ 0.3	6.4 $\pm$ 0.4
MCH (pg/cell)	35.9 $\pm$ 8.2	33.6 $\pm$ 5.3	34.3 $\pm$ 2.8
MCV (fL)	247.9 $\pm$ 56.7	182.1 $\pm$ 16.4	190.5 $\pm$ 15.7
MCHC (g/dL)	14.6 $\pm$ 0.4	18.1 $\pm$ 1.6	18.3 $\pm$ 1.6
RBA (OD 540)	0.5 $\pm$ 0.0	0.4 $\pm$ 0.1	0.5 $\pm$ 0.0
LYZ (U/mL)	770.4 $\pm$ 52.1	777.8 $\pm$ 33.9	637.0 $\pm$ 114.6

Abbreviations: RBC: red blood cell count; WBC: white blood cell count; HCT: hematocrit; HB: hemoglobin; MCH: mean cell hemoglobin; MCV: mean cell volume; MCHC: mean cell hemoglobin concentration; RBA: respiratory burst activity; LYZ: lysozyme.

Table 5. Plasma biochemical parameters of *L. calcarifer* juveniles fed different experimental diets for 63 days. Data are presented as the mean $\pm$ pooled SE of three replicates

Treatments	Control	MIX5%	MIX10%
GLU (mg/dL)	84.9 $\pm$ 9.5 b	163.3 $\pm$ 21.6 a	112.0 $\pm$ 20.6 ab
CHOL (mg/dL)	151.8 $\pm$ 11.7 a	127.1 $\pm$ 8.1 b	134.2 $\pm$ 10.5 b
TRIG (mg/dL)	83.1 $\pm$ 9.2 a	87.7 $\pm$ 9.2 a	47.9 $\pm$ 8.9 b
TP (g/dL)	2.8 $\pm$ 0.2 a	2.5 $\pm$ 0.3 a	1.6 $\pm$ 0.3 b
ALB (g/dL)	1.0 $\pm$ 0.1	0.9 $\pm$ 0.1	0.8 $\pm$ 0.0
GLOB (g/dL)	1.8 $\pm$ 0.2 a	1.4 $\pm$ 0.3 a	0.8 $\pm$ 0.0 b
Ca (mg/dL)	6.6 $\pm$ 0.8 c	12.3 $\pm$ 2.3 a	8.4 $\pm$ 0.6 b
ALP (U/L)	5.5 $\pm$ 0.4 a	5.3 $\pm$ 1.4 a	3.7 $\pm$ 0.3 b
AST (U/L)	0.7 $\pm$ 0.1	0.6 $\pm$ 0.0	0.5 $\pm$ 0.1

Abbreviations: GLU: glucose; CHOL: cholesterol; TRIG: triglyceride; TP: total protein; ALB: albumin; GLOB: globulin; Ca: calcium; ALP: alkaline phosphatase; AST: aspartate aminotransferase.

The highest and lowest plasma GLU were in fish fed MIX5% and the control groups, respectively (Table 5). Plasma CHOL significantly decreased in fish fed MIX5% and MIX10% diets. Plasma TRIG in fish fed MIX10% was significantly lower than the other treatments. Plasma TP, GLOB, and ALP was significantly reduced in fish fed MIX10% diet ($P < 0.05$). Plasma calcium in fish fed MIX5% diet was higher than the other groups. Other biochemical parameters, including ALB and AST did not change among treatments.

Discussion

Growth

In the current study, replacement of dietary FM with a mixture of SP and *Sargassum* meals at 5% level significantly enhanced growth rate that was associated with increment of feed intake, digestive enzymes activity (e.g. amylase, ALP, total protease, and trypsin), liver SOD activity and GSH content in this species. It seems

that the mixture of micro- and macroalgae could provide a balanced and highly nutritional blend, by combining *Sargassum* meals that are poor in proteins and lipids with SP meal that has high amounts of protein and lipid content. Furthermore, a mixture could provide synergistic immunostimulatory and antioxidant effects derived from the distinct species (Amaro et al., 2019; Batista et al., 2020 a, b). Moreover, the high level of macro and trace elements in both macro- and microalgae may promote growth at 5% FM replacement level; however, higher replacement level at 10% may decrease growth due to the presence of toxic elements (e.g. aluminum, arsenic, and copper), high fiber and ash content, particularly in *Sargassum* meals and may be deficient in some EAA such as lysine, methionine, histidine, arginine, and threonine (Olvera-Novoa et al., 1998; Rosas et al., 2019 a, b). In agreement to our results, Shapawi and Zamry (2016) reported that supplementing diet with 5% *S. polycystum* meal did not affect growth and FCR in *L. calcarifer*, but increased feed intake in this species. In addition, dietary inclusion of *Gracilaria* spp. and *Sargassum* spp. meals (1:1) mixture up to 12% did not compromise growth and FCR in *L. calcarifer*, but 15% inclusion level drastically reduced growth (Seyedalhosseini et al., 2023). On the other hand, Siddik et al. (2022) reported that dietary FM substitution with selenium enriched SP meal up to 10% did not affect growth performance in *L. calcarifer*, but 20% replacement level reduced growth. In a study, supplementing a plant protein-rich diet with a mixture of micro- (*Chlorella vulgaris* and *Nannochloropsis oceanica*) and macroalgae (*Ulva* sp. and *Gracilaria gracilis*) at 6% significantly enhanced growth and feed utilization in European seabass (*Dicentrarchus labrax*) that was associated with better feed and protein efficiency and higher feed intake (Mota et al., 2023). In contrast, Batista et al. (2020 b) reported that dietary single or blended seaweed *G. gracilis* and microalga *N. oceanica* supplementation at 8% did not affect growth and feed utilization in European seabass. These findings are species-specific and depend on micro- and macroalgae species, feed biochemical composition, the FM replacement level among the other factors.

Digestive enzymes

In the present study, dietary FM replacement with algal mixture at 5% significantly enhanced amylase, total protease, alkaline phosphatase and trypsin in *L. calcarifer*, but at 10% replacement level significantly suppressed digestive enzymes activities. In this regard, Morshedi et al. (2018) reported that protease and amylase activities in *L. calcarifer* were significantly decreased by replacement of dietary FM with 9% *G. pulvinata* meal. These negative effects on the digestive enzymes could be attributed to fiber present in seaweeds (Oliveira et al., 2009). In fact, high level of fiber in seaweeds may reduce gut transit time and decrease the time available for digestion, and negatively influence the effectiveness of digestive enzyme activity. Moreover, seaweeds contain

several anti-nutrients (e.g. lectins and proteinase inhibitors) (Dvir et al., 2009) that interfere with digestion and nutrients utilization processes (Francis et al., 2001). In contrast, Zeynali et al. (2020) reported that 6% dietary FM replacement with *S. ilicifolium* meal enhanced amylase and lipase activities in *L. calcarifer*, but 9% replacement level reduced protease activity in this species. On the other hand, it has been suggested that microalgae have prebiotic effects that stimulate the propagation of beneficial microbes in the gut and enhance endogenous digestive enzymes synthesis (Anand et al., 2013; Adel et al., 2016). In this regard, protease activity increased in Oscar fish (*Astronotus ocellatus*) fed a diet supplemented with 5.5% SPM [70]. Furthermore, supplementing diet with 5–10% SP meal significantly elevated lipase and protease activities in great sturgeon (*Huso huso*) (Adel et al., 2016). In addition, supplementing diet with 8% blends of *G. gracilis* and *N. oceanica* meals significantly enhanced maltase, sucrose-isomaltase and alkaline phosphatase activities in the pyloric caeca brush boarder of European seabass compared to those fed diets solely supplemented with *G. gracilis* or *N. oceanica* (Batista et al., 2020 b). The ALP is a valuable marker for enterocytes brush border maturation and intestinal cell differentiation (Hinnebusch et al., 2004) and its increment in the gut suggesting the inclusion of algal mixture can modulate gut digestive activity.

Antioxidant activity

In the current study, replacement of dietary FM with a mixture of micro- and macroalgae at 5% level increased liver SOD activity and GSH level that was associated with better growth rate in *L. calcarifer*. This could be because of the synergistic effects of micro- and macroalgae supplementation, which leads to greater antioxidant capacity against oxidative stress. In agreement with our results, Batista et al. (2020 b) reported that supplementing diet with 8% of a mixture of *Gracilaria gracilis* and *Nannochloropsis oceanica* meals significantly reduced total glutathione and GPx activity, but it did not affect CAT in European seabass. SP meal is a rich source for bioactive compounds such as catechins, phycobiliproteins, allophycocyanin, phycocyanin, vitamins A, E, pigments and phenolic components that have vital role in stimulation of the enzymatic antioxidant pathway and immunocompetence in the body (Abdel-Tawwab and Ahmad, 2009; Zhang et al., 2020; Alagawany et al., 2021). The bioactive compounds in SPM can actively scavenge and neutralize reactive free radicals and chelate metal ions (Sagaram et al., 2021). In this context, it has been shown that dietary FM replacement with selenium-enriched SP meal significantly enhanced serum GPx activity, but it did not affect CAT in *L. calcarifer* (Siddik et al., 2022). Superoxide dismutase and total antioxidant capacity increased in juvenile gibel carp (*Carassis auratus gibelio* var. CAS III) by replacement of dietary FM with SP (Cao et al., 2018). Also, supplementing a diet with 0.08% SPM significantly enhanced CAT and TAC in Yellow River

carp that was associated with MDA reduction (Ren et al., 2021). Moreover, supplementing diet with 10% SP meal significantly enhanced SOD activity and GSH content in hepatopancreas of grass carp (*Ctenopharyngodon idella*) (Faheem et al., 2022). In contrast, Yu et al. (2018) reported that supplementing diet with 10% SP meal significantly enhanced TAC but reduced SOD, CAT, GPX and MDA in the liver of coral trout *Plectropomus leopardus*. Seaweeds contain numerous antioxidants, including ascorbic acid, selenium, sulfated polysaccharides, carotenoids, phytosterols, flavonoids, fucoxanthin, and fucoidan (Ahn et al., 2004; Wan et al., 2019) and brown seaweeds exclusively have a potent polyphenol antioxidant namely phlorotannin which has UV radiation-absorbing properties (Soleimani et al., 2018). In this regard, Zeynali et al. (2020) reported that 6 to 9% dietary FM replacement with *S. ilicifolium* meal significantly increased liver SOD activity in *L. calcarifer*.

Hemato-immunological parameters

In the present study, replacement of dietary FM with a mixture of micro- and macroalgae at 5% level significantly increased WBC compared to other groups, suggesting immunostimulatory effects and anti-infection properties of the algal mixture. It has been reported that the presence of phycocyanin in spirulina increased WBC in mice (Zhang et al., 1994). In addition, polysaccharides (e.g. carrageenan, alginates, β -glucans, and sodium alginate) in seaweeds have great immunostimulatory effects and could increase diseases resistance in fish (Wan et al., 2019; Moreira et al., 2022; Vijayaram et al., 2024). In this context, Zeynali et al. (2020) reported that replacement of dietary FM with *Sargassum ilicifolium* meal significantly enhanced RBC and WBC counts in *L. calcarifer*. Also, dietary inclusion of SPM significantly increased WBC in *Labeo rohita* (Andrews et al., 2011) and rainbow trout (Yeganeh et al., 2015). In the present study, dietary FM replacement with algal mixture reduced blood HCT in *L. calcarifer*, but did not affect other hematological factors. In this regard, Yong et al. (2017) reported that replacement of dietary FM with SPM over 10% significantly reduced HCT and HB in *L. calcarifer*. In contrast, Siddik et al. (2020) reported that replacement of dietary FM with selenium enriched spirulina meal did not affect hematological parameters in *L. calcarifer*.

Plasma biochemical parameters

In the current research, plasma glucose increased in fish fed algal mixture supplemented diets that may be related to higher feed intake in these groups. In this context, it has been reported that increasing feeding frequencies increased serum glucose in grass carp (Wu et al., 2021). Similarly, Batista et al. (2020 b) reported that replacement of dietary FM with *Gracilaria gracilis* and microalga *Nannochloropsis oceanica* or their mixture enhanced serum glucose in European seabass. Also, inclusion of 1% SP meal in diet increased serum glucose in Nile tilapia (*Oreochromis niloticus*) (Belal et al., 2012).

In contrast, increasing dietary macroalgae (*G. cornea* and *Ulva rigida*) levels reduced serum glucose level in gilthead seabream (*Sparus aurata*) (Vizcaino et al., 2016).

It seems the hypolipidemic effect of brown seaweeds is related to their phytosterols content (Chen et al., 2021) that abate the availability of cholesterol for the gut's absorption (Meinita et al., 2021). In addition, fucoidan in brown seaweeds has been shown to decrease blood cholesterol and alleviate hypercholesterolemic syndrome (Cherry et al., 2019). It should be mentioned that high soluble fibers levels, such as pectin which is naturally present in seaweeds, have hypocholesterolemic effects (Castro et al., 2005), because they inhibit digestion and absorption of dietary lipids (Matanjun et al., 2010). Moreover, spirulina contains bioactive compounds (e.g. phycocyanin, β -carotene and phenolic components), with hypocholesterolemia effects (Colla et al., 2008).

In this study, plasma CHOL and TRIG significantly decreased in fish fed algal mixture supplemented diets, especially in MIX10 group suggesting hypolipidemic effect of algal mixture. This hypolipidemic effect was associated with lower VSI value in MIX10 diet, too. Similarly, supplementing a diet with single or a mixture of *G. gracilis* and microalga *N. oceanica* significantly reduced serum TRIG in European seabass (Batista et al., 2020 b). Morshedi et al (2018) reported that 9% FM replacement with *G. pulvinata* significantly reduced serum triglycerides and cholesterol in *L. calcarifer*. Velasquez et al. (2016) reported that supplementing diet with SM significantly reduced serum TRIG in Nile tilapia. Furthermore, supplementing a diet with *Pyropia columbina* abated plasma cholesterol and triglycerides in juvenile Pacú (*Piaractus mesopotamicus*), indicating hypolipidemic effects of seaweeds (Cian et al., 2019). Moreover, SP meal inclusion in diet of stinging catfish (*Heteropneustes fossilis*) fingerling significantly reduced CHOL, TRIG, and GLU (Rahman et al., 2023). On the other hand, Sabzi et al. (2023) reported that supplementing diet with 0.75–1.5% *Sargassum vulgare* extract significantly reduced cholesterol and triglycerides in common carp (*Cyprinus carpio*) juveniles.

An increase in the serum proteins could be associated with stronger immunity in fish (Wiegertjes et al., 1996). In our experiment, fish fed the MIX10% diet showed reduced total protein and globulin, suggesting a dose-dependent response in these immune parameters. It should be mentioned that plasma lysozyme was also reduced in this treatment but it was not statically significant. Similar results were reported in European seabass fed diet containing 7.5% *Ulva* spp. that reduced lysozyme activity compared to those fed control or 2.5% *Ulva* diets (Peixoto et al., 2016). Morshedi et al (2018) reported that 6–9% FM replacement with *Gracilaria pulvinata* significantly reduced total protein, immunoglobulin, alternative complement pathway and lysozyme activities in *L. calcarifer*, suggesting immunosuppression of seaweeds at high supplementation levels due to the presence of the

antinutritional factors. Also, Gora et al., (2018) reported that supplementing diet with 3–6% *Sargassum wightii* dry meal significantly reduced GLOB and total protein in *Labeo rohita* compared to those fed 1–2% fucoidan-rich extract. In the present study, plasma calcium increased in fish fed algal mixture supplemented diets, may be due to higher bioavailability of calcium in these ingredients leading to higher their absorption (Xu et al., 2020). In the present study, plasma ALP was reduced in fish fed MIX10% which may indicate health promoting effects of algal mixture on the liver. Also, the substitution of dietary FM with SP meal over 20% significantly increased serum AST in *L. calcarifer* (Yong et al., 2017). In contrast, inclusion of SP meal (0.1%) in a diet reduced serum AST in Nile tilapia (Shalata et al., 2021). In addition, Siddik et al. (2022) reported that dietary FM replacement with selenium enriched SP meal at 5% significantly reduced serum ALT and AST in *L. calcarifer*.

Conclusion

In conclusion, the results of the current research showed that replacement of dietary FM with micro- and macroalgal mixture at 5% level increased growth performance that was associated with higher feed intake and increasing amylase, ALP, total protease and trypsin activities in *L. calcarifer*. Also, the SOD activity and GSH level increased in the liver of fish with 5% FM replacement level. The liver GSH level and plasma LYZ activity increased with increasing the amount of SPM in diet. In addition, at 5% replacement level blood WBC increased, suggesting modulatory effects of micro and macro mixture on immunocompetence of fish. In addition, the inclusion of algal mixture showed distinct hypocholesterolemia effects by lowering plasma CHOL in *L. calcarifer*. Plasma calcium level increased with the increment of algal mixture in diet. Finally, plasma ALP level decreased by inclusion of algal mixture in the diet, suggesting health promoting effects of this alternative protein source on the liver tissue. More studies are required at molecular level such as transcriptomics to find the effects of various micro- and macroalgae blends on growth, antioxidant capacity and disease resistance of *L. calcarifer*.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' contributions

TM: project administration and methodology, and supervision. MTM: conceptualization, methodology, project administration interpretation of data, and writing original draft, and supervision. PM: project administration and methodology, MM: project administration and methodology, MK: project administration and methodology.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Availability of data and materials

Data will be available on reasonable request.

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Received: 24 V 2024

Accepted: 3 IX 2024