Nutritional comparison and evaluation of two macroalgae, Sargassum ilicifulium and Padina australis, as partial substitution with fish meal in practical diets of Asian sea bass juvenile (Lates calcarifer)

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Abstract

In this study, we used Asian seabass (*Lates calcarifer*) with initial weight of 29.0 ± 1.0 g as the model organism to investigate the effects of dietary *Sargassum ilicifolium* and *Padina australis* on various aspects of growth and immune response. We formulated three diets in which fish meal (FM) was gradually replaced by *S. ilicifolium* (SIM) and *P. australis* meal (PAM) at a substitution level of 6% (SIM 6 (S6) and PAM 6 (P6)). The control diet (C) did not contain any macro algae. Our findings revealed that the group given the P6 diet exhibited significantly higher growth and feed utilization than the control group ($P < 0.05$). Our findings indicate that the inclusion of *P. australis* in the diet had a significant impact on red blood cells (RBC), haemoglobin (Hb), hematocrit (Hct), white blood cells (WBC), lymphocytes, neutrophils, and cholesterol ($P < 0.05$). Furthermore, the *S. ilicifolium* diet resulted in significantly higher levels of total protein and triglyceride in the fish as compared to the other groups ($P < 0.05$). Our study showed that the group fed with 6% *P. australis* had significantly higher levels of immunoglobulin and lysozyme in both skin mucus and serum compared to the control group ($P < 0.05$). Furthermore, the group fed with 6% *S. ilicifolium* exhibited significantly higher levels of serum immunoglobulin ($P < 0.05$). However, there were no significant differences in alternative complement activity and serum lysozyme among all treatment groups ($P > 0.05$). After evaluating the activity of digestive enzymes, including protease, lipase, and amylase, we observed no significant differences among the treatment groups ($P > 0.05$). Additionally, we measured the expression levels of growth-related genes, such as insulin-like growth factor I (IGF-I), lysozyme (LZ), and interleukin-1β (IL-1β). Our findings indicate that the P6 group had significantly higher expression levels of these genes compared to all other treatment groups ($P < 0.05$). In conclusion, our research demonstrates that incorporating *Padina australis* into the diet of Asian seabass juveniles and partially replacing fish meal has positive effects on their immune system and growth performance.

Introduction

Nowadays the increasing fish production leads to an increase in fish feed production as well as the demand for feed raw materials and additives. Feed manufacturing are looking for alternatives to replace the declining fish stocks and raw material sources. Developing feed technologies helped to increase the rate of substitution of terrestrial or marine vegetable protein sources instead of fish flour-based feeds.

Being among marine plant protein sources, seaweed and microalgae can be used in fish feeds at certain rates. Seaweed has lower protein content than microalgae. On the other hand, seaweed, especially when used at low rates (< 10%) in feeds (Norambuena et al. 2015; Peixoto et al. 2016), have shown many beneficial effects on fish health and development thanks to metabolites such as peptides, carotenoids, phenolics, essential amino acids, essential fatty acids, functional carbohydrates, vitamins and minerals in their content (Wan et al. 2019).

Beneficial properties of seaweed such as antibacterial, antiparasitic, antiviral, antioxidant, anti-inflammatory, and hepatoprotective make them favourable fish feed additives. Research have shown that the addition of seaweed to fish feeds below 10% provides an increase in the activation of digestive

enzymes, growth performance, disease resistance, resistance to different stressors, improves meat quality and/or lipid metabolism (Ergün et al. 2009; Yıldırım et al. 2009; García-Vaquero and Hayes 2016; Morshedi et al. 2018; Wan et al. 2019; Fumanal et al. 2020; Jyotsna et al. 2021; Zuo et al. 2022; Xie and Niu 2022; Ragunath and Ramasubramanian 2022).

The production of Asian sea bass, *Lates calcarifer* which is an important fish species for increasing the aquaculture potential in the Persian Gulf and Oman Sea (Ashouri et al. 2018), has reached around ~ 117 thousand tons per year (FAO 2020). Being among brown seaweed, *Sargassum ilicifolium* and *Padina australis* are widely grown in the Persian Gulf, and the source of the additives should be ideally close to the feed production factories.

Digestive enzymes, hematological parameters, immunological responses, serum biochemical variables, immune and growth-related gene expression responses are important markers routinely used to evaluate the impacts of feed additives on fish health. Previous studies have reported beneficial effects of *S. ilicifolium* meal in some fish species like *Oncorhynchus mykiss* (Zamannejad et al. 2016), *Huso huso* (Yeganeh and Adel 2019), *Rastrelliger kanagurta* (Akbary 2019)d *calcarifer* (Zeynali et al. 2020). However, no study is available on the effect of the *P. australis* meal on the growth performance and/or health status of fish. Therefore, in the present study, dietary *S. ilicifolium* and *P. australis* meal were administered to *L. calcarifer*, aiming to compare the effects of two marco algae on growth performance, body proximate composition, digestive enzyme activities, hematological parameters, serum biochemical variables, serum and mucus immune parameters, and immune and growth related gene expression responses.

Materials and Methods

Experimental design and feed formulation

The Asian sea bass (*Lates calcarifer*) were purchased from Ramooz Company (Bushehr, Iran) and transported to the laboratory. 180-piece fish were acclimated to laboratory conditions for two weeks in two 4000-L tanks and fed on a commercial diet (Byza, Iran) containing 40% crude protein, 17% crude fat, 2% crude fiber, and 14% ash before starting the experiment. After the adaptation phase, fish with an average weight of 31.0 ± 0.3 g were randomly selected and stocked in nine 300-L tanks (triplicate groups per dietary treatment) at 20 fish per tank density. They were fed with dietary supplements for six weeks, twice a day (at h 10am and 5pm). *Sargassum ilicifolium* and *Padina australis* (SIM and PAM) were collected from the Persian Gulf coast area, spread out after washing in the tray (extending the surface to dry), and dried in the shade for 24 hours. Then the product was collected and dried in a furnace at 60°C for 24 hours and then milled. The basal diet was formulated, containing 40% crude protein and 20% crude lipid (Table 1). The two test diets were formulated by adding the SIM and PAM to the basal diets. Fish meal was replaced at levels of 6% with dried SIM (S6) and PAM (P6), and a control test diet was free of algae (C) (Table 1).

Table 1

Ingredient of the experimental diets (g kg^{-1}).

Ingredient	Control	S6	P6
Fish meal [1]	480	420	420
PAI/DSI[3]	0	60	60
Soybean meal	100	100	100
Corn gluten meal	100	145	145
Fish oil[2]	67.5	67.5	67.5
Soybean oil[4]	67.5	67.5	67.5
Wheat meal	90	50	50
Vitamin premix[5]	15	15	15
Mineral premix[5]	15	15	15
Squid meal	5	5	5
Antioxidant	2	2	2
Gelatin	45	45	45
Carboxymethyl cellulose	13	8	8
Crude protein	40.92	39.48	39.19
Crude fat	20.92	20.20	20.65
Ash	9.07	10.34	9.59
Moisture	9.54	8.63	9.15

[1]Pars kilka (Mazandaran, Iran) (635 g kg^{-1} crude protein, 177 g kg^{-1} crude lipid).

[2]Havorash (Bushehr, Iran)

[3]Dried *Sargassum ilicifulium* (protein, 5.54; lipid, 1% dry matter) and *Padina australis* (protein, 5.78; lipid, 1% dry matter)

[4]Product of Kesht Va Sanat Shomal Vegetable Oil Factories Complex (Neca, Iran).

[5]Vitamin and mineral premix (supplied by Beyza Feed Mill, Fars, Iran), and covered known requirements for Asian sea bass.

The physicochemical parameters of water containing temperature, pH, salinity and saturation dissolved oxygen were 27.0 ± 1.0 ºC, 7.3 ± 0.5, 45.0 ± 1.0 ppt and 70–80%, respectively (WTW model B3223 / set 1). The light period was 12 hours of light and 12 hours of darkness. All seawater used during the rearing process was collected from the Persian Gulf and was filtered after that it was used.

Sample collection and analysis

At the end of the growth trial, the fish in each of the 9 tanks were individually weighed and growth performance was evaluated by specific growth rate (SGR), weight gain (WG), feed conversion ratio (FCR), protein efficiency ratio (PER), K: Fulton's condition factor (K) and feed intake (FI) according to Azodi et al. (2016).

A total of nine fish per feeding group (three individuals per replicate tank) were randomly collected and stored at − 20°C for whole body proximate composition analysis. The moisture, crude protein, crude lipid, crude fiber and ash of experimental diets and whole body composition were measured according to the standard methods of the Association of Analytical Chemists (AOAC 1995).

At the end of the experiment (after 24 h starvation), the fish were collected for digestive enzyme analyses. Samples of the fish intestines (three fish from each replicate tank or nine fish per treatment) were homogenized immediately in 100 mM Tris–HCl buffer with 0.1 mM EDTA and 0.1% Triton X-100, pH 7.8, followed by centrifugation (30000×g; 12 min at 4°C). After centrifugation, the supernatant was collected and frozen at -80°C (Gisbert et al. 2016). Total protease, lipase and amylase were assayed according to the methods for total proteases (Walter 1984), bile salt-activated lipase (Iijima et al. 1998), α-amylase (Métais and Bieth 1968) and the soluble protein of homogenized samples was quantified (Bradford 1976).

Three fish from each replicate tank were randomly collected and sedated with 2-phenoxyethanol (0.5 mL per liter of water) before blood and mucus samples were withdrawn from the caudal vein using heparinized syringes, transferred into two eppendorf tubes. One of them was centrifuged (5000 × g) for 10 min to obtain blood serum, and another was used for blood counts according to Blaxhall and Daisley (1973) and Dacie and Lewis (2001). A polyethylene bag with 10 mL saline buffer (50 mM NaCl) was used to collect mucus (Ross et al. 2000). Serum and mucus lysozyme activity was determined by the method described by Ellis (1990) based on the lysis of the lysozyme- sensitive gram- positive bacterium *Micrococcus lysodeikticus* (Sigma, USA), and the results of lysozyme activity are given as units per milliliter. The serum total immunoglobulin level was measured according to Siwicki et al. (1994). The alternative complement activity (ACH50) was measured using rabbit red blood cell hemolysis (RaRBC) according to Yano (1992). Serum total protein, cholesterol and triglycerides were analyzed using an ACCENT 200 automated chemistry analyzer (PZ Cormay S.A., Warszawa, Poland).

For evaluation of relative expression of IL-1β, LZ and IGF-I genes, three fish from each replicate were sampled. The primers were designed using Primer 3 online software, and Ef1a was used as housekeeping gene (Table, 2). Relative targeted gene expression was calculated for each reaction by the ΔΔCt method (Livak and Schmittgen 2001).

Table 2
Primers sequences and amplification efficiencies

Gene name	Sequences of primers	Accession number	Efficiency
IGF-I	Forward: ACGCTGCAGTTTGTATGTGG Reverse: CCTTAGTCTTGGGAGGTGCA	XM_018697285.1	98%
LZ	Forward: GGTGTTTCTGCTCTTGGTGG Reverse: GCCGTAGTCAGTGGATCCAT	XM_018667849.1	99%
IL-1β	Forward: CCTGTCGCATTTCAGTACGG Reverse: ATTTCCACCGGCTTGTTGTC	XM_018669006.1	95%
Ef1a	Forward: AAATTGGCGGTATTGGAAC Reverse: GGGAGCAAAGGTGACGAC	GQ507427.1	97%

Abbreviations are as follow: IGF-1: insulin-like growth factor I; LZ: lysozyme; IL-1β: interleukin-1β and Ef1α:

Elongation factor 1 α.

Statistics

Data were analyzed using SPSS ver.22.0 statistical software. All the data are presented as means ± SE determined from three replicates. One-way ANOVA was performed with a significance level of 0.05 following confirmation of normality and homogeneity of variance. Tukey's procedure was used for multiple comparisons.

Result

Fish growth performance

Results of the growth performance of juvenile *Lates calcarifer* fed by *Sargassum ilicifolium* and *Padina australis* are shown in Table 3. Fish growth parameters were improved significantly with macro algae diet ($P < 0.05$). The final body weight (BW_f), WG, SGR, FCR, and PER in P6 group were significantly higher than those of control group ($P < 0.05$). However, FCR and PER in S6 group were no significant differences with relative to the control group ($P > 0.05$). There was no significant change in K, FI, and survival in all groups ($P > 0.05$).

Table 3
Growth performance and feed utilization in *L. calcarifer* juveniles fed diet containing different macro algae *Sargassum ilicifolium* and *Padina australis* for six weeks (mean ± SE). A different superscript in the same row denotes statistically significant differences ($P < 0.05$).

	Diets		
Parameters	Control	S6	P6
BW_i (g)[a]	31.00 ± 0.11[a]	31.25 ± 0.14[a]	31.50 ± 0.28[a]
BW_f (g)[a]	54.35 ± 0.31[c]	59.08 ± 0.25[b]	62.61 ± 0.46[a]
WG (%)[b]	75.34 ± 1.01[b]	89.14 ± 3.15[a]	98.84 ± 3.30[a]
SGR (% BW day^{-1})[c]	1.40 ± 0.01[b]	1.59 ± 0.04[a]	1.71 ± 0.04[a]
K (%)[d]	0.71 ± 0.02[a]	0.69 ± 0.01[a]	0.66 ± 0.01[a]
Survival (%)[e]	100 ± 0.00	100 ± 0.00	100 ± 0.00
FI (g day^{-1})[f]	0.22 ± 0.00[a]	0.22 ± 0.00[a]	0.22 ± 0.00[a]
FCR[g]	2.09 ± 0.04[a]	1.87 ± 0.06[ab]	1.81 ± 0.06[b]
PER[h]	1.21 ± 0.02[b]	1.36 ± 0.04[ab]	1.41 ± 0.05[a]
[a] BW_i and BW_f::initial and final body weight			
[b]WG: weight gain (%) = $((BW_f - BW_i)/BW_i) \times 100$			

[c] SGR: specific growth rate (%) = $((\ln BW_f - \ln BW_i) / t) \times 100$, where t is experimental period = 42 days.

[d] K: Fulton's condition factor = $(BW_f /$ standard lenrth$^3) \times 100$

[e] SUR: survival (%) = number of fish in each group remaining on day 42/initial number of fish) × 100

[f] FI: feed intake = total feed intake per tank (g) / number of day

[g]FCR: feed conversion ratio = feed intake (g) / weight gain (g)

[h] PER: protein efficiency ratio = protein intake (g) / weight gain (g)

Body proximate composition

The body composition of *L. calcalifer* fed with of *S. ilicifolium* and *P. australis* macroalgae in different experimental diets are shown in Table 4. There was no significant difference in the contents of moisture,

protein, lipid, ash, and fiber among all treatment groups (P > 0.05).

Table 4
Body proximate composition (%, mean ± SE, n = 3) of *L. calcarifer* juveniles fed *Sargassum ilicifolium* and *Padina australis* supplemented diets for six weeks. No significant changes were observed (*P* < 0.05).

	Diets		
Parameters	Control	S6	P6
Moisture	71.06 ± 0.10	69.42 ± 0.89	71.36 ± 0.28
Protein	16.66 ± 0.18	17.32 ± 0.75	16.42 ± 0.37
Lipid	5.62 ± 0.25	5.65 ± 0.26	5.53 ± 0.13
Ash	4.79 ± 0.27	4.87 ± 0.09	4.50 ± 0.31
Fiber	0.51 ± 0.16	0.24 ± 0.04	0.31 ± 0.08

Hematological and serum biochemical parameters

As shown in Table 5, hematological and serum biochemical parameters were improved significantly with macro algae diet (P < 0.05). The RBC, Hb, Hct, WBC, lymphocytes, neutrophils, and cholesterol contents significantly increased as a consequence of feeding fish in P6 group compared to control (P < 0.05). It was observed that the levels of total protein and triglyceride in fish fed S6 diet significantly increased compared to the other groups (P < 0.05). There was no significant difference in the contents of monocytes and eosinophil among all treatment groups (P > 0.05).

Table 5
Hematological and serum biochemical parameters (%, mean ± SE, n = 3) of *L. calcarifer* juveniles fed *Sargassum ilicifolium* and *Padina australis* supplemented diets for six weeks. A different superscript in the same row denotes statistically significant differences ($P < 0.05$).

	Diets		
Parameters	Control	S6	P6
RBC ($\times 10^6$ μl^{-1})	2.54 ± 0.00^{b}	2.69 ± 0.00^{ab}	2.88 ± 0.00^{a}
Hb (g dL^{-1})	6.66 ± 0.03^{b}	7.06 ± 0.21^{b}	7.65 ± 0.02^{a}
Hct (%)	27.66 ± 0.33^{b}	29.00 ± 0.57^{ab}	30.50 ± 0.28^{a}
WBC ($\times 10^3$ μl^{-1})	8.03 ± 0.12^{b}	8.66 ± 0.17^{ab}	9.10 ± 0.34^{a}
Lymphocytes (%)	75.66 ± 0.88^{a}	76.00 ± 1.15^{a}	71.00 ± 0.57^{b}
Neutrophils (%)	20.00 ± 0.57^{b}	20.00 ± 0.57^{b}	23.00 ± 0.00^{a}
Monocytes (%)	4.00 ± 0.00^{a}	3.66 ± 0.66^{a}	5.00 ± 0.57^{a}
Eosinophils (%)	0.33 ± 0.33^{a}	0.33 ± 0.33^{a}	1.00 ± 0.00^{a}
Total protein (g dL^{-1})	5.04 ± 0.05^{b}	5.58 ± 0.16^{a}	5.37 ± 0.02^{ab}
Cholesterol (mg dL^{-1})	187.67 ± 5.81^{b}	206.33 ± 4.37^{a}	209.50 ± 0.86^{a}
Triglyceride (mg dL^{-1})	115.33 ± 0.88^{ab}	123.67 ± 2.90^{a}	113.50 ± 2.02^{b}

Serum and mucus immune parameters

As shown in Table 6, serum total Ig contents significantly increased as a consequence of feeding fish with macro algae diets compared to control ($P < 0.05$), and the S6 group had the highest serum total Ig ($P < 0.05$). There was no significant difference in the contents of ACH50 and serum lysozyme among all treatment groups ($P > 0.05$). Mucus immune parameters were improved significantly with macro algae diet ($P < 0.05$). The mucus total Ig and lysozyme contents significantly increased as a consequence of feeding fish in P6 group compared all treatment groups ($P < 0.05$).

Table 6
Serum and mucus immune parameters (mean ± SE, n = 3) of *L. calcarifer* juveniles fed *Sargassum ilicifolium* and *Padina australis* supplemented diets for six weeks. A different superscript in the same row denotes statistically significant differences ($P < 0.05$).

	Diets		
Parameters	Control	S6	P6
Serum Total Ig (mg ml^{-1})	26.40 ± 0.45^{c}	28.40 ± 0.45^{b}	27.35 ± 0.05^{a}
ACH50 (U ml^{-1})	123.67 ± 0.33^{a}	124.67 ± 0.33^{a}	124.00 ± 0.57^{a}
Serum Lysozyme (U ml^{-1})	25.00 ± 0.57^{a}	25.00 ± 0.57^{a}	26.50 ± 0.28^{a}
Mucus Total Ig (mg ml^{-1})	3.90 ± 0.11^{b}	4.06 ± 0.08^{ab}	4.35 ± 0.02^{a}
Mucus Lysozyme (U ml^{-1})	4.53 ± 0.20^{b}	5.23 ± 0.24^{ab}	5.75 ± 0.02^{a}

Digestive enzymes

The effect of dietary *S. ilicifolium and P. australis* level on the digestive enzyme activities is shown in Table 7. There was no significant difference in the contents of protease, lipase, and amylase among all treatment groups ($P > 0.05$).

Table 7
Digestive enzymes of *L. calcarifer* juveniles fed diet containing different macro algae *Sargassum ilicifolium* and *Padina australis* for six weeks (mean ± SE). No significant changes were observed ($P < 0.05$).

	Diets		
Parameters	Control	S6	P6
Protease (U mg $protein^{-1}$)	1.36 ± 0.55	1.38 ± 0.59	1.50 ± 0.28
Lipase (U mg $protein^{-1}$)	0.21 ± 0.00	0.16 ± 0.01	0.18 ± 0.00
Amylase (U mg $protein^{-1}$)	2.76 ± 0.11	2.80 ± 0.10	2.97 ± 0.07

Expression of immune-related and growth-related genes

The effect of dietary *S. ilicifolium* and *P. australis* level on the expression of immune-related and growth-related genes (IGF, IL1ß, and LZ) is shown in Table 8. Immune-related and growth-related genes were increased significantly with macro algae diet ($P < 0.05$). The IGF, IL1ß, and LZ in P6 group were significantly higher than among all treatment groups ($P < 0.05$).

Table 8
Expression of immune-related and growth-related genes of *L. calcarifer* juveniles fed diet containing different macro algae *Sargassum ilicifolium* and *Padina australis* for six weeks (mean ± SE). A different superscript in the same row denotes statistically significant differences ($P < 0.05$).

	Diets		
Parameters	Control	S6	P6
IGF-I	1.01 ± 0.16^{c}	4.74 ± 0.14^{b}	8.31 ± 0.32^{a}
IL1ß	1.04 ± 0.22^{c}	4.48 ± 0.25^{b}	6.29 ± 0.48^{a}
LZ	1.25 ± 0.23^{c}	2.25 ± 0.17^{b}	3.36 ± 0.18^{a}

Discussion

According to the results of the present study, *Sargassum ilicifolium* and *Padina australis* supplementation significantly improved growth and feed efficiency in Asian seabass, *Lates calcarifer*. Similarly, *S. ilicifolium* was added to the feed of Juvenile Asian seabass at different rates (3, 6 and 9%), six weeks after feeding; all *S. ilicifolium* supplemented groups were improved growth performance (Zeynali et al. 2020). The improvabilities of *S. ilicifolium* and *P. australis* on the fish growth parameters may be related to their improving the gut morphology and its structure and stimulating the secretion of digestive enzymes (Zeynali et al. 2020). Previous studies have reported that inclusion of different marine seaweed meals such as *Kappaphycus alvarezii* (Shapawi et al. 2015), *Gracilaria pygmaea* (Farhoudi et al. 2020) *Sargassum polycystum* (Nazarudin et al. 2020) improved growth performance and/or protein efficiency ratio of Asian seabass. However, addition of *Gracilaria pulvinata* meal (Morshedi et al. 2018)d *alvarezii*, *Eucheuma denticulatum* and *Sargassum polycystum* (Shapawi and Zamry 2016) to Asian seabass feeds did not show any effect on growth performance.

The present study found that the fish fillet proximate composition was the same among all experimental groups. Similarly, many other seaweed species also not effected the fillet or whole body proximate composition of fish species, such as *Dicentrarchus labrax* (Peixoto et al. 2016), *Oncorhynchus mykiss* (Ribeiro et al. 2017), L. *calcarifer* (Morshedi et al. 2018; Zeynali et al. 2020), *Paralichthys olivaceus* (Ragaza et al. 2021), *Labeo rohita* (Shambhulingaiah et al. 2021) and *Micropterus salmoides* (Liao et al. 2022). Differently, several studies reported that the fillet or whole body proximate composition significantly changed in different fish species fed on some seaweed-incorporated diets (Ergün et al. 2009; Güroy et al. 2013; Abdel-Warith et al. 2016). These differences might be attributed to several factors such as different fish species, seaweed species, feeding period, experimental design, and specific formula of the diet or others.

Hematological parameters, white blood cells and serum biochemical parameters are a significant parameter benefitted in the evaluation of general health status in fish. The present study showed that

dietary 60 g kg^{-1} *P. australis* supplementation significantly improved the hematological profiles. This can be explained the higher bioavailability of iron for the erythropoiesis and haematosynthesis. Cian et al. (2016) reported that chelating peptides obtained from seaweed *Pyropia columbina* effectively enhance the bioaccessibility of iron. Earlier studies have reported that seaweed significantly enhanced the RBC counts, Hb values and/or Hct ratios of *L. calcarifer* (Zeynali et al. 2020), *Cirrhinus mrigala* (Ragunath and Ramasubramanian 2022) and *Oreochromis niloticus* (Nur et al. 2020; Fredrick Raja et al. 2022). In this study, an increase was observed in the granulocyte ratio and a decrease in the lymphocyte ratio was observed in fish when fed diets supplemented with P6. Similar results were recorded in *S. ilicifolium* (Zeynali et al. 2020) and *Pangasianodon hypophthalmus* (Abdelhamid et al. 2021) fed with seaweed incorporated diets. The decreasing number of circulating lymphocytes can be related to the induced transfer of lymphocytes from the blood into lymphoid organs (Yilmaz and Ergün 2018). On the other hand, the effect of seaweed to increase circulating neutrophil counts may be related to their effectiveness in inhibiting neutrophil migration through the capillary endothelial barrier by increasing *TNF-α* and *IL-8* gene expressions, as has been reported earlier in fish fed with different feed additives (Kumari et al. 2003; Yilmaz and Ergün 2018).

In the present study, no difference occurred in the RBC count, Hb value, Hct ratio lymphocyte and neutrophil percentages in S6 group. Differently, Zeynali et al. (2020) found that *S. ilicifolium* meal especially at 6 and 9% significantly increased the RBC count and neutrophil ratio and decreased lymphocyte ratio of *L. calcarifer* compared with the control group after a 6 week feeding experiment. These different results might be associated with differences in experimental conditions and/or physiological status of fish.

In this study, serum total protein levels and WBC counts of fish fed with 6% *S. ilicifolium* containing feeds were the highest level whereas it did not significantly change in fish fed with 6% *P. australis* containing feeds. Increases in serum protein values and WBC counts are usually thought to be associated with a stronger innate immune response in fish (Yilmaz and Ergün 2018). Parallel with our study, increased serum protein values and/or WBC counts have been recorded in *L. calcarifer* fed with *Sargassum polycystum* (Nazarudin et al. 2020), L. *calcarifer* fed with *S. ilicifolium* (Zeynali et al. 2020), A. *persicus* fed with *Gracilaria persica* (Adel et al. 2021) and *Sparus aurata* fed with *Gracilaria gracilis* (Passos et al. 2021).

In our study, fish fed the S6 and P6 diets showed an increased cholesterol levels compare to fish fed on the control diet, while the incorporation of seaweed additives did not show any influence on serum triglyceride levels of experimental fish. However, in different studies, when seaweeds were added to diets of fish, it was reported that they decreased serum cholesterol or triglyceride levels of fish (Cian et al. 2019; Ale et al. 2021). Unlike our study, Zeynali et al. (2020) reported inclusion of *S. ilicifolium* at 3, 6 or 9% levels did not affect serum cholesterol and triglyceride in *L. calcarifer*. Differences between studies can be explained with the differences in feed processing, experimental condition, and physiological status of fish.

Increased serum and mucus immune parameters and/or immune related gene expression responses in *L. calcarifer* by seaweed additives could be due to immunomodulatory effects. No study on the effects of *P. australis* meal on fish immune responses has been found in the literature so far. However, *P. astraulis* polysaccharide extract has been reported to improve some non-specific immune parameters in shrimp (Akbary and Aminikhoei 2018; Kilawati et al. 2021).

In this study, digestive enzymes were not affected by the dietary treatments. There are contradictory data in the literature on the effect of macro algae on digestive enzymes of fish. A similar effect has also been reported in the level of total alkaline protease activity in intestinal extracts of juvenile *S. aurata* fed on diets supplemented with G. cornea or *U. rigida* (Vizcaíno et al. 2015). Moreover, seaweed supplementation (*Gracilaria* spp., *Ulva* spp. *and Fucus* spp.) in practical diets for European seabass and Asian seabass juveniles have no significant impact on digestive enzyme activities (Peixoto et al. 2016; Morshedi et al. 2018). In contrast with the results of this study, dietary supplementation of *Ulva rigida* (Akbary et al. 2018) and *Padina astraulis* (Hauk) (Akbary and Shahraki 2019) extracts significantly increased pancreatic digestive enzymes in grey mullet, *Mugil cephalus*. Moreover, the study of Zeynali et al. (2020) revealed that fish fed on the diet supplemented with 6% SIM had higher pancreatic digestive enzymes activities than other groups. The presence of soluble non starch polysaccharides (NSP) in seaweeds and the limited capacity in fish intestinal microflora to degrade NSP may reduce the rate of diffusion of digestive enzymes to substrates (Shpigel et al. 2017). In addition, the presence of several antinutrients in seaweeds may negatively affect the nutritional quality and nutrients digestion/absorption of seaweeds (Silva et al. 2015).

IGF-1 gene is mainly secreted by the liver via activation of the GH receptor. In addition, a pro-inflammatory cytokine *IL1ß* is produced mainly by monocytes/macrophages leading to proliferation of leucocytes, and it plays an important role in the fish immunological defenses against infections and mediates the secretion of other cytokines (Corripio-Miyar et al. 2007). Similar to our findings, it has been reported *S. ilicifolium* meal significantly increased serum total immunoglobulin, and liver *IGF-I*, *IL1ß* and *LZ* levels in *L. calcarifer* (Zeynali et al. 2020). Choi et al. (2014) added 0.5 and/or 1% brown macroalgae (*Hizikia fusiformis*) to the feeds of *Paralichthys olivaceus* and plasma IGF-I levels increased with increasing liver pro-inflammatory cytokine (*IL-2* and *IL-6*). Parallel with our study, feeding *L. calcarifer* with 0.1 and 0.2% of *Sargassum sp*. extract resulted in increased total immunoglobulin and head kidney *lysozyme* gene expression (Yangthong et al. 2016). However, unlike our study, serum lysozyme levels decreased with increasing dietary *G. pulvinata* in *L. calcarifer* (Morshedi et al. 2018). Therefore, additional research experiments are still required to illuminate the roles of ingredients in seaweeds in the modulation of the immune response.

In conclusion, the results of the current study showed that the replacement of a small portion (6%) of dietary FM with *P. australis* and *S. ilicifolium* improved growth performance and innate immune parameters in *L. calcarifer* juveniles. Furthermore, whole body proximate composition and pancreatic digestive enzymes did not significantly change in different fish species fed on the seaweed-incorporated diets. At transcriptional level, our findings demonstrated that supplementing diet with *P. australis* and *S.*

ilicifolium up-regulated liver IGF-1, lysozyme and IL-1β gene expression that could be resulted in higher growth rate and immunomodulatory effects of seaweeds in these groups. Overall, according to the findings of this study 6% of dietary FM could be replaced with *P. australis* to improve of growth and health status in *L. calcarifer* juveniles compared to 6% of dietary FM replaced with *S. ilicifolium*.

Declarations

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Authors' contributions

All persons listed as authors have read and contributed to preparing the manuscript as given below:

Vahid Morshedi, Reza Gamoori and Shirin Hamedi carried out fish maintenance and sample collection

Seyed Hossein Moradian carried out body composition analysis

Vahid Morshedi and Sevdan Yilmaz wrote the article

Vahid Morshedi, and Shirin Hamedi the experimental design and statistical analyses.

Ahmad Ghasemi carried out gene expression

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Ethics approval

This study was carried out by the principle of the Basel Declaration and recommendations of the Faculty of Veterinary Medicine at the University of Tabriz, the FVM.REC.1396.939. The protocol was approved by the FVM.REC.1396.939.

Conflicts of interest/Competing interests

The authors declare that there is no conflict of interest regarding the present data and manuscript.

Availability of data and material (data transparency)

The supporting data is accessible from the corresponding author upon reasonable request.

Consent to participate

The authors declare that they have every consent to participate

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