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Effect of Dietary Seaweed Supplementation on Physiological Responses of Genetically Improved Farmed Tilapia

A. Priyatharshni[1], Cheryl Antony[1], A. Uma[1], B. Ahilan[1], P. Chidambaram[1], P. Ruby[1], E. Prabu[1] 10.18805/IJAR.B-5281

ABSTRACT

Background: The present study aimed to determine the immune and stress responses of juvenile GIF tilapia fed with *Ulva* seaweed meal supplemented diet.

Methods: The study was undertaken with different inclusion levels of *Ulva* seaweed meal (USM) such as raw (UR) and fermented (UF) *Ulva* meal (UR-5, UR-10, UR-15, UF-5, UF-10 and UF-15% of diet) and control feed (C) for a period of 60 days.

Result: GIF tilapia fed with fermented *Ulva* meal at a 10% inclusion level in their diet showed the highest growth (41.43 g). Immune parameters such as catalase activity (CAT), super oxide dismutase (SOD), respiratory burst activity (RBA), myeloperoxidase activity (MPA) and serum protein value (SPV) showed significant increases in fish fed with *Ulva* meal incorporated diet followed by UF-5, *Ulva* Raw (UR) 5%, control (C), UF-15, UR-10 and UR-15 diet respectively. Decreased glucose level was recorded in GIFT fed with fermented *Ulva* meal at 10% inclusion level followed by UF5, UR5, control diet, UF15, UR10 and UR15 diet. Increased plasma cholesterol, triglyceride, GPx activity, GRA activity and GST activity were recorded in GIFT tilapia fed with fermented *Ulva* meal at 10% inclusion level followed by UF5 diet, UR5 diet, control diet, UF15 diet, UR10 diet and UR15 diet. GIFT fed with fermented *Ulva* meal at a 10% inclusion level in their diet exhibited the highest growth performance (41.43 g) followed by UF5 (41.07±0.43 g), UR5 (39.65±0.36 g), control (39.09±0.20 g), UF15 (38.67±0.26 g), UR10 (38.47±1.47 g) and UR15 (35.77±0.35 g) diet. GIF tilapia fed with UF10 diet showed lowest FCR (1.34±0.00), highest SGR (0.99±0.00) and PER (2.37±0.07) followed by UF5 diet, UR5 diet, control diet, UF15 diet, UR10 diet and UR15 diet. One-way ANOVA data analysis and followed by Tukey's test for conducting pair wise comparisons at significance level of 0.05. GIFT tilapia fingerlings had significant differences ($P<0.05$) among the different experimental diets. From the present experiment, it could be concluded that, *Ulva* meal incorporation at 10% in the diets increases immune responses in GIF tilapia.

Key words: GIF tilapia, Immune parameters, Physiological response, Seaweed, Stress parameters.

INTRODUCTION

Aquaculture emerges as the most rapidly growing sector in the domain of food production on a worldwide level. The role of nutrition, feed and feed management is significant in advancement of sustainable aquaculture. Seaweeds are marine macroalgae that proliferate abundantly in shallow waters of the sea, estuaries and backwaters up to a depth of 118 meters where 0.1% photosynthetic light is accessible (Chapman, 1970; Okazaki, 1971). Global cultivation of algae, dominated by marine macroalgae known as seaweeds, grew by half a million tonnes in 2020, up by 1.4 per cent from 34.6 million tonnes in 2019 (FAO, 2022). Some major producing countries including China and Japan experienced growth in 2020, while seaweed harvests decreased in Southeast Asia and the Republic of Korea. During the last decade, substantial improvements have been achieved in reducing the percentage of fishmeal and fish oil in pelleted feeds (Naylor *et al*., 2021). Marine resources continue to have an important role in aquafeed, the use of plant-based ingredients has been increasing steadily, creating connections between land and sea (Hardy and Lee, 2010). The inclusion of seaweed as dietary constituent for fish has led to an increased immune response in various species, including Olive flounder (Pham *et al*., 2006; Choi *et al*., 2015), Red seabream (Gakkaishi *et al*., 1987), Trout (Valente *et al*., 2015) and White-spotted spinefoot (Xu *et al*., 2011). Nader *et al.* (2010) carried out the study on the effect of green seaweeds (*Ulva* sp.) as feed supplements in fish diet on growth performance, feed efficiency and body composition of Red Tilapia (*Oreochromis sp.*). Llias *et al.* (2015) studied that the enrichment of nutritional value could be achieved by using solid state fermentation when using seaweed and palm kernel cake as alternative protein ingredients in fish feed. The present study was aimed to evaluate the efficacy of incorporating seaweed into the diet on growth performance and immune and stress responses during intensive farming of GIF tilapia.

[1]Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Dr. M.G.R. Fisheries College and Research Institute, Ponneri-601 204, Tamil Nadu, India.

Corresponding Author: A. Priyatharshni, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Dr. M.G.R. Fisheries College and Research Institute, Ponneri-601 204, Tamil Nadu, India.
Email: priyaarivoli@gmail.com

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MATERIALS AND METHODS

Experimental diet

The *Ulva* seaweed species were collected from the near-shore waters of the Indian Ocean Coast at Pamban, Rameshwaram, Tamil Nadu, India. Fermentation of seaweed meal was done using method described by Siddik *et al*. (2018). After fermentation process, seaweed was dried in hot air oven at 40°C for 48 h and later used as fermented seaweed meal which was the test ingredient. Six different isonitrogenous and isoenergetic experimental diets were prepared utilizing green seaweeds, with crude protein of 320 g/kg. These diets included varying amounts of *Ulva* meal, labelled as raw (UR) at 5.0, 10.0, 15.0% levels and fermented (UF) at 5.0, 10.0 15.0% levels within the diet. The control diet (C) without *Ulva* meal.

Proximate analysis of experimental feed

The proximate analysis of experimental feed was estimated using standard method (AOAC, 2010). The feed ingredients, *Ulva* seaweed (Raw and Fermented) proximate composition and proximate composition of experimental diets were presented in Table 1, 2 and 3.

Experimental setup and experimental fish

The experimental fish GIF tilapia (*Oreochromis niloticus*) was procured from Centre for Sustainable Aquaculture (CeSA), TNJFU, Barur, Tamil Nadu. The experimental study comprised of six sets of treatments and one set of control with three replicates. FRP tanks (water volume: 350 l; length: 94 cm, height: 61 cm, breadth: 70 cm) were used for experiment. The fish seeds were properly acclimatized in FRP tanks and were nursed for 30 days with commercial diet. All fishes were graded according to their weight prior to the experiment. 2.51 g weight of GIFT tilapia juveniles were stocked @ 20/m^2 in each experimental tank. The fishes were fed @ 5% of their body weight every day. The feeding ration was divided into three equal quantities and given thrice a day *viz*., morning, afternoon and evening. The faecal matter and uneaten feeds were siphoned out daily and water exchange was done @ 10% in order to maintain water quality in all tanks, growth sampling was done at fortnightly. During feeding trial, water quality parameters were monitored daily and mean values were recorded as per standard procedure (APHA, 2005).

Immuno-physiological responses

At the end of the feeding trial, six fish from each treatment group were selected and anaesthetized with clove oil @ 50 µ LL^{-1} of water before taking blood from fish. Blood was taken from caudal vein region using a medical syringe which was previously rinsed with 2.7% EDTA solution. Blood were collected then transferred immediately to test tube containing thin layer of EDTA powder. Further serum was collected when blood was collected without using anticoagulant and transferred into the tubes without containing anticoagulant and keeps the tubes in slanting position for around 2 h and centrifuged at 3500 rpm at 4°C followed by collection of yellow coloured serum with micropipette and samples were stored at -20°C till use (Ruby *et al*., 2022).

Respiratory burst activity analysis

Respiratory burst activity was performed following the modified method of Anderson and Siwiki (1995). 0.2% Nitroblue tetrazolium solution was added to 0.1 ml of blood sample and incubated for 30 min at room temperature. 1.0 ml N, N-dimethyl formamide was added to the 0.05 ml of the NBT blood cell suspension and centrifuged for 5 min at 5000 rpm. The supernatant was collected and read on a spectrophotometer at 540 nm.

Serum protein value analysis

The protein estimation in serum was done by Lowry's method (1951). Serum total protein was estimated according to Biuret method using the kit (Erba, India). Protein present in the serum binds with copper ions an analkaline medium of the biuret reagent and produces a purple-coloured complex, absorbance is proportional to the protein concentration. Three test tubes were labelled as blank (B), standard (S) and test (T) were taken. In to all the tubes, 1 mL of biuret reagent and 2 mL of distilled water were added. A volume of 0.05 mL protein standard was taken in the test tube labelled as S and 0.05 ml of serum was added into the test tube labelled as T. It was mixed well and incubated at 37°C for 10 minutes. The absorbance of S and T were measured against B in spectrophotometer at 630 nm. The calculation was done as follows:

$$\text{Total protein (g dL}^{-1}) = \frac{\text{Absorbance of test (T)}}{\text{Absorbance of standard (S)}} \times 6$$

Myeloperoxidase activity (MPO) analysis

MPO was measured according to standard method of Quade and Roth (1997) with slight modification. About 10 µL of serum was diluted with 90 µL of hank's balanced salt solution (HBSS). Then 35 µL of 20 mM 3, 3′-5,5′-Tetramethyl Benzedrine Hydrochloride and 5 µL of 5 m MH_2O_2 (freshly prepared) were added to the serum. The colour change reaction was stopped after 2 minutes by adding 35 µL of 4M sulphuric acid (H_2SO_4) and OD was read at 450 nm.

Units/ml enzyme = (A 470 nm Test at 1 min - A 470 nm Blank at 1 min) (df)/(1.0) (0.035)
Where,
df = Dilution factor.
1.0 = The increase in A 470 nm/minute per unit of enzyme.
0.035 = Volume of enzyme (ml).

Catalase enzyme assay (CAT) analysis

The measurement of CAT was performed following the procedure outlined by Takahara *et al.* (1960). The blood sample (10-50 µl) was added to 2.5 ml phosphate buffer (50 mM/pH7). One microlitre of 0.3% H_2O_2 (freshly prepared) was added to above suspension. The decrease in absorbance was read at 240 nm for 3 mins at 30-second intervals. The enzyme blank activity was expressed as

nanomoles of H_2O_2 solution. Enzyme activity was expressed as nano-moles H_2O_2 decomposed/min/mg protein.

CAT (units/mg protein) =

$$\frac{[\text{OD/min (3)} \times \text{Total volume}]}{[34 \times \text{Sample volume} \times \text{Protein}]} \times 1000$$

Superoxide dismutase assay (SOD) analysis

Superoxide dismutase of serum samples was assayed according to the method described by Mishra and Fridovich (1972) based on the oxidation of epinephrine adrenochrome transition by the enzyme. The blood sample (10-50 µl) was added to 1.5 ml of carbonate buffer (0.1 M/ pH 10.2). A 0.5 ml of epinephrine (freshly prepared) was added to the above suspension. The increase in the absorbance was read at 480 nm for 3 mins at 30 sec interval.

$$\text{Inhibition \%} = \frac{\text{OD Blank-Change in OD/min}}{\text{OD blank}} \times 100$$

$$\text{SOD units} = \text{Inhibition \%/50} \times \text{Sample vol} \times 18$$

Analysis of glucose, cholesterol and triglycerides

Blood glucose, serum cholesterol and triglyceride were estimated using a semi-automatic blood biochemical analyzer (Alpha chem. 100 i). Three test tubes were labelled as Test (T), Blank (B) and Standard (S). To each test tube 10 µL of blood sample Test (T), R-reagent and 10 µL of distilled water in blank and same volume of reagent to standard solution in Standard (S) were added. Test tubes were shaken well and incubated for approximately 10 min at 37°C. The absorbance was read by using a semi-automatic blood biochemical analyzer (Alpha technologies).

$$\text{Glucose content (mg dL}^{-1}) = \frac{\text{Absorbance (sample)}}{\text{Absorbance (standard)}} \times 100$$

Analysis of glutathione-S-transferase

The enzymatic activity of glutathione-S-transferase was determined utilizing spectrophotometric method at a temperature of 25°C, following the procedure outlined by Habig *et al*., 1974. This evaluation entailed the measurement of the production of a GSH conjugate in conjunction with a wavelength of 340 nm, 1-chloro-2, 4-dinitrobenzene was employed with an extinction coefficient of 9.6 mM^{-1} cm^{-1}.

Analysis of glutathione-reductase activity

The determination of GR concentration was conducted in a spectrophotometer by measuring the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at a specific wavelength of 340 nanometers, in accordance with the method established by Carlberg and Mannervik, (1975).

Analysis of GPx activity

The quantification of GPx activity was based on the rate of NADPH oxidation through its interaction with GR at a wavelength of 340 nm, as per the guidelines outlined by Flohe and Gunzler, (1984). The activities of GR and GPx were quantified by assessing the quantity of NADPH consumed per minute per milligram of protein.

Water quality parameters

During the course of experiment, the water quality parameters were monitored and the mean values were as follows: Dissolved oxygen (DO) 6.09±0.03 mg/l, temperature 28.42±0.01°C, pH 8.28±0.02, alkalinity 167±0.26 mg/l, hardness 341±0.06 mg/ l, nitrite 0.032± 0.05 mg/l, nitrate 11.06±0.05 mg/l and ammonia 0.02±0.03 mg/l were observed. All the water quality parameters were estimated using standard procedures (APHA, 2005).

Statistical analysis

All the data were presented as the mean values ± standard deviation (SD) of three replicates. One-way ANOVA, followed by Tukey's test for multiple comparisons at the significance level of 0.05 was used to compare the differences between dietary groups. The data were statistically analyzed by SPSS 20.0 for windows (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Influence of *Ulva* seaweed meal on immune responses in GIF tilapia

The immuno physiological responses of GIF tilapia fed *Ulva* meal are given Fig 1-5. Significant differences in the catalase activity, super oxide dismutase, respiratory burst activity, serum protein values and myeloperoxidase activity were observed between the treatments and control. The increased values of catalase, SOD, respiratory burst activity, serum protein values and myeloperoxidase activity were observed in fermented *Ulva* supplemented diet at 10% inclusion level when compared with control and other treatments.

Increased catalase activity (0.88±0.02 u/mg of protein), SOD (3.18±0.22 u/mg of protein), RBA (0.88±0.02 units/ml), SPV (0.68±0.01 mg/ml) and MPA (6.72±0.04 u/ml of enzyme) values were recorded in GIF tilapia fed with fermented *Ulva* meal at 10% inclusion level followed by UF5 diet (CAT-0.82±0.01 u/mg of protein, SOD-3.13±0.12 u/mg of protein, RBA-0.81±0.02 units/ml, SPV-0.67±0.03 mg/ml and MPA-6.53±0.18 u/ml of enzyme), UR5 diet (CAT -0.66±0.04, SOD-2.62±0.06 u/mg of protein, RBA-0.69±0.02 units/ml, SPV-0.67±0.03 mg/ml and MPA-5.39±0.15 u/ml of enzyme), control diet (CAT-0.56±0.28 u/mg of protein, SOD-2.32±0.09 u/mg of protein, RBA-0.64±0.04 units/ml, SPV-0.63±0.05 mg/ml and MPA-5.04±0.15), UF15 diet (CAT-0.38±0.03 u/mg of protein, SOD-1.94±0.00 u/mg of protein, RBA-0.46±0.03 units/ml, SPV-0.24±0.02 mg/ml and MPA -3.48±0.22 u/ml of enzyme), UR10 diet (CAT -0.35±0.04 u/mg of protein, SOD-1.66±0.02 u/mg of protein, RBA-0.59±0.02 units/ml, SPV-0.17±0.03 mg/ml and MPA-2.30±0.16 u/ml of enzyme) and UR15 diet (CAT -0.31±0.02 u/mg of protein, SOD-1.29±0.05, RBA-0.44±0.06 units/ml, SPV-0.12±0.01 mg/ml and MPA -1.05±0.02 u/ml of enzyme).

Similarly, Shrimp fed with *U. pinnatifida* at 6% inclusion showed enhancement in SOD activity (Niu *et al*., 2015). Grey mullet fed with *Ulva rigida* extract at 10 mg/kg inclusion showed increased immune reponses (Akbary and Aminikhoei, 2018). Red seabream fed with *Ulva* meal at 5%

inclusion showed enhancement in complement activity and disease resistance (Satoh *et al*., 1987). Nile tilapia fed with *Ulva* spp. (*Ulva rigida* and *Ulva lactuca*) at 10% inclusion showed improved lysozyme or peroxidase activity (Valente *et al*., 2016). Rainbow trout fed with *Ulva intestinalis* extract at 1.5% inclusion showed enhancement in lysozyme and complement activity (Safavi *et al*., 2019). Nile tilapia fed with *Ulva fasciata* extract at 100 mg/kg inclusion showed enhanced antioxidant enzyme activities of superoxide dismutase and catalase activity (AboRaya *et al*., 2021). Shrimp fed with *U. pinnatifida* at 6% inclusion showed enhanced SOD activity (Niu *et al*., 2015). Nile tilapia fed with *U. clathrata* extract inclusion showed increased immune responses (Del Rocío Quezada-Rodríguez and Fajer-Avila, 2017). Juvenile grey mullet (*Mugil cephalus)* fed with *Ulva rigida* extract at 10% inclusion showed stimulated lysozyme, phagocytic and respiratory burst activity (Akbary and Aminikhoei, 2018).

SOD and CAT are important enzymes in antioxidant defence system as they play a key role in removing free radicals and toxicity of drugs and chemicals (Farombi *et al*., 2007). Increased SOD with sodium alginate due to the high presence of superoxide anions in polysaccharides (Yeh *et al*., 2008; Lee *et al*., 2017). Polysaccharides stimulate immune systems and can improve different parts of the innate immunity such as lysozyme, ACH_{50}, anti-protease activities, phagocytic activity, respiratory burst and phenoloxidase enzyme activities (Mohan *et al*., 2019). Presence of some bioactive compounds such as palmitic acid (Aparna *et al*., 2012; Raman *et al*., 2012; Wong *et al*., 2017), oleic acid (Hashimoto *et al*., 2006), clionasterol (Raman *et al*., 2012), phytol (McGinty *et al*., 2010; Santos *et al*., 2013) and neo-phytadiene (Raman *et al*., 2012) improved immune response in fishes. Improving immune system of fish may act indirectly to promote growth performance (Hindu *et al*., 2019; Mohan *et al*., 2019). Sulfated polysaccharides can stimulate the immune response by binding to cellular receptors of the immune system (Teruya *et al*., 2009; Jiao *et al*., 2011). Extracts derived from seaweeds are natural sources of antioxidants and they neutralize free radicals (Goiris *et al*., 2012). *Ulva* spp. contains carotenoids, minerals and vitamins, which affect the health of and improve the immunity fish.

Table 1: Formulation of experimental diets with varying inclusion levels of *Ulva* meal (g/kg of diet).

Ingredients	Control	UR5	UR10	UR 15	UF 5	UF10	UF15
Fish meal	18	18	18	18	18	18	18
Soy bean meal	22	22	22	22	22	22	22
Corn gluten meal	9	9	9	9	9	9	9
Seaweed meal	-	5	10	15	5	10	15
Wheat flour	18	18	18	18	13	8	3
Corn flour	20	14	9	4	19	19	19
Cassava meal	8	8	8	8	8	8	8
Soy lecithin	1	1	1	1	1	1	1
Fish oil	1	1	1	1	1	1	1
Dicalcium phosphate	1	1	1	1	1	1	1
Vitamin premix	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mineral premix	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Salt	1	1	1	1	1	1	1
Chromium oxide	1	1	1	1	1	1	1

UR- *Ulva* raw, UF- *Ulva* fermented.

Table 2: Proximate composition of *Ulva* seaweed (Raw and fermented).

	Ulva sp.					
	Crude protein (%)	CHO (%)	Crude lipid (%)	Crude fiber (%)	Ash (%)	Moisture (%)
Raw seaweed	11.6	49.26	0.19	11.85	11.2	15.9
Fermented seaweed	15.7	53.35	1.94	2.56	10.05	16.4

Table 3: Proximate composition of experimental diets with varying inclusion levels of *Ulva* meal (g/kg of diet).

	Control	UR5	UR10	UR 15	UF 5	UF10	UF15
Fibre	9.24	9.60	10.01	9.98	8.63	9.02	9.67
Moisture	2.35	2.00	3.54	3.66	0.17	0.61	2.21
Protein	32.96	32.69	32.90	32.33	32.6	32.61	32.12
Ash	8.54	8.65	9.79	9.97	8.5	8.78	9.38
Fat	1.12	1.19	1.39	1.05	0.77	0.74	0.82

Similarly this study also proved GIF tilapia fed with fermented *Ulva* meal reflected positive immune responses performance as per the result given in Fig 1-5.

Influence of *Ulva* meal on the stress responses of GIF tilapia

The stress responses observed in GIF tilapia fed *Ulva* based diets given in Table 4. Decreased glucose level (83.18±0.61) was recorded in GIF tilapia fed with fermented *Ulva* meal at 10% inclusion level. The triglycerides (196.85±0.41) and cholesterol (151.67±1.52) were observed highest in fish fed fermented *Ulva* meal at 10% diet. Increased glutathione peroxidase values (14.13±0.17) were observed in GIF tilapia fed with fermented *Ulva* meal at 10% incorporated diet.

Lower glucose level represents lower stress level in fish. Plasma cholesterol and triglyceride reflect nutritional status of fish (Pourmozaffar *et al*., 2019). Similarly, the significant decreases in glucose level in the blood of yellow catfish, rohu and red sea bream (*Pagrus major*) fed with dietary fucoidan (Yang *et al*., 2014; Mir *et al*., 2017; Sajina *et al*., 2019; Sony *et al*., 2019) have been reported. 5% *Ulva lactuca* fed fish showed improved stress response after

Table 4: Stress responses in GIFT juveniles fed varying inclusion levels of *Ulva* supplemented diets.

	Control	UR5	UR10	UR15	UF5	UF10	UF15
GLU	93.33±2.08^{c}	90.33±0.94^{c}	104.68±0.61^{a}	105.41±1.05^{a}	85.19±1.47^{d}	83.18±0.61^{d}	99.41±0.90^{b}
CHOL	115.19±0.36^{f}	135.68±0.68^{c}	124.74±1.48^{e}	126.44±1.00^{e}	139.7±1.32^{b}	151.67±1.52^{a}	130.34±0.91^{d}
TRG	172.82±0.84^{f}	190.28±0.95^{bc}	181.23±0.55^{de}	179.43±0.51^{e}	192.95±0.42^{ab}	196.85±0.41^{a}	186.15±0.25^{cd}
GPx	8.39±0.68^{cd}	10.27±0.49^{bc}	7.87±1.64^{d}	6.75±0.32^{d}	11.13±0.20^{b}	14.13±0.17^{a}	8.65±0.90^{cd}
GST	1.61±0.03^{b}	1.65±0.09^{b}	1.46±0.06^{b}	1.21±0.03^{b}	1.91±0.02^{ab}	2.59±0.78^{a}	1.52±0.01^{b}
GRA	3.08±4.57^{a}	5.54±0.21^{a}	4.92±0.05^{a}	4.55±0.02^{a}	6.09±0.02^{a}	6.19±0.02^{a}	5.49±0.23^{a}

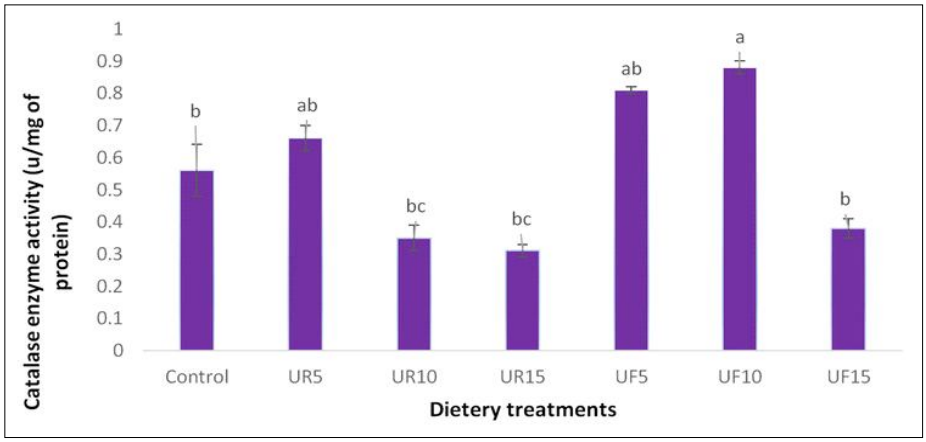


Fig 1: CAT in GIF tilapia juveniles fed varying inclusion levels of *Ulva* supplemented diets.

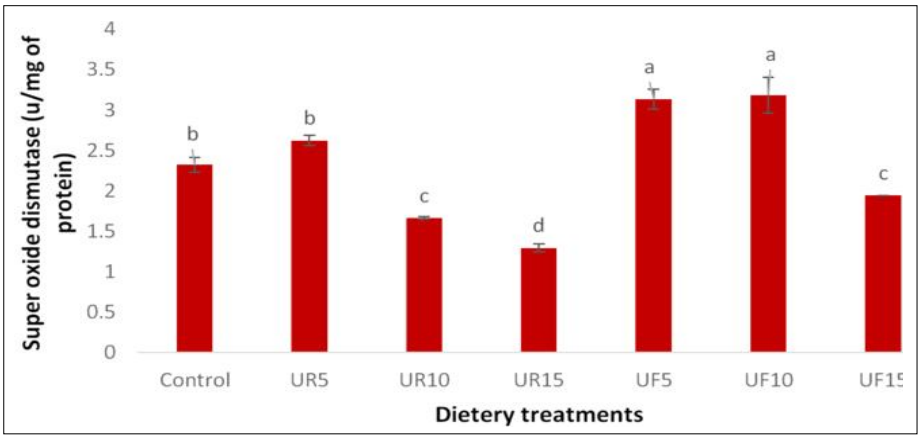


Fig 2: SOD (u/mg of protein) activity in GIF tilapia juveniles fed varying inclusion levels of *Ulva* supplemented diets.

a 5-min air exposure test, prior to termination of the feeding trial (Wassef *et al*., 2013). Rainbow trout fed with *Ulva intestinalis* sulfated polysaccharide extract *at* 1.5% inclusion showed decreased amount of cortisol (Safavi *et al*., 2019). Cortisol and glucose are indicator of fish status during acute or chronic stress (Barton and Iwama, 1991). Similarly this study also proved that GIF tilapia fed with fermented *Ulva* meal reflected reduced stress responses and positive enhancement in antioxidant enzyme activities.

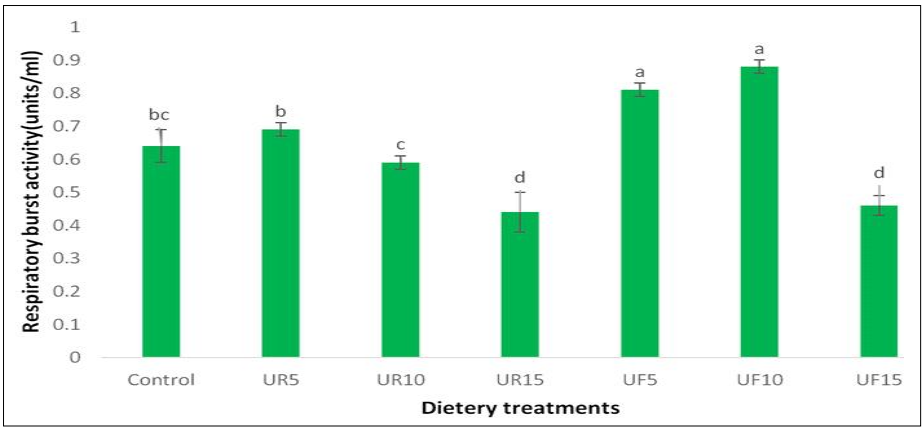


Fig 3: RBT (units/ml) activity in GIF tilapia juveniles fed varying inclusion levels of *Ulva* supplemented diets.

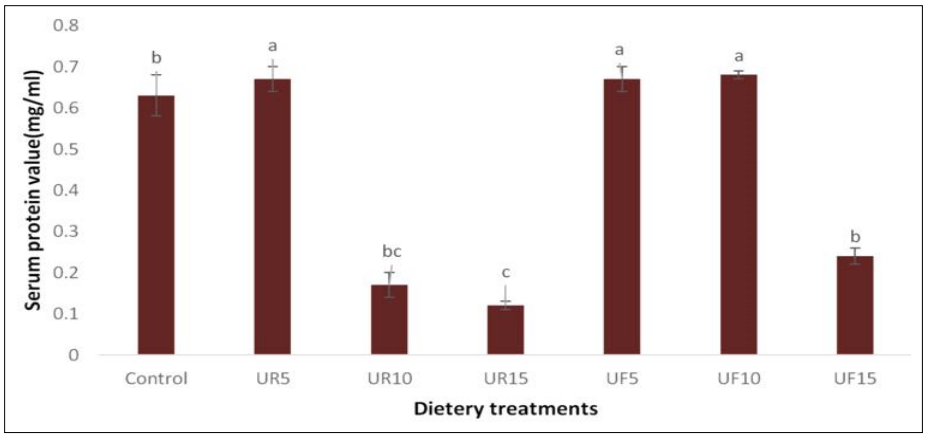


Fig 4: SPV (mg/ml) in GIF tilapia juveniles fed varying inclusion levels of *Ulva* supplemented diets.

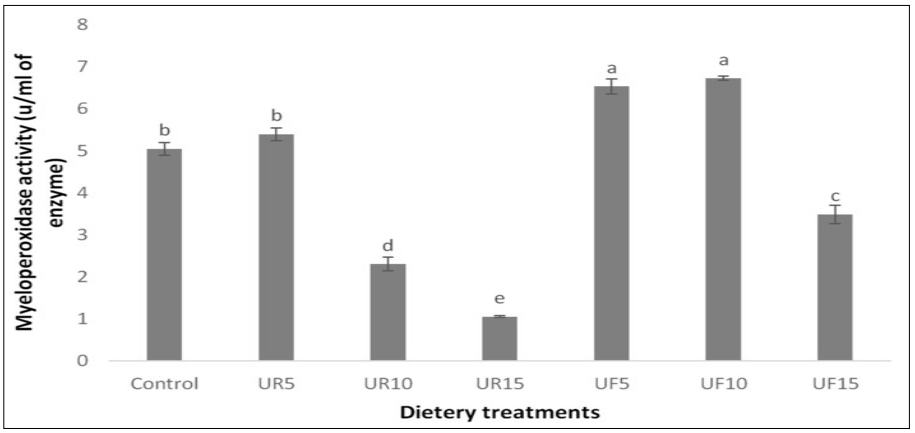


Fig 5: MPA (u/ml of enzyme) in GIF tilapia juveniles fed varying inclusion levels of *Ulva* supplemented diets.

CONCLUSION

The present study concluded that, fermented *Ulva* meal at 10% supplementation increased immune responses and decreases the stress responses in GIF tilapia which was evident from the improved catalase activity, super oxide dismutase, respiratory burst activity, serum protein values and myeloperoxidase activity with increasing concentrations when the fishes fed with 10% fermented *Ulva* meal supplementation.

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Conflict of interest

The authors declare that they have no conflict of interest.

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