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Enhancements of non-specific immune response in *Mugil cephlus* by seaweed extract against *Vibrio alginolyticus* (BRTR07)

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ABSTRACT

Objective: To focus on the growth rate and feed utilization of fish by using trash fish feeds supplement with marine seaweeds.

Methods: Selected seaweed was extracted using hot-water and its extract was mixed with trash fish feed at different concentrations (0.5%, 1% and 2% for 1-30 days) and the non-specific immune response in fish was studied and challenged with *Vibrio alginolyticus* at 1 × 10^6 CFU/fish. The hot-water extract of seaweeds was analysed by gas chromatography-mass spectrometry.

Results: The average body weight (5.320 ± 0.018), percent weight gain (227.66 ± 0.28), specific growth rate (2.080 ± 0.015), hepatosomatic index (1.197 ± 0.00) and viscerosomatic index (4.421 ± 0.150) were significantly increased in the fish feed with seaweed containing 5% of *Sargassum wightii* (*S. wightii*) when compared with other seaweeds and control diet. Hot-water extract of *S. wightii* (1%) was significantly enhanced the immune response in fish when compared with other diets (0.5% and 2%). *S. wightii* showed good immunostimulation properties. Gas chromatography-mass spectrometry result showed that the hot-water extract of *S. wightii* seaweed contained fatty acids.

Conclusions: Trash fish feed will reduce the production cost and also provide evidence that aqueous leaf extract of *S. wightii* (1%) was added to a formulated fish diet which could activate the non-specific immune response and disease resistance against *Vibrio alginolyticus* in *Mugil cephalus*.

1. Introduction

Fish nutrition is a matter of great importance in aquaculture industry worldwide[1]. The long-term sustainability of aquaculture may be threatened by its present over-dependence on fish meal and fish oil[2]. Moreover, fish feeding represents over 50% of operating costs in intensive aquaculture, with protein being the most expensive dietary source[3]. Therefore, an intensive effort during these last decades has been made in order to evaluate the potential of alternative protein sources in aquafeeds[4]. There is one way of reducing protein production cost to substitute high price fish meal in aquafeed with less expensive protein source.

Several studies evaluated the incorporation of various seaweeds species in aquafeeds including *Ascophyllum nodosum* (*A. nodosum*) [5], *Porphyra*[6], *Ulva*[7], *Sargassum* sp.[1], *Hizikia fusiformis*[8], *Gracilaria bursa-pastoris*, *Gracilaria cornea* and *Ulva rigida*[4], and *Padina arborescens* and *Sargassum siliquastrum*[9]. Trash fish, shrimp waste, acetes and seaweeds were used as the alternative protein feed for fish[1]. *Vibrio* sp. is one of the most important pathogen in aquaculture which causes greater economic loss. Disease outbreaks occur when fish are exposed to infectious agents in the presence of stress factors[10]. However, it has also been suggested that this species is a pathogen of several marine animals[11]. There is controversy about the precise role of *Vibrio alginolyticus* (*V. alginolyticus*) as a fish pathogen[12]. This species has been reported to be the causal agent of outbreaks of *Vibrio*sis.

Many plant-derived compounds are known to have non-specific immunostimulatory properties in animals, of which more than a dozen have been evaluated in fish and shrimp[13]. Even though

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glucan and other immunostimulants have positive effects on fish and prawn[14], some disadvantages have been found with the application of these natural immunostimulants like being intolerant to heat and indigestion. Hence, it is advisable to continue searching for alternative immunostimulant products from plants. All forms of stress activate responses through diverse physiological processes, and among them, energy metabolism is of prime importance for physiological compensation by organisms[15]. Stress can also suppress the defense system to such an extent that susceptibility to disease is increased[16]. Since aquatic organisms are constantly subjected to environmental fluctuations and are under challenges from potential pathogens in the aquatic environment, reciprocal changes in the physiological and immune processes are anticipated.

Immunostimulants can increase resistance to infectious diseases, not by promoting specific immune responses, but by enhancing non-specific defense mechanisms. Use of immunostimulants is an effective means of increasing immunocompetency and disease resistance in fish. In contrast to vaccines, immunostimulants enhance the innate (or non-specific) immune response[17]. The major components of the innate immune system are macrophages, monocytes, granulocytes and humoral elements, like lysozyme or complement system.

One of the possible solutions of this problem is to transform processing waste into either silage or flour and use this material in the formulation of fish feeds. Trash fish and seaweeds can reduce the production cost of the fish feed. The present study was to evaluate three cultivated marine seaweeds such as *Gracilaria edulis* (*G. edulis*), *Sargassum wightii* (*S. wightii*) and *Kappaphycus alverizi* (*K. alverizi*) which are used as a dietary ingredient in partial substitution of trash fish feed for growth performance, and nutrient utilisation of *Mugil cephalus* (*M. cephalus*). The present study is focused on potential seaweed extract used as an immunostimulatory effect in *M. cephalus* and it is resistance against *V. alginolyticus*.

2. Materials and methods

2.1. Collection of sample

Fresh marine seaweeds such as *S. wightii*, *G. edulis* and *K. alverizi* were collected from Mandapam (latitude 8°35'–9°25' N; longitude 78°08'–79°30' E), Rameshwaram, South East Coast of Tamil Nadu, India. Collected samples were washed with tap water in order to remove epiphytes and other marine organisms and then washed with distilled water and dried at 45 ℃ and ground.

2.2. Preparation of feeds

Trash fish (trash fishes, shrimp waste, acetes) were collected from seashore and local market (Parangipettai, Tamil Nadu, India). Fish meals were prepared with trash fishes, shrimp waste, acetes and seaweeds. All the materials were shadow-dried and partially sterilized in hot air oven (for laboratory scale) at 100 ℃ for 30 min. The dried materials were ground into fine powder in a hammer mill and proximate compositions were estimated. All the ingredients were finely weighed and mixed manually for 10 min and fish oil was added slowly than mixing for 10 min. Sterilized distilled water was added to the mixture to form dough. The mixture was autoclaved for 2 min. After autoclaving, vitamin and mineral were added into the mixture and mixing for 5 min. Dough passed through a pelletizer (with 2 mm diameter filter). The diets were dried in an oven at 50 ℃ until the moisture was reduced. The dry pellets were broken into small pieces and placed in covered plastic bag and stored in a refrigerator at -4 ℃ until used as a feed. When needed, the diets were thawed at room temperature and fed to the fish. Two types of control diet were used: one was commercial diet (CD-diet 1) and another one was FD (FD-diet 2) without inclusion of any seaweed. Six experimental diets were formulated to substitute 2% and 5% of three seaweed weeds *G. edulis* (GE2 and GE5), *S. wightii* (SW2 and SW5), and *K. alverizi* (KA2 and KA5). Compositions of experimental diets were summarized in Table 1.

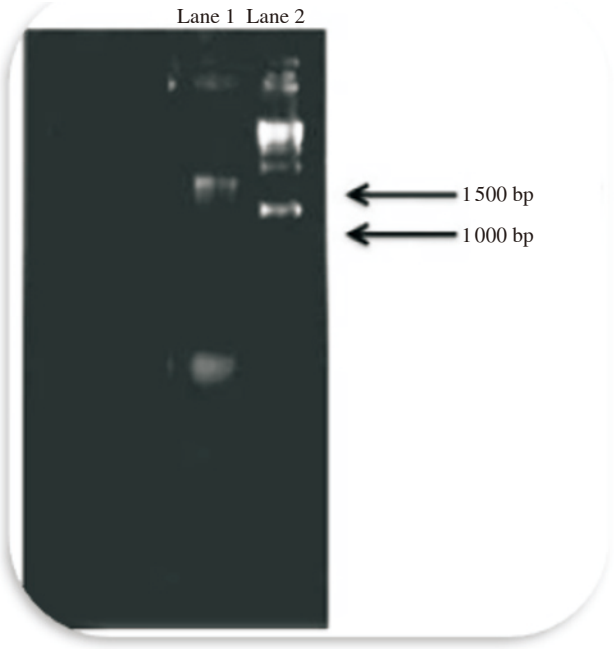

Figure 1. PCR.
Lane 1: PCR amplicons of 16S rDNA gene of the bacterial isolates BDTR07; Lane 2: Marker (molecular weight DNA ladder).

Table 1
Composition of experimental diets. % dry matter basis.

Ingredients	G1	G2	G3	G4	G5	G6	G7	G8
	Diet 1	Diet 2	GA2	GA5	SW2	SW5	KA2	KA5
Fish meal	29	-	-	-	-	-	-	-
Shrimp meal	28	-	-	-	-	-	-	-
Soya been meal	11	-	-	-	-	-	-	-
Trash fish	-	17	17	16	17	16	17	16
Trash shrimp	-	14	14	14	14	14	14	14
Trash acetes	-	16	15	15	15	15	15	15
Wheat flour	13	23	23	22	24	22	24	22
Corn flour	13	22	22	20	20	20	20	20
Fish oil	3	3	3	3	3	3	3	3
Other fish premix	3	3	3	3	3	3	3	3
Binder	2	2	2	2	2	2	2	2
Seaweeds	0	0	2	5	2	5	2	5

Diet 1: CD; Diet 2: FD; Group 3: FD with *G. edulis* (2%); Group 4: FD with *G. edulis* (5%); Group 5: FD with *S. wightii* (2%); SW5: FD with *S. wightii* (5%); Group 7: FD with *K. alverizi* (2%); Group 8: FD with *K. alverizi* (5%). Fish premix contained vitamins and minerals.

2.3. Proximate composition analysis of feed

Total protein was determined with the method used by Association of Official Analytical Chemists (AOAC)[18]. Total crude lipid was determined gravimetrically by AOAC method[18]. The moisture content (%) of seaweeds was determined by drying 2 g of samples in a thermo regulated incubator at 105 ℃ until constant weight[19]. Ash content was determined by heating the samples for 4 h in a muffle

furnace at 500 ˚C (AOAC)[18]. Nitrogen-free extract = Carbohydrate content = [100 - (% protein + % fat + % ash + % fiber)]. After feed preparation, proximate analysis (on dry matter basis) was done.

2.4. Experimental sites and study period

2.4.1. Collection and acclimatization of fish

Healthy mullet (*M. cephalus*) fishes were collected from Vellar estuary (latitude 11˚29' N and longitude 79˚46' E), Parangipettai, southeast coast of Tamil Nadu, India. All the fishes were transported to laboratory at the wet condition and stocked randomly in triplicate groups in 200 L circular plastic tank (fiber reinforce plastic) fitted with a continuous flow through system.

2.4.2. Feeding trial

The fishes were fed with experimental diets over two feeding schedule at 7:00 and 18:00. No feed was offered to the fish on the day and they were weighed. The feeding trials were done for 8 weeks. After 15 days of acclimation, fishes were divided into eight experimental groups (n = 15). Group 1 was fed with CD-diet 1, Group 2 was fed with FD-diet 2, Groups 3 and 4 were fed with GE2 and GE5, Groups 5 and 6 were fed with SW2 and SW5 and Groups 7 and 8 were fed with KA2 and KA5. All groups were maintained in triplicate. Every day, fishes were fed with 3% of the body weight twice a day (7:00 and 18:00). Every week, body weights were recorded. Any uneaten feed was siphoned off immediately.

2.4.3. Fish analysis

The day after the final weighing, fishes were anaesthetized and fish withdrawn 6 h after the meal were then killed by cervical section and liver was quickly excised, weighed and frozen in liquid nitrogen and stored at -80 ˚C before analyses.

2.4.4. Growth and feed utilization of fish

Some formulas were calculated as follows. Feed efficiency = Wet weight gain/Dry matter intake; Protein efficiency ratio = Wet weight gain/Crude protein intake. Percent weight gain (%) = 100 × [Final body weight - Initial body weight/Initial body weight]. Average body weight (ABW) = [Final weight (g) + Initial weight (g)]/2. Survival (%) = 100 × (Number of fish surviving on last day/Number of fish initially stocked). Specific growth rate (SGR) (%) = (Ln final fish weight – Ln initial fish weight) × 100/Time in days, where Ln = the natural log. Percent weight gain (PWG) = Body weight gain × 100/Initial body weight. Protein efficiency ratio (PER) = Wet weight gain (g)/Total protein fed (g). Condition factor (%) = 100 × [Body weight (g)/Body length]. Hepatosomatic index (HSI) (%) = 100 × (Liver weight/Whole body weight). Viscerosomatic index (VSI) (%) = 100 × [Viscera weight/Body weight (g)].

2.5. Isolation of Vibrio sp. from infected fish

Vibrio species was isolated from infected live fishes (*M. cephalus*). The *Vibrio* sp. was identified by biochemical and molecular characterization.

2.6. Sequencing and phylogenetic analysis

The amplified PCR products were purified using a Genei PCR purification kit (Genei, Bangalore, India). Nearly full length sequences of the amplified 16S rRNA genes (BDTR07) were obtained by automated sequencer (Bioserve, Hyderabad). The sequences were edited by using Clustal X mega software and a basic local alignment search tool search was performed in the National Center for Biotechnology Information database to identify the nearest neighbour of the amplified sequence. The results of the sequencing were used for homology searches. Phylogenetic trees were inferred using the neighbour-joining method. Nucleotide sequence of the partially complete 16S rDNA sequences of identified species were deposited in the Genbank database[19].

2.7. Preparation of bacterial suspension

Isolated *V. alginolyticus* (BRTR07) was cultured in tryptone soy broth (Hi media) for 24–48 h at 28 ˚C. After incubation, the nutrient broth was centrifuged at 6000 r/min for 10 min and the pellet was suspended into sterile phosphate buffered saline (PBS) and injected intraperitoneally with 0.2 mL of suspension (6×10^5 CFU/mL) per fish. This bacterial suspension was used for the challenge test[20].

2.8. Feed formulation

2.8.1. Preparation of feeds

Hot-water extract of *S. wightii* was prepared by adopting method of Fujiki *et al*.[21]. FD (trash fish meal) was prepared at four different concentrations. FD 1, 2 and 3 were prepared with seaweed at the three different concentrations of 0.5%, 1% and 2% respectively, where only diet 4 was prepared without seaweed. All the ingredients were mixed in a domestic mixer together with minerals and vitamins premix dissolved in a small quantity of water. The mixture was slowly mixed with hot-water (80 ˚C) in a proportion of 50:50 (v/w) to accomplish agglutination. The dough was passed through a meat chopper (Brand-Filizola) to obtain pellets of 2 mm diameter and dried in a forced circulation air drier at a temperature of (65 ± 2) ˚C for 36 h. The dried pellets were stored in plastic bags at -4 ˚C

2.8.2. Experimental design

Healthy gray Mullet (*M. cephalus* 10–25 g) was collected from Vellar estuary, Parangipettai, southeast coast of Tamil Nadu. All the fishes (*M. cephalus*) were transported to the wet laboratory and fishes were too stocked in circular plastic tank (200 L and 100 L) with continuous aeration for 15 days for acclimatization. During this period, the fishes were fed with FD (without seaweed) at the rate of 4% of the body weight twice a day at 6:00 and 17:00. After 15 days of acclimatization, the *M. cephalus* were stocked randomly and divided into five experimental groups (n = 15) in triplicate in 200 L and 100 L circular plastic tank water volume 150 L and 75 L respectively and tanks were fitted with a continuous flow-through system. Groups 1, 2 and 3 were fed with diet 1, 2 and diet 3 respectively, Groups 4 and 5 with diet 4. The respective diets were provided till the end of the experiment. On the 15th day of feeding, all the groups were injected intraperitoneally with 100 µL of PBS containing *V. alginolyticus* at 1×10^8 CFU/mL. Mortality and symptoms were recorded during experiments.

2.8.3. Non-specific immune response in M. cephalus

2.8.3.1. Haematological parameters

Red blood cell (RBC) and white blood count (WBC) counts were determined using a haemocytometer with Neubauer counting chamber as described by Blaxhall[22]. The following formula was used to calculate the number of erythrocytes and leucocytes per milliliter of the blood sample: Number of cells = (Number of cells counted × dilution)/(Area counted × depth of fluid). Thin blood smears were prepared from fresh heparinized blood on microscope

slides and stained with Wright-Giemsa.

2.8.3.2. Hematocrit

Blood was drawn into heparinized hematocrit pipette up to the graduation mark. The lower opening of the pipette was closed up to 2 cm depth using sealant and heating it carefully over the spirit lamp which closed the upper opening. The pipettes were centrifuged for 3 min with a speed of 3000 r/min and placed on the reading device and read-off. The hematocrit value was expressed as % blood cells in total volume of blood[23].

2.8.3.3. Nitroblue tetrazolium (NBT) assay

The production of oxidative radicals by neutrophils in blood during the respiratory burst was measured via NBT assays, in accordance with the description of Anderson and Siwicki[24]. In brief, blood and 0.2% NBT were mixed in equal proportion (1:1), incubated for 30 min at room temperature and then 50 μL was extracted and dispensed into Eppendorf tubes. For the solubilization of the reduced formazan product, 1 mL of dimethyl formamide was added and centrifuged at 2000 r/min for 5 min. Finally, the supernatant was acquired and the extent of reduced NBT was determined at an optical density of 540 nm with a microreader. Dimethyl formamide was used as the blank.

2.8.3.4. Lysozyme activity

Lysozyme activity was measured by adapting the turbidimetric method described. Fifty microliters of serum was placed in triplicate in a 96-well plate with 50 μL PBS, pH 5.8. After mixing, the serum was serially diluted until the last well. Finally, 50 μL of sample was discarded in the last well. To each well, 125 μL of *Micrococcus lysodeikticus* was added. The reduction in the absorbance at 450 nm was measured from 0–15 min at room temperature in an ELISA reader. The lysozyme activity was converted to lysozyme concentration using hen egg white lysozyme as standard[25].

2.8.3.5. Disease resistance and survival experiment

The diseases resistant experiment was conducted by slightly modify method of Harikrishnan *et al*.[26]. The pathogenic *V. alginolyticus* were inoculated into nutrient broth and incubated at 28 ℃ for 24 h. The culture was centrifuged at 2500 r/min for 15 min at 4 ℃. The packed cells were washed and the required dose was prepared in PBS. Groups of 15 fish each in triplicate were fed with seaweed supplemented feed at 4% of body weight on Day 1. After 10 days administration, the fishes were challenged with *V. alginolyticus* (6×10^5 CFU/mL). Once the fishes were exposed with pathogen, they were observed for the clinical symptoms and mortality.

2.8.3.6. Statistical analysis

The values of the each experimental parameter were expressed as mean ± SD and probabilities of $P < 0.05$ were considered significant. The effects of the seaweeds diet on haematological and immunological parameter were tested and a statistical package origin 6.1 for Windows 7 was used for these statistical analyses.

3. Result

3.1. Proximate composition of feed

Control and experimental diets were prepared for different ingredient, but the concentration of all the feed was nearly 40%–41%. Fiber content with all feed was between 4% and 2.5% of dry weight. Lipid content of the feed was 10%–9%. Ash content of the feed was 7%–8.5%. Moisture content of the feed was 9%–10%. Nitrogen-free extract content of the feed was 35%–40% (Table 2).

3.2. Growth performance of M. cephauls

Growth performance of the *M. cephalus* was studied by using three different seaweeds extract with two different concentrations (Table 3). The ABW, PWG, SGR were increased in the experiments when compared with control. Fish fed with SW5 diet showed the highest weight gain (5.32 g/fish) and the lowest weight gain was observed in diet 1 (3.61 g/fish). The highest PWG was observed in SW5 diet (227.66) and followed by KA2 (212.90). When compared with artificial diet, in the experimental feed showed the highest weight gain. The highest SGR was observed in SW5 diet and SW2 was 2.08% and 1.87% respectively.

3.3. Feed utilization of M. cephauls

Feed utilization of *M. cephalus* was summarised in Table 4. The feed conversion ratio (FCR) and PCR were improved during the experiment in all groups but very low in control group. The highest FCR was observed in SW5 diet (3.17) and SW2 diet was 2.49 and the lowest FCR observed was in GA2 diet and GA5. The lowest FCR was found in the control of artificial diet was 1.18, when compared with FD diet (1.75). PER was calculated during the experiment and it was high in SW5 (3.162) and SW2 (4.172) the artificial diet showed the lowest PER (2.774) (Table 4).

The effect of dietary feed with seaweeds levels on HSI and VSI was given in Table 4. The experimental group showed the lowest HSI and VSI values, when compared with the commercial diet group. The HSIs (%) of SW5 and SW2 were 1.197 and 1.206 respectively. The VSIs (%) of SW2 and SW5 was 4.587 ± 0.060 and 4.421 ± 0.150 respectively. The highest survival rate was observed in SW5 (80.33%) and SW2 (85.33%) followed by KA2 (75.66%) and KA5 (80.33%). The lowest survival rate was recorded in Group 1 (artificial diet-70%). The control group also showed less survival rate (75%) during the experiment.

Table 2

Proximate analysis of feed (dry matter basis*). %.

Constituents	G1-Diet 1	G2-Diet 2	G3 (GA2)	G4 (GA5)	G5 (SW2)	G6 (SW5)	G7 (KA2)	G8 (KA5)
Crude protein	41.45	40.25	41.94	41.36	41.28	41.50	41.43	40.97
Fiber	2.64	3.71	3.92	3.22	3.19	3.25	3.55	3.88
Crude lipid	9.40	9.50	9.70	10.30	9.50	9.60	9.30	9.60
Ash	7.25	7.20	8.20	8.12	8.32	8.35	7.19	8.11
Moisture	10.00	10.00	9.00	10.00	10.00	10.00	9.00	9.00
Non-fiber carbohydrate	39.26	39.34	36.24	37.00	37.71	37.30	38.53	37.44
Dietary energy (kj/100 g)	3504.00	3585.00	3590.60	3741.42	3666.20	3564.00	3734.20	3702.80

Table 3

Growth and survival rate of *M. cephauls*.

Parameters	Feed without seaweeds				Feed with seaweeds			
	G1-Diet 1	G2-Diet 2	G3 (GA2)	G4 (GA5)	G5 (SW2)	G6 (SW5)	G7 (KA2)	G8 (KA5)
Initial body weight (IBW) (g/fish)	2.260 ± 0.053^{a}	2.360 ± 0.151^{a}	2.183 ± 0.246^{a}	2.230 ± 0.057^{a}	2.300 ± 0.110^{a}	2.496 ± 0.005^{a}	2.330 ± 0.029^{a}	2.533 ± 0.057^{a}
Final body weight (FBW) (g/fish)	5.070 ± 0.052^{d}	6.150 ± 0.050^{c}	5.236 ± 0.055^{d}	6.430 ± 0.057^{c}	6.450 ± 0.050^{c}	8.166 ± 0.057^{a}	7.280 ± 0.056^{b}	6.560 ± 0.057^{c}
ABW (g/fish)	3.610 ± 0.090^{d}	4.060 ± 0.007^{c}	3.670 ± 0.063^{d}	4.280 ± 0.076^{b}	4.380 ± 0.014^{b}	5.320 ± 0.018^{a}	4.770 ± 0.062^{b}	4.560 ± 0.028^{b}
PWG (g/fish)	125.770 ± 0.190^{f}	207.510 ± 0.300^{b}	141.040 ± 0.830^{e}	189.700 ± 0.250^{c}	181.440 ± 0.480^{c}	227.660 ± 0.280^{a}	212.900 ± 0.080^{b}	160.390 ± 0.520^{d}
SGR (%)	1.570 ± 0.061^{e}	1.730 ± 0.056^{c}	1.650 ± 0.030^{d}	1.860 ± 0.030^{b}	1.870 ± 0.022^{b}	2.080 ± 0.015^{a}	1.800 ± 0.090^{bc}	1.760 ± 0.140^{c}

Data were mean of triplicate determinations. Mean in the same column sharing a same superscript letter were not significantly different. ABW = (IBW + FBW)/2; PWG = [(IBW/body weight gain) ×100]; Survival (%) = 100 × (number of fish surviving on last day/number of fish initially stocked).

Table 4

Feed utilization of *M. cephauls*.

Parameters	Feed without seaweeds				Feed with seaweeds*			
	G1-Diet 1	G2-Diet 2	G3 (GA2)	G4 (GA5)	G5 (SW2)	G6 (SW5)	G7 (KA2)	G8 (KA5)
FCR	1.180 ± 0.020^{a}	1.750 ± 0.020^{b}	1.390 ± 0.010^{a}	1.690 ± 0.080^{b}	2.490 ± 0.160^{c}	3.170 ± 0.100^{d}	2.480 ± 0.160^{c}	1.850 ± 0.040^{b}
PER	2.774 ± 0.020^{c}	3.797 ± 0.006^{b}	2.895 ± 0.080^{c}	3.423 ± 0.100^{b}	4.172 ± 0.020^{a}	3.162 ± 0.050^{b}	3.881 ± 0.070^{b}	3.797 ± 0.008^{b}
HSI (%)	1.376 ± 0.109^{a}	1.258 ± 0.102^{b}	1.262 ± 0.050^{b}	1.252 ± 0.080^{b}	1.206 ± 0.000^{ab}	1.197 ± 0.000^{a}	1.241 ± 0.080^{b}	1.235 ± 0.047^{b}
VSI (%)	5.294 ± 1.078^{e}	4.868 ± 0.579^{d}	4.646 ± 0.127^{c}	4.746 ± 0.810^{d}	4.587 ± 0.060^{b}	4.421 ± 0.150^{a}	4.657 ± 0.370^{c}	4.587 ± 0.075^{b}
Survival rate (%)	70.330 ± 2.880^{d}	75.330 ± 2.880^{c}	75.330 ± 2.880^{c}	80.330 ± 1.150^{b}	85.337 ± 1.150^{a}	80.331 ± 2.880^{b}	75.661 ± 1.150^{c}	80.330 ± 2.880^{b}

Data were mean of triplicate determinations. Mean in the same column sharing a same superscript letter were not significantly different. Feed conversion efficiency = Wet weight gain (g)/Total dry weight of diet fed; PER = Wet weight gain (g)/Total protein fed (g); SGR (%) = (Ln final fish weight – Ln initial fish weight) × 100/time); HSI (%) = 100 × (Liver weight/Whole body weight); VSI (%) = 100 × [Viscera weight/Body weight (g)].

3.4. Biochemical characteristics

The isolated bacterium was Gram-negative short rods and motile. Oxidase, indole, methyl red and urease were negative. Voges Proskauer and Citrate test were positive. Glucose and sucrose fermentation was positive and lactose fermentation was negative. No growth was observed in media containing 0% NaCl but well growth appeared in 6% and 10% of NaCl concentration (Table 5).

Table 5

Identification for *V. alginolyticus* by cellular morphology and biochemical test.

Morphology		Test organisms (07)	Bergey's manual**
Colour of colonies on media	Nutrient agar	Yellow colour	Yellow colour
	TCBS	Yellow colour	Yellow colour
	Cellular morphology	Short rods	Short rods
	Aerotolerance	Aerobic	Aerobic
	Motility	Motile (+)	Motile (+)
Staining methods	Gram stain	Purple colour (-)	Purple colour (-)
Growth in NaCl	0%	No growth (-)	No growth (-)
	6%	Growth (+)	Growth (+)
	10%	Growth (+)	Growth (+)
Biochemical test	Oxidise	Positive (+)	Positive (+)
	Catalase	Positive (+)	Positive (+)
	Indole	Red colour (+)	Red colour (+)
	Methyl red	Negative (-)	Negative (-)
	Voges Proskauer	Positive (+)	Positive (+)
	Citrate utilization*	Green (-)	Green (-)
	Urease detection*	Negative (-)	Negative (-)
	Lysin utilization*	Negative (-)	Negative (-)
	Ornithine utilization*	Positive (+)	Positive (+)
	Phenylalanine deamination*	Negative (-)	Negative (-)
	Nitrate reduction*	Positive (+)	Positive (+)
	H_2S on TSI*	Negative (-)	Negative (-)
Carbohydrate utilization test	Glucose*	Positive (+)	Positive (+)
	Lactose	Negative (-)	Negative (-)
	Sucrose	Positive (+)	Positive (+)
	Arabinose*	Positive (+)	Negative (-)
	Sorbitol*	Negative (-)	Positive (+)
	Adonitol*	Negative (-)	Negative (-)

*: Hi-media biochemical test (KA002); **: Bergey's manual of systematic bacteriology.

3.5. 16s rDNA sequencing analysis

Molecular identification of the 16S rRNA genes showed that the identified strain belonged to the genera *Vibrio*. The sequenced 16S rRNA genes of strain BDTR07 (Genebank accession no KF758571) that was identified as *V. alginolyticus* was 630 bp in the length and exhibited high similarity 99% (Figure 2) with the 16S rRNA genes of *V. alginolyticus* from Genbank database. This sequences showed 99% similarity with the sequences of Gene bank accession number JX976307 (*V. alginolyticus*), JQ780446 (*V. alginolyticus*). Results from colony morphology, biochemical tests and 16S rDNA indicated that the isolates were *V. alginolyticus* (BDTR07).

3.6. Phylogenetic analysis

The partial sequences of 16S rRNA genes were used to construct phylogenetic tree that was generated with sequences from same group of *Vibrio* strains [*Vibrio* sp. (KC777294), *V. alginolyticus* (AB680916), *V. alginolyticus* (JQ780446), *Vibrio parahaemolyticus* (EU814515) (*V. parahaemolyticus*), *V. alginolyticus* (JX976307), *Vibrio natriegen* (EU636230) and *Streptomyces* sp. (AB845420) (out group)] got from National Center for Biotechnology Information database (Figure 3).

3.7. Non-specific immune response

3.7.1. Haematological parameters

RBC counts of Groups 1, 2 and 3 were significantly higher than control. In the infected fishes (diet 5), RBC counts were decreased in the fish. In the 4th week of the experiment, diet 2 showed the highest RBC count than other diets. Exact variations were observed in the treatments, infected and control group (Figure 4). During the study, the control group of WBC count was normal. In the diet 5 infected group, WBC counts were increased in the 1st two week of the experiments and the WBC count was decreased after 3 and 4th weeks. In the experimental group, the highest blood count was found in diet 2. In the diet 2, infection fish showed an increased blood

count throughout the experiments when compared with control and other group (Groups 1 and 3), but in diet 5, infection fish showed RBC decreased week by week (Figure 5).

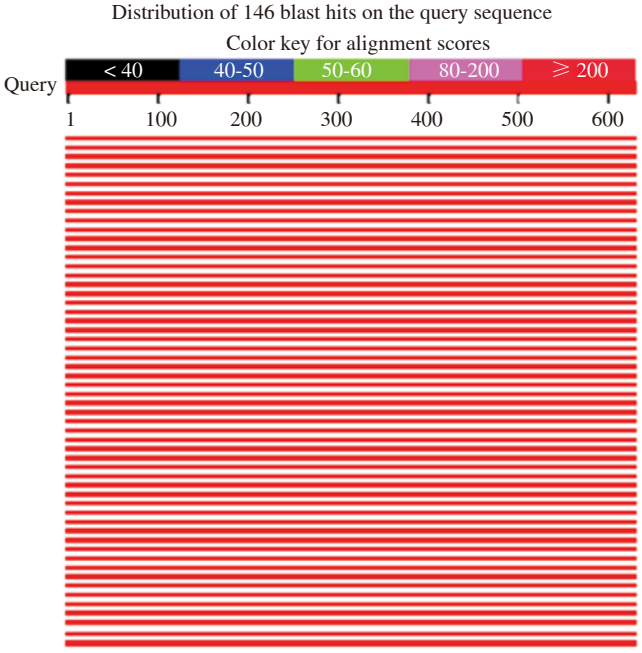

Figure 2. Basic local alignment search tool-graphic summary.

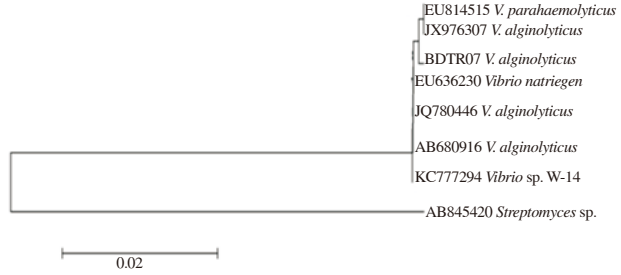

Figure 3. Phylogenetic analysis.

3.7.2. Hematocrit

In control group, hematocrit (%) was in the normal condition. Due to infection, the hematocrit percentage was decreased in infected groups, when compared with control (Figure 6). In this experimental group, hematocrit percentage was increased in the diet 1, diet 2 and diet 3 when compared with feed diet 5 and control. Diet 2 showed an increased hematocrit (%)

3.7.3. NBT and lysozyme

Studies on neutrophil activity showed the enhancing effect of dietary supplements on neutrophil respiratory burst as evidences from the increased NBT reduction (Figure 7). The respiratory burst activity in infected fish fed with normal diet significantly increased when compared with the control. The seaweeds supplement diet 2 (1%) enhanced the respiratory burst activity in infected *M. cephalus*. The lysozyme activity was progressively increased in experiment and in Group 2 lysozyme activity increased in the first week onwards. In the infected fish, lysozyme activity was very low when compared with seaweeds feed diet. After the third week, the lysozyme in infected group was decreased (Figure 8).

3.7.4. Disease resistant

The cumulative survival was shown in Figure 9. Experiments were checked the resistance against *V. alginolyticus* in fish (*M. cephalus*).In the 2nd day, mortality was started in the entire group. In the 18th day, the Group 5 showed 20% survival rate and Groups 1, 2 and 3 showed a 68%, 78% and 66% survival rates. At the end of the experiment, the Group 5 showed 0% survival rate and Group 4 showed no mortality. Group 2 showed the highest survival rate (73%), followed by Group 1 (63%) and Group 3 (55%) .

3.8. Gas chromatography-mass spectrometer (GC-MS) chromatogram

The GC-MS chromatogram of the hot-water extracts of the seaweed *S. wightii* showed the main fatty acids compound (Figure 10). *S. wightii* which included palmitoleic acid, *n*-hexadecanoic acid, oleic acid, octadecanoic acids, cis-13-Octadecenoic acid and cyclooctaneacetic acid was presented in Table 6.

Table 6
GC-MS analysis of cured hot-water extract of seaweeds *S. wightii.*

Chemical name	Retention time	Area (%)
Palmitoleic acid	17.63	4.05
n-Hexadecanoic acid	17.80	42.49
Oleic acid	19.47	20.34
Octadecanoic acids	19.63	28.11
Cis-13-octadecenoic acid	20.83	2.06
Cyclooctaneacetic acid	22.48	2.42

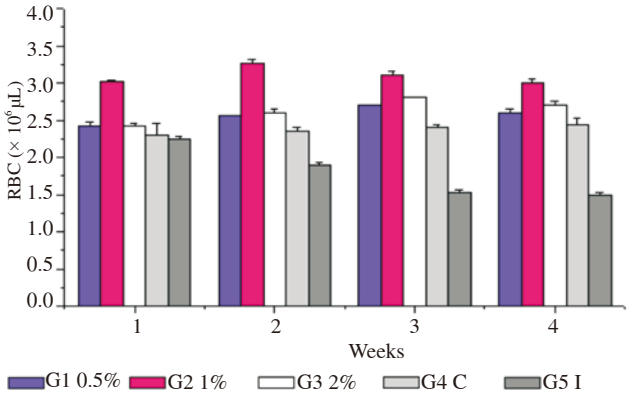

Figure 4. RBC count.
M. cephalus fed with hot-water extract of *S. wightii* at 0.5%, 1% and 2%. C: Control without seaweeds; I: Infection without seaweeds. Values were presented as mean ± SD of triplicates.

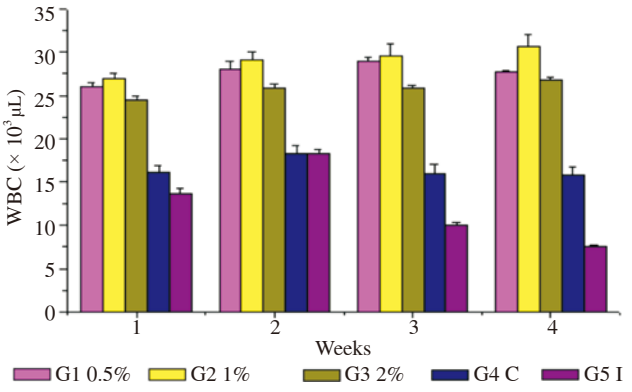

Figure 5. WBC count.
M. cephalus fed with hot-water extract of *S. wightii* at 0.5%, 1% and 2%. C: Control without seaweeds; I: Infection without seaweeds. Values were presented as mean ± SD of triplicates.

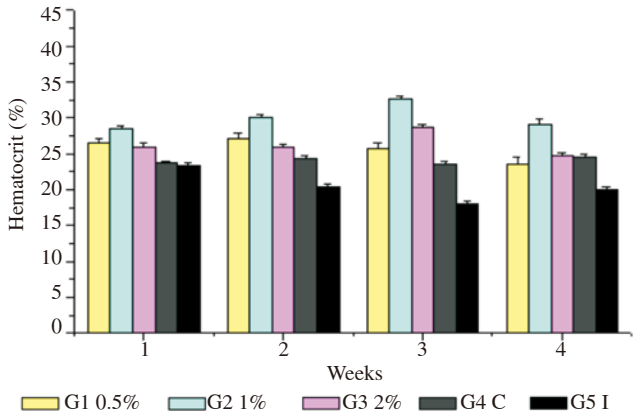

Figure 6. Hematocrit.
M. cephalus fed with hot-water extract of *S. wightii* at 0.5%, 1% and 2%. C: Control without seaweeds; I: Infection without seaweeds. Values were presented as mean ± SD of triplicates.

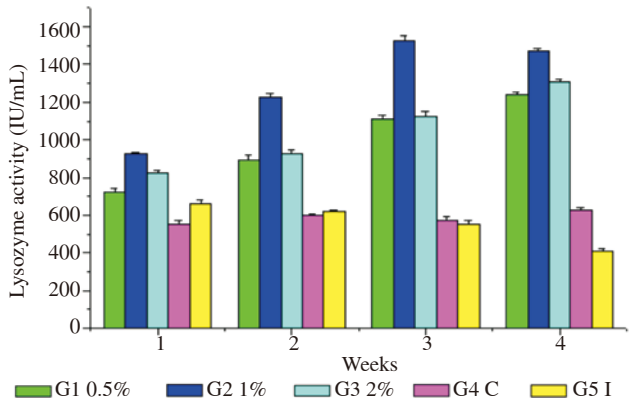

Figure 8. Lysozyme activity.
M. cephalus fed with hot-water extract of *S. wightii* at 0.5%, 1% and 2%. C: Control without seaweeds; I: Infection without seaweeds. Values were presented as mean ± SD of triplicates.

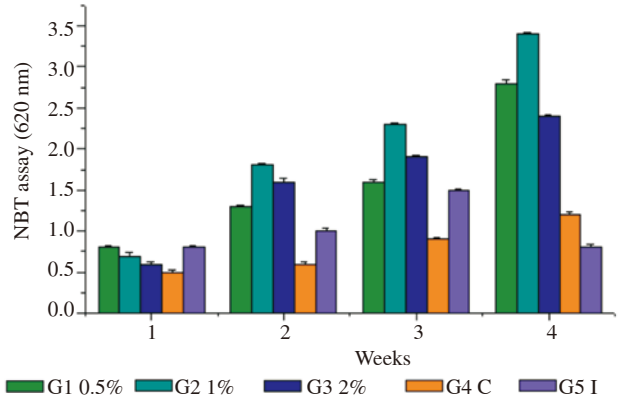

Figure 7. NBT assay.
M. cephalus fed with hot-water extract of *S. wightii* at 0.5%, 1% and 2%. C: Control without seaweeds; I: Infection without seaweeds. Values were presented as mean ± SD of triplicates.

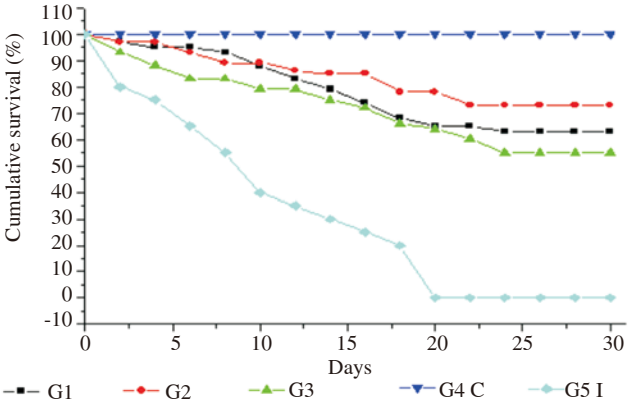

Figure 9. Cumulative survival (resistance against *V. alginolyticus* in *M. cephalus*).
M. cephalus fed with hot-water extract of *S. wightii* at 0.5%, 1% and 2%. C: Control without seaweeds; I: Infection without seaweeds. Values were presented as mean ± SD of triplicates.

4. Discussion

The potential use of seaweeds in fish feeds depends on the costs involved in their production, harvesting and processing prior to their inclusion in fish diets. Studies on the biochemical constituents such as protein, carbohydrate and lipid in green and brown marine algae have been carried out from different parts of Indian coast[27]. Manivannan *et al*. reported that high protein content was present in the brown seaweed *S. wightii*[28], whereas in the present study, the high amount protein content was found in brown seaweed *S. wightii* when compared with red seaweed. In this present study, analyses of protein, carbohydrates, lipid, ash, moisture and mineral presented in three different seaweeds were carried out. Manivannan *et al*. reported that carbohydrate content was highest in *S. wightii* but low amount of carbohydrates in *Gracilaria folifera*[28].

Carbohydrates were the major components in the proximate composition of all the seaweeds examined. In the present study, high amount of carbohydrates were present in *S. wightii*, when compared to the other two seaweeds. Manivannan *et al*. reported that lipid content in *Gracilaria folifera* was high when compared with *S. wightii*[28]. In this study, results showed that *G. edulis* contained high amount of lipid.

Sánchez-Machado *et al*.[29] reported that ash contents of the seaweeds ranged from 19.07 to 34.00 (100 g dry weight) and it was considerably higher than the 21.3%–22.8% reported by Wong *et al*.[30] but lower than the 20.6%–39.3% reported by Rupérez and Saura-Calixto[31]. In this study, the ash contents were 12–19 g/ dry weight of the seaweeds. Similar results were also showed by Wong *et al*.[30]. The ash contents of seaweeds, which are generally much higher than those of terrestrial vegetables other than spinach, vary between species, between geographical locations and between seasons[32].

Moisture content is an important criterion in determining the shelf-life and quality of processed seaweed meals as high moisture may hasten the growth of microorganisms. The present results showed the high amount of moisture present in *K. alverizi* (20.33%) but different ranges of moisture contents were reported. Matanjun *et al*. observed that the range of moisture content among the various seaweed samples varied from 6.8% (*Ulva lactuca* powder) to 11.5% (*Colpomenia sinuosa* powder)[33]. The higher and lower moisture absorption was observed in *Encephalitozoon intestinalis* (8.5%) and *Ulva lactuca* (0.2%), respectively.

Mustafa and Nakagawa found that the inclusion of three different seaweeds (*A. nodosum*, *Porphyra yezoensis* and *Ulva pertusa*) at a level of 5% increased body weight, feed utilization and muscle protein deposition in red sea bream fingerlings (*Pagrus major*) [34]. Nonetheless, Appler reported similar growth performances and protein utilization efficiencies in *Oreocromis niloticus* and

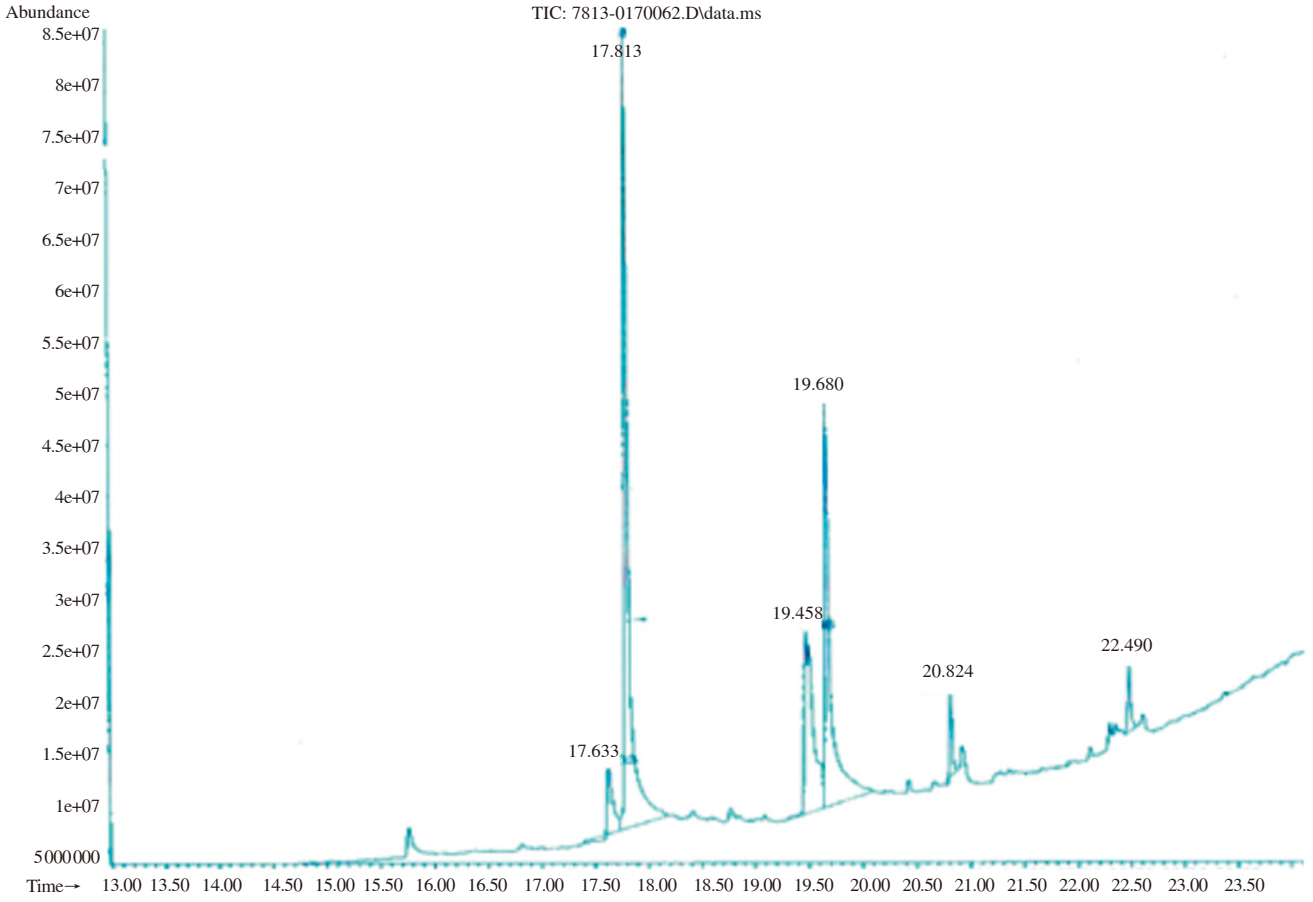

Figure 10. GC-MS analysis of cured hot-water extract of seaweeds *S. wightii.*

Tilapia zillii when fed diets containing 5% of a freshwater algae *Hydrodictyon reticulatum*, but reduced growth performances with increasing dietary inclusion levels[35]. Growth performances of *M. cephalus* were studied. The ABW, PWG, SGR were significantly increased in all the experiment, when compared with the control. Fishes fed with SW5 diet showed the highest weight gain (5.32 g/fish) and the lowest weight gain were observed in diet 1 (3.61 g/fish). The highest PWG was observed in SW5 diet (227.66) followed by KA2 (212.90), when compared with artificial diet and the experimental feed showed the highest weight gain.

Catacutan *et al*. found that SGR of 1.80% seemed to be the best for red snapper *Lutjanus argentimaculatus* being fed fishmeal-based diets at the rate of 4.5% to 5% body weight per day for 100 days[36]. In the present study, SGR was observed in SW5 (2.08%) diet and SW2 (1.87%). Similar SGR (2.17%) was reported by Abbas and Siddiqui when fish was fed with 42% fishmeal cum soybean meal-based diet for 75 days[37]. Luo *et al*. reported that SGR varied between 1.1% to 1.5%[38]. In contrast, Millamena reported higher values of SGR ranging between 2.8 to 3.2[39]. Abbas *et al*.[40] reported that the highest SGR of 2.65% was obtained by the diet containing 40% and Siddiqui and Khan[41] reported that *Heteropneustes fossilis* (*H. fossilis*) fed with 40% of protein showed 1.76% of SGR.

Davies *et al*. demonstrated that the use of the macroalgae *Porphyra purpurea* at high inclusion levels (16% and 33%), as an ingredient for mullet (*Chelon labrosus*) diets suppressed growth performance and feed utilization efficiency[6]. Contradictory results were obtained by El-Sayed who evaluated the use of a microalga meal, *Spirulina*, as protein source for silver sea bream (*Rhabdosargus sarba*) fingerlings at inclusion levels up to 100% and concluded it could successfully replace up to 75% of the fish meal protein without any adverse effects on growth performance and feed utilization efficiency[42]. These results suggests that the response of fish to algae seems to be species-specific and indicates a general beneficial effect of low level supplementation of seaweeds in fish diets. The relative low nutritive value of seaweeds could explain their deleterious effect on overall growth performance at high inclusion levels and could also explain the lowest performance of fish fed[4].

The FCR and PCR were improved during the experiment in all groups except for control group. Zehra and Khan reported that growth performance of *Corydoras punctatus* fingerlings in response to varying levels of dietary protein levels, at 450 g/kg FCR was 1.48[43]. In this present study, the highest FCR was observed in SW4 diet (3.17) and SW2 diet (2.49) and the lowest FCR was observed in GA2 diet and GA5. The lowest FCR was found in the control of artificial diet (1.18) when compared with FD (1.75). Siddiqui and Khan reported that growth parameters of young *H. fossilis* fed diets containing graded levels of protein FCR was 1.42[41].

PER of the present study was increased when fed with *S. wightii* (SW5 and SW2). PER was calculated during the experiment and it was highest in group SW5 (3.162) and SW2 (4.172), while the artificial diet showed low PER (2.774). Siddiqui reported that 40% of the dietary protein increased PER level to 1.75 in *H. fossilis*[41].

Shapawi *et al*. reported that higher hepato- and VSI were observed in fish fed the commercial feeds[44]. HSI is related to the nutritional state of fish and may directly relate to energy requirements for growth. Catacutan *et al*. showed that HSI of the snapper was found to be between 1.88% and 2.4%[36]. HSI is known as an indicator for assessing the nutritional status of fish[45,46]. In the present study, HSIs of SW5 and SW2 were 1.197 and 1.206, respectively. Jobling

reported that HSI was around 1.41% to 2.57% as it is normal[47]. The VSIs of SW2 and SW5 were 4.58 ± 0.069 and 4.42. Peres and Oliva-Teles[48] reported that VSI found in European seabass was 6.9 to 13.4 and Martino *et al*.[49] reported that VSI was 5.7 to 7.5, but higher amount of VSI was observed by Luo *et al*.[38] who showed that VSI ranged between 8.37 and 9.36, which was generally similar to that seen in other fish, such as, rainbow trout (VSI 8.1–10.5)[50], whitefish (VSI 7.3–8.8)[51], red sea bream (VSI 9.1–10.3)[52].

The highest survival rate was observed in SW5 (80%) and SW2 (85%) followed by KA2 (75%) and KA5 (80%). The lowest survival rate was recorded in Group 1 (artificial diet). The control group also showed less survival rate (75%) during the experiment. *Gynostemma pentaphyllum* is a traditional Chinese herbal medicine incorporated in fish feed resulted in an increased weight gain, feed conversion efficiency and specific growth rate in grass carp *Ctenopharyngodon idella*[53]. Most of the pathogenic bacteria are Gram-negative, aerobic and facultative anaerobic bacteria and belong to the genus *Vibrio, Yersinia, Pasteurella* and *Edwarsiella*. Members of the genus *Vibrio* are opportunistic pathogens that have been associated to infections of marine animals[54].

In the phylogenetic tree analysis, *V. alginolyticus* and *V. parahaemolyticus* are closely related. Similarly Blake *et al*. reported that *V. alginolyticus* and *V. parahaemolyticus*, which are closely related, have similar flagellar systems[55]. The occurrence of *V. alginolyticus* in the marine water samples, as well as in the fish samples was strongly determined by sampling season, *i.e.* with water temperature.

The variation degree on the haematological response is an important tool for fish health diagnosis and may vary according to stressor stimulus, treatment, parasitic or infectious diseases[56]. In the present study, infected fish RBC count were decreased in the fish. Decreased RBC counts and hematocrit concentration indicate that RBCs are being destroyed by the leucocytosis activity in an erythrocytic anemia with subsequent erythroblastosis.

A decline in RBC and hematocrit combined with signs of anaemia was also described by Hoffmann and Lommel in cases of proliferative kidney disease[57]. Diet 1, diet 2 and diet 3, showed an increased blood count when compared with control diet. WBC, RBC, haemoglobin B and thrombocytes were decreased in the infected fish when compared with control group. Waagbo *et al*. reported that in Atlantic salmon, *Salmo salar* with the 'Hitra disease' decreased in the values of RBC, hematocrit and haemoglobin B, associated with symptoms of severe anaemia[58]. Cardwell and Smith did find a progressive effect on hematocrit and haemoglobin B in juvenile chinook salmon with vibriosis[59]. Harbell *et al*.[60] recorded the same in coho salmon, *Oncorhynchus kisutch* experimentally infected with a highly virulent *Vibrio anguillarum*.

In our present study, the values of WBC, RBC, hemoglobin and hematocrit were lower in the normal condition. Similarly, Hrubec *et al*. reported that the values of hematocrit, RBC and thrombocytes were lower in the tilapia in normal conditions[61,62]. Sarder *et al*. did not observe any alteration in the differential counts of white blood cells in tilapia challenged with *Aeromonas hydrophila* (*A. hydrophila*)[63]. Carp experimentally was infected with *A. hydrophila*. Harikrishnan *et al*. showed related increased WBC counts and also reported that decreased RBC counts and hematocrit indicated that erythrocytes were being affected or destroyed with the infection[64].

Destruction of ingested microorganisms may be due to degranulation or metabolic activation, when toxic intermediates of oxygen reduction are produced. Since superoxide anion is the first product to be released from the respiratory burst, the measurement of O_2 has been accepted as a precise way of measuring respiratory burst[65]. Fish fed with 0.1% and 1.0% *Prunella vulgaris* extract enriched diet showed significantly enhanced phagocytic activity from weeks 1 to 4 compared to the control[66]. In this study, *S. wightii* (1%) fed with diet 2 increased the NBT activity from 1st week to 4th week when compared with control.

A total lysozyme level is a measurable humoral component of the non-specific defence mechanism. Lysozyme is a fish defence element, which causes lysis of bacteria, parasite and viral infection and fish blood is considered as a good indicator of its immune function[67].

Hot-water extract of *S. wightii* seaweeds enhanced the lysozyme activity in *M. cephalus*. In *Oreochromis niloticus* fed with 0.1% and 0.5% *Astragalus radix* root for 1 week, lysozyme activity was enhanced[25]. In the present study, all the doses of hot-water extract of *S. wightii* incorporated in the fed diet significantly enhanced the lysozyme activity in the first week of the experiments. Similarly seabass *Dicentrarchus labrax* which had been fed a diet containing sodium alginate from brown algae *Laminaria digitata* and *A. nodosum* after 15 days showed increased alternative complement and lysozyme activity[68].

All treated groups showed a reduced mortality compared to the control group. The best survival rate was observed in the diet 2 treated with 1% of seaweeds extract by oral administration. Survival rates of infected fish are usually increased after treatment with various immunostimulants, vaccinesand probiotics[69-71]. In the present study, diet 2 (1%) feed showed the best survival rates when compared with Group 5 (infection). Feeding carp with chitosan and levamisole reduced mortality of common carp after challenge with *A. hydrophila*[72]. A similar result was reported after feeding large yellow croaker with glucan and challenging with *Vibrio harveyi*[73].

The GC-MS chromatogram of the hot-water extracts of the seaweed *S. wightii* showed that the main contained phenolic compounds. This result agrees with the report of Khotimchenko and Vaskovsky who suggested that fatty acids are a vital constituent of both terrestrial and marine plants[73,74]. The immunostimulation observed in the present study might be due to phytoconstituents of *S. wightii* which include palmitoleic acid, *n*-hexadecanoic acid, oleic acid, octadecanoic acids, cis-13-octadecenoic acid and cyclooctaneacetic acid. Christybapita *et al*. reported that phytoconstituents of *Eclipta alba* containing eclalbatin, α-amyrin, urosilic acid and oleanolic acid[75]. It was suggested that seaweed poly phenolic constituents might directly activate innate defence mechanisms by acting on receptors and trigger gene activation, which might result in production of anti-microbial molecules[76].

The present study also supported that seaweed extract mixed with feed, increased weight gain, feed conversion efficiency and specific growth rate in *M. cephalus*. The present study concluded that the utilization of the trash fish, shrimp waste and acetes with seaweed enhanced the growth of *M. cephalus*. *V. alginolyticus* was one of the causative agents of tail and skin haemorrhagic infection in the fish *M. cephalus*. Aqueous extract of *S. wightii* added to a formulated fish diet could activate the non-specific immune mechanisms and disease resistance against *V. alginiolyticus* in *M. cephalus*. However, the most effective doses, application methods and administration regimes for different age groups of fish have to be investigated and confirmed before application of this product in culture situations.

S. wightii are potential sources of bioactive compounds. Fish nutrition is a matter of great importance in aquaculture industry worldwide. Seaweeds with elevated protein content and production rates are receiving increasingly attention as novel feeds with potential nutritional benefits. Prevention and control of infective diseases using the natural immunostimulatory compounds is one of the safer and eco-friendly approaches.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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