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Effect of Dietary Lysine and Methionine Supplementation on the Growth and Physiological Responses of Pearlspot Fingerlings, *Etroplus suratensis*

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ABSTRACT

Background: Successful and sustainable culture of finfish and shellfish depends on the use of nutritionally balanced, low-cost and eco-friendly feeds. This experiment was conducted to evaluate the effect of dietary supplementation of lysine and methionine on the growth and physiological responses of juvenile pearlspot, *Etroplus suratensis*.

Methods: Fingerlings with an average initial body weight of 8.7±0.04 g were distributed to the 300 L FRP tank (n=30 pieces/tank). The pearl spot fingerlings were fed with isonitrogenous (25% Crude protein) feeds supplemented with methionine (1.8, 2.2 and 2.6 g/100 g of diet) and lysine (0.5, 0.9 and 1.3 g/100 g of diet). The growth performance and physiological response of the fishes were assessed. The feeding trial was conducted for a period of 60 days.

Result: The methionine supplemented diets at 2.2 g/100 g (T2) and Lysine supplemented diet at 0.9 g/100 g (T5) yielded the best results within the treated groups in terms of average body weight gain, FCR (feed conversion ratio), SGR (specific growth rate), PER (protein efficiency ratio) and FER (feed efficiency ratio). The maximum mean body weight gain recorded in methionine supplemented diet was 20.68±0.20 g in T2 and the maximum mean body weight gain recorded in lysine supplemented diet was 19.6±0.15 g in T5. The mean body weight gain in control was recorded as 12.12±0.20 g. The present study revealed that methionine incorporation at 2.2 g/100 g (T2) and lysine incorporation at 0.9 g/100 g (T5) resulted in better growth performance of the pearl spot *Etroplus suratensis* fingerlings. Results of challenge tests confirmed that *V. anguillarum* could act as a primary pathogen for pearlspot reared in freshwater. In the present study, the Hb, RBC, MCV, MCH and MCHC were also affected by dietary methionine and lysine supplementation and were found significantly ($p<0.05$) higher in fish fed methionine at 2.2% and lysine at 0.9% of diet, indicating that dietary methionine and lysine supplementation activates immune response and promotes fish health. Second-order polynomial regression analysis of weight gain against dietary methionine and lysine levels indicated that the optimum dietary methionine and lysine requirement for maximum growth and feed utilization of pearlspot fingerlings was methionine at 2.2% and lysine at 0.9%.

Key words: Growth performance, Lysine, Methionine, Pearlspot, Supplementation.

INTRODUCTION

Fisheries and aquaculture remain as an important sources of food, nutrition, income and livelihood for coastal fisherfolks around the world. Global fish production is estimated to have reached about 178.5 million tonnes in 2018 (FAO, 2020). *Etroplus suratensis*, is the largest among the cichlids indigenous to peninsular India and Srilanka. The pearlspot is popularly known as “Karimeen” in Kerala, is an excellent table fish of delicacy, fetching a good market price. It is a low-fat, high-protein food, rich in omega 3 fatty acid (Padmakumar *et al*., 2002). The expansion of global aquaculture is increas-ing the demand for aquaculture feed, which is the prime input in fish culture practices (Singh *et al*., 2011). The supplied diet must contain the necessary nutritional require-ments for the fish to ensure the best growth (Ghomi *et al*., 2012). World aquaculture is the fastest growing food-producing sector in the world. Globally, aquaculture is expanding into new directions, identifying and diversifying. With increasing demand for environment friendly aquaculture, the use of growth promoters in fish nutrition is new widely accepted (Shreeja *et al*., 2018).

Aquaculture has thus become more integrated into the global food system, with rapid growth in production and major transformations in feed ingredients and production technologies (Rosamond *et al.,* 2021). To ensure maximal growth, fish species need ten indispensible amino acids (IAA) in their diet. A common alternative ingredient used in commercial feed replacing fish meal is soybean meal, a plant protein source which approximately reduce the cost of dietary protein to 25% (Pike, 2006). Its use in feeds as a

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primary source of protein often requires supplementation with synthetic forms of essential amino acids such as L-methionine, D-L methionine or analogs (Forster and Dominy, 2006). Soybean meal currently represents the predominant choice as an alternate protein source in commercial feed formulations for aquatic species. Methionine is a sulfur containing essential amino acid (EAA) required by fish as well as terrestrial vertebrates for normal growth and metabolic functions. Methionine deficiency resulted in reduced growth and feed efficiency. Lysine is another one of the most limiting amino acid in ingredients used for production of commercial fish feeds, especially when fishmeal is replaced by plant protein sources (Mai *et al*., 2006). In addition to meet the basic metabolic requirements for maximum growth, dietary lysine supplementation has been shown to have other positive effects on various animals (Borlongan and Benitez, 1990; Cheng and Hardy, 2003). Chinu *et al*. (1986) reported that the addition of crystalline lysine significantly improved the biological value of corn gluten meal as a protein source for milk fish fry. Successful and sustainable culture of finfish and shellfish depends on the use of nutritionally balanced, low-cost and eco-friendly feeds (Joseph and Ignatius, 2016). Dietary nutrient requirements in fish are usually estimated empirically by feeding graded levels of a specific nutrient, in a basal diet containing a different level of that nutrient and then measuring growth, feed intake, body nutrient stores or other variables (Lekshmi and Prasad, 2014). The main aim of this study is to investigate the effect of lysine and methionine supplementation on the growth performance and physiological responses of juvenile pearlspot.

MATERIALS AND METHODS

Experimental fish and feeding trial

Pearlspot fingerlings were collected from Pulicat lake by local fishermen. Prior to experiment, the fishes were graded to select an individual average weight of 8.71±0.04 g and were stocked in FRP tanks. All the FRP tanks of 300 L capacity were equipped with aeration facility. Totally 21 (6 treatments and 1 control with triplicates) numbers of individual fish tanks were arranged. Fishes were fed thrice a day @ 3% of fish body weight. The feeding ration was divided into three equal quantities and given thrice a day *viz*., 10.00 am, 2.00 pm and 5.00 pm. Water exchange was done at the rate of 40% every day in each tank. 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 28.421°C, pH 7.68 ±0.02, TDS 0.89± 0.02, Conductivity 2.05±0.01, Resistivity 458±0.04, ORP 222±0.03. Alkalinity 123±0.26 mg/l, Hardness 343±0.06 mg/l, Calcium 37±0.03 mg/l, Magnesium 110±0.02 mg/l and Ammonia Nil.

Experimental diets

Seven experimental isoprotein diets containing 25% levels of crude protein (CP 25%) were formulated. The experimental diets were supplemented with methionine @ 1.8, 2.2 and 2.6% and lysine @ 0.5, 0.9 and 1.3% respectively. The experimental diets were prepared by mixing the dry ingredients, followed by the addition of oil and water. This mixture was formed into dough and dry pellets were made by passing the dough through a hand screw pelleting machine and pellets were air dried for 48 hours. After drying, the pellets were broken up and stored at air tight container.

Fish sampling

The sampling was carried out once in 15 days. At the end of the feeding trial, all the experimental fishes were weighed and analyzed to estimate their survival rate, food conversion ratio, protein efficiency ratio and specific growth rate using standard procedures.

Proximate, aminoacid and fatty acid analysis

Seven fishes from each tank were randomly selected for the whole body composition. The amino acid profile of the diets and whole body were analyzed by Ultra Pressure Liquid Chromatography (UPLC) (Model- Waters ACQUITY-UPLC, Waters, Massachusetts, USA), following the method of Ishida, Fujita and Asai (1981) at TNJFU Referral Laboratory for Fish Quality Monitoring and Certification, FC and RI, Thoothukudi. About, 50 mg of sample was transferred to an ampule (Borosil Glass Works Ltd., Ahmedabad, India) sealed under a stream of nitrogen gas and hydrolyzed using 6 N HCl for neutralization and then filtered through 0.2 µm PTFE filter. The samples were derivatized using AccQ Tag Ultra derivatization kit and separated using a Waters ACQUITY UPLC fitted with 2.1 × 100 mm column 1.7 µm fitted with AccQ Tag Ultra C18 following stepwise gradient elution. Amino acid standard was used and the amino acids were quantified based on the absorbance values at 260 nm measured by a tunable UV detector and analyzed using Empower 2. Amino acid standards were also run simultaneously for calibration. The proximate composition of experimental diets (50 g sample) and fatty acid profile of experimental animal muscle (50 g sample) was analyzed in Animal feed analytical and Quality Assurance Laboratory, Veterinary College and Research Institute, Namakkal.

Digestive enzyme analysis

The dissected tissues such as intestine and liver were homogenized with 0.25M sucrose solution on a wet weight basis (pH 7.0, 1:20 w/v) in a hand-held homogenizer. The samples were stored the ice box (4-5°C). After centrifugation, the supernatant was stored in a 15 ml sterile tube. The samples were stored at -20°C until further analysis.

The reducing sugars were estimated using Dinitro salicylic acid (DNS) method (Rick and Stegbauer, 1974). The reaction mixture consists of 1% (w/v) starch solution, phosphate buffer and the tissue homogenate. The reaction mixtures were incubated at 37°C for 30 minutes. DNS was added after incubation and kept in boiling water bath for 5 minutes. After cooling, the reaction mixture was diluted with distilled water and absorbance was measured at 540 nm.

Protease activity was determined by the method of Moore and Stein (1948), using bovine serum albumin as the substrate. For blank 0.8 ml of 0.5 M Tris Hcl with pH 7.5 was added to 0.2 ml of 0.1% BSA solution followed by 2.1 ml trichloro-acetic acid (TCA). For test samples, 0.8 ml of buffer, 0.1 ml of BSA followed by 0.1 ml of enzyme solution were added. TCA was added just after adding the enzyme solution to the tubes. All the solution were mixed well using the vortex mixer. After incubating for 30 min at 37°C, 2.0 ml of TCA were added to all the solutions. The solutions were centrifuged again in refrigerated centrifuge. 0.3 ml of supernatant was taken to which 1.2 ml of ninhydrin reagent was added. The samples were cooled and OD was read at 570 nm.

The lipase activity was assayed by the method of cherry and crandell (1932). Two test tubes labeled as test (T) and control (C) were taken. Each of the two tubes, 3 ml of the distilled water and 1ml of the homogenate were added. One of the tube (C) was placed in boiling water served to inactivate the lipase in control. Then 0.5 ml of buffer solution (Phosphate buffer pH 7.0) and 2 ml olive oil emulsion was added to both the tube, shaken well and incubated at 37°C for 24 hr. Then 3 ml of 95% alcohol and 2 drops of phenolphthalein solution were mixed. The both tubes were titrated with 0.05N NaOH upto the appearance of permanent pink colour.

Haemato- biochemical responses

0.5 ml of blood was drawn from the caudal vein with the help of a sterile 2 ml hypodermal syringe and 24 gauge needles. The syringes were pre-coated with EDTA (2.7%) as an anticoagulant. Before drawing the blood, fishes were anesthetized using clove oil. The blood was then transferred to 1 ml EDTA coated vials and used for NBT assay. The red blood cell (RBC) and white blood cell (WBC) counts were determined by haemacytometer. Haemoglobin (Hb) concentrations were determined by Cyanmethaemoglobin method (Drabkin,1946) and Haematocrit (Ht) was determined by the microhematocrit method (Nelson andMoris, 1989). The erythrocyte indices were calculated such as MCV, MCH and MCHC by Wintrobe (1934).

For serum preparation, 0.5 ml of blood was collected in a syringe without anticoagulant and transferred to serological tubes. The blood samples were allowed to clot and stored in a refrigerator for overnight. The clot was then spun down at 6000 rpm for 10 min. The collected serum was stored in sterile tubes at -20°C until used for further analysis. Blood glucose level was analyzed using commercial kit (Sigma Diagnostics, India) according to Trinder (1969). Total serum cholesterol was analyzed by Zlatkis (1953) method.

Histology analysis

Histological investigations on different tissues of fish gills, liver and intestine were collected by dissecting the fingerlings and it was preserved in 10% NBF solution for 24 to 48 h. Gills were specifically taken for histology study just to find out the morphological response due to osmotic changes effected by fresh water. Tissues were processed using routine methods and embedded in paraffin wax. The sections cut 4 µM thickness were then stained using haematoxylin and eosin.

Disease resistance against *V. anguillarum*

Vibrio anguillarum is an opportunistic fish pathogen that is common to marine and estuarine environments. It has been identified as the main cause of *vibriosis*, a potentially fatal septicemia that affects fish and shellfish in marine aquaculture, with consequent economic loss. This pathogen mainly affect in brackishwater species of pearlspot. At the end of growth trial, the experiment animals (*E. suratensis*) were challenged with *V. anguillarum* obtained from State Referral Laboratory, Madhavaram. Isolation of *V. anguillarum* generally involves plating on nutrient medium containing 1 to 2% NaCl and selective medium, mainly thiosulfate-citrate-bile salts-sucrose agar (TCBS) they were incubated at 25°C for 48 h was centrifuged at 4500 rpm for 5 to 10 min followed by pellet resuspension in phosphate buffered saline (PBS, pH -7.2). The suspension in sterile 0.1 ml of phosphate-buffered saline supplemented with 1.5% NaCl (PBSS) containing 4.6×10^3 CFU of *V. anguillarum* per fish was injected intramuscularly (0.1 ml) in healthy *Etroplus suratensis* fed with lysine and aminoacid supplemented diets and stocked in stock tank for 14 days for post infection studies and resistance against *V. anguillarum* infection. The infected moribund fish was sacrificed for histopathological study after 14 days post infection.

Statistical analysis

All the data of this study were examined to one-way analysis of variance (ANOVA) utilizing the statistical software program SPSS version 16.0 (SPSS Inc., IL, USA). Duncan's post-hoc test was used to compare the averages of data at significance level of $p<0.05$.

RESULTS AND DISCUSSION

Growth performances and feed utilization parameters of pearlspot after 60 days of feeding trial are given in Table 1. Among the methionine supplemented diets, the highest mean body weight gain was recorded in the diet T2 (17.67± 0.20 g) than other treatments. Methionine deficiency in the diet leads to poor growth and reduced feed efficiency in juvenile rockfish *Sebastes schlegeli* (Yan *et al*., 2007). Liang *et al*., 2016 found that optimal dietary methionine level of pre-adult blunt snout bream of 0.74-0.76% of the diet (2.24-2.30% of dietary protein). In this present study, dietary methionine supplementation significantly affected PER in pearlspot and found highest in fish fed with methionine at 2.2% of diet, which showed that protein intake was efficiently utilized for fish growth at this dietary level of methionine. Second-order polynomial regression analysis of weight gain against graded dietary methionine levels results were depicted in Fig 1. Among the lysine supplemented diets, the highest mean body weight gain was recorded in the diet T5 (17.6±0.15 g) than other treatments. Second-order

polynomial regression analysis of weight gain against graded dietary lysine levels results were depicted in Fig 2. The deficiency of lysine in the feed will result in reduced growth and feed efficiency of fish. El Saidy and Gaber (2002) found that diet with 55% SBM supplemented with 0.5% L-lysine can totally replace fish meal in a diet for Nile tilapia fingerlings, without adverse effect on fish performance. Similar to this study improved growth performances, protein efficiency and feed utilization were observed in Nile tilapia adequately provided with dietary tryptophan, methionine and lysine (Zaminhan *et al.*, 2017; 2018; Nguyen, *et al.*, 2019) and phenylalanine (Xiao *et al.*, 2019).

Similarly, FCR values in the present study were significantly affected by the dietary treatments and best FCR (T2-1.53±0.03) was obtained in fish fed methionine at 2.2% compared to other diets, which indicated that juvenile pearlspot are able to effectively assimilate methionine at this level for enhancing growth performance. The best FCR (T5-1.61±0.03) was obtained in fish fed with lysine at 0.9% of diet compared to other diets, which indicates juvenile pearlspot are able to effectively assimilate lysine at this level for enhancing growth performance. SGR values in the present study were significantly affected by dietary treatments and best SGR value was obtained in fish fed methionine at 2.2% (T2-3.35±0.01) and lysine at 0.9% (T5-3.3±0.01) compared to other diets. It is generally considered that the excessive amount of amino acid supplementation may become toxic and may have an adverse effect on the growth performances and feed utilization because the imbalanced intake of one amino acid may have synergetic effect on other amino acids (Ahmed and Khan, 2005). This statement is in agreement with the present study, where the feed utilization was reduced with increasing methionine and lysine supplementation. In the present study, whole body composition of pearlspot fingerlings was not significantly ($p>0.05$) affected by graded levels of methinine and lysine supplementation, which might be due to the isonitrogenous and isoenergetic diets used in this study. Significant differences ($p<0.05$) were observed in weight gain, FCR and SGR. Fish fed with 2.2% methionine and 0.9% lysine diet showed higher growth performance and feed utilization compared to other treatment groups. Results of whole-body composition (g/kg of protein wet weight) of pearlspot fingerlings fed graded level of methionine and lysine was depicted in Fig 3.

Fatty acids have important roles in human nutrition, disease prevention and are in general beneficial to health. When comparing wild and farmed fish, higher lipid contents are found in farmed fishes due to the accessible and well formulated diets (Alasalvar *et al.*, 2002). Increase in the lipid supplementation in diet appears to have negative effect on growth, FCR and PER indicating that pearlspot does not seem to utilize lipid energy source for growth at tropical temperature (Cho *et al.*, 1985). In the present study, the lipid content in *Etroplus suratensis* was found to be 2.98%.

Table 1: Bio-growth parameters of pearlspot fed with different inclusion levels of methionine and lysine incorporated diets.

Parameters	Control	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
Mean initial length (cm)	6.81±0.02^{a}	6.82±0.07^{a}	7.04±0.08^{b}	5.68±0.05^{a}	6.82±0.04^{a}	6.93±0.09^{a}	6.81±0.01^{a}
Mean final length (cm)	11.02±0.05^{bc}	10.20±0.01^{a}	12.60±0.5^{b}	10.83±0.05^{a}	12.63±0.04^{bc}	12.10±0.01^{b}	11.0±0.02^{a}
Mean length (cm)	7.06±0.02^{b}	7.04±0.03^{ab}	7.07±0.04^{bc}	5.1±0.04^{a}	5.8±0.04^{b}	5.16±0.05^{b}	4.20±0.03^{a}
Mean initial weight (g)	8.62±0.01^{a}	9.04±0.01^{b}	8.93±0.05^{ab}	8.73±0.04^{ab}	8.78±0.06^{a}	8.72±0.04^{a}	8.76±0.05^{a}
Mean final weight (g)	20.73±0.03^{a}	24.56±0.00^{b}	29.61±0.05^{c}	25.33±0.03^{b}	25.40±0.01^{b}	28.32±0.00^{c}	23.16±0.05^{b}
Mean weight gain (g)	12.11±0.00^{a}	15.52±0.00^{b}	20.68±0.00^{c}	16.6±0.00^{b}	16.62±0.01^{b}	19.6±0.05^{c}	14.4±0.05^{a}
Average daily growth rate (g/day)	0.20±0.00^{b}	0.25±0.00^{b}	0.34±0.00^{c}	0.27±0.00^{b}	0.27±0.00^{b}	0.32±0.00^{c}	0.24±0.00^{a}
ADG Specific growth rate (%) / day (SGR)	2.99±0.01^{a}	3.16±0.01^{ab}	3.35±0.01^{c}	3.19±0.01^{b}	3.19±0.01^{b}	3.3±0.01^{c}	3.10±0.00^{a}
Feed conversion ratio (FCR)	1.8±0.03^{c}	1.79±0.03^{b}	1.53±0.03^{a}	1.69±0.06^{b}	1.62±0.03^{b}	1.61±0.03^{a}	1.67±0.02^{c}
Feed efficiency ratio (FER)	0.55±0.00^{a}	0.55±0.01^{ab}	0.65±0.01^{c}	0.59±0.00^{b}	0.61±0.01^{b}	0.62±0.01^{c}	0.57±0.00^{a}

Values in the same row with different superscript differ significantly ($p<0.05$) between the treatments for each parameters. One way ANOVA was used following Duncan multiple ranges testing SPSS 20.0. Values are presented as mean±Standard error.

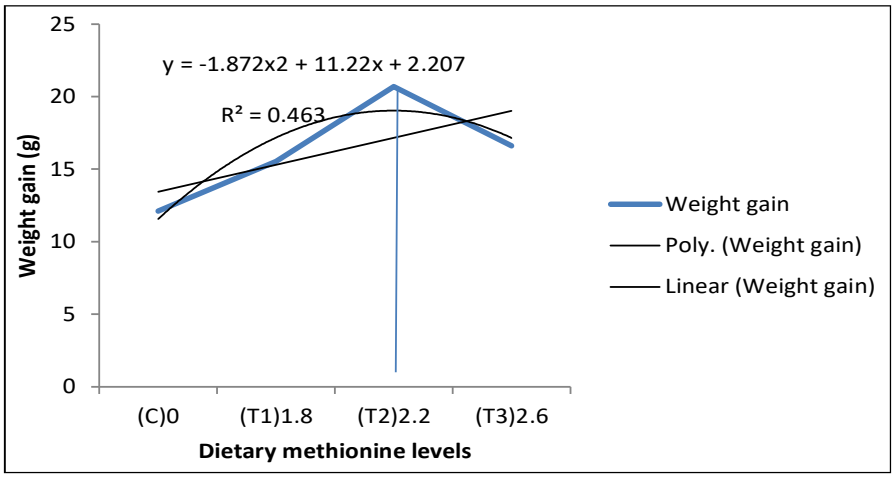

Fig 1: Second-order polynomial regression analysis of weight gain against graded dietary methionine levels.

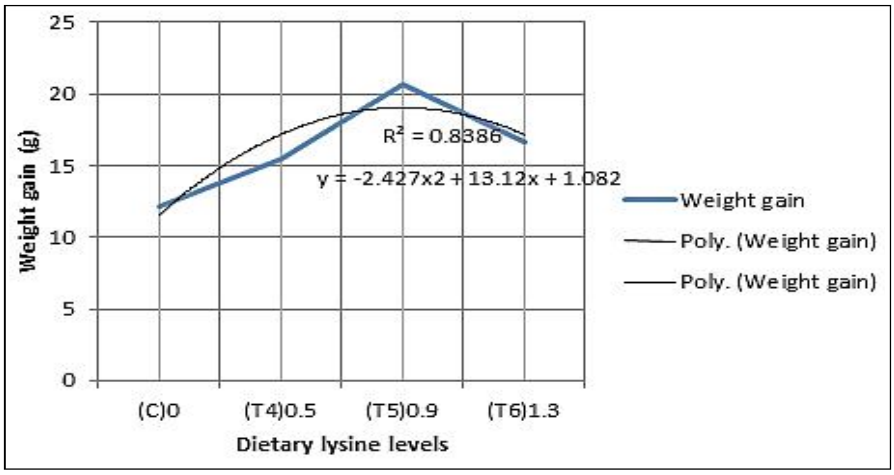

Fig 2: Second-order polynomial regression analysis of weight gain against graded dietary lysine levels.

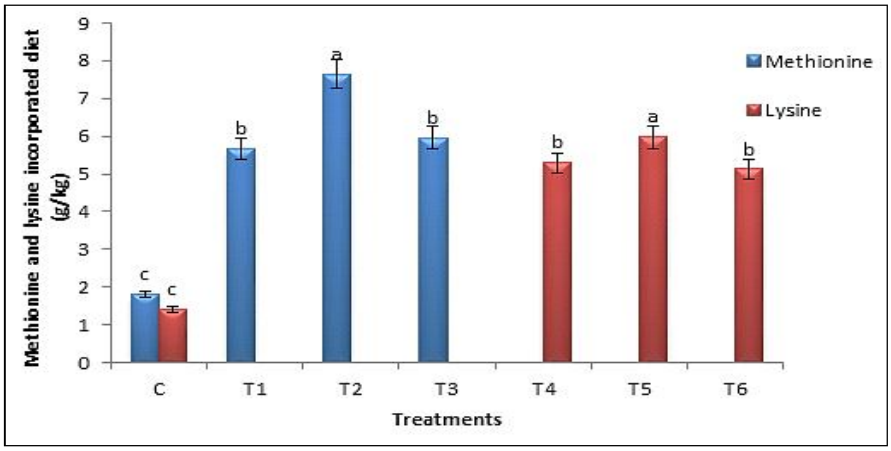

Fig 3: Whole-body composition (g/kg of protein wet weight) of pearlspot fingerlings fed graded level of methionine and lysine supplemented diets.

Fish muscle obtained high EPA (6.36%) and DHA (4.13%) content. The present study is in agreement with findings in milkfish and is also within the range of lipid levels found in the diets of different teleost species (Satoh, 1991). The results of fatty acid profile in animal muscle depicted in Fig 4.

The literature with reference to digestive enzyme profile of pearlspot is inadequate for the formulation of efficient compound feeds. Distribution and activity of intestinal digestive enzymes along the intestinal tract varies with feeding habit and intestinal morphology (Kuz'mina, 1984; Sabapathy, 1993 and Kolkovski, 2001). The amylase activity is greater in omnivorous and herbivorous fish than in carnivorous fish. *E. Suratensis* has a short and less coiled intestine and the alkaline protease activity that gradually decreases (Hidalgo *et al*.,1999; Fange *et al*., 1979 and Ugolev *et al*., 1983). Similarly, *Mugil cephalus* exposed to higher salinity (>10 ppt) showed lower digestive enzyme activity (Barman *et al*., 2012). In the present study, high protease activity, amylase activity and a low lipase activity has been exhibited by *E. suratensis.* The enzyme activities were lower in control. The enzyme activity in the liver and intestine were of *Etroplus suratensis* fingerlings depicted in Fig 5.

Hematological analysis is considered as one of the main indicators of salinity stress as it alters the blood parameters including blood components (Boeuf *et al*., 2001). The efficiency of oxygen transport from gill to tissues was evaluated based on the RBCs, haemoglobin and hematocrit content (Cook *et al*., 2013). The findings of present study are in agreement with results reported in Guinean tilapia (Akinrotimi *et al*., 2012), mullet (Fazio *et al*., 2013), blue

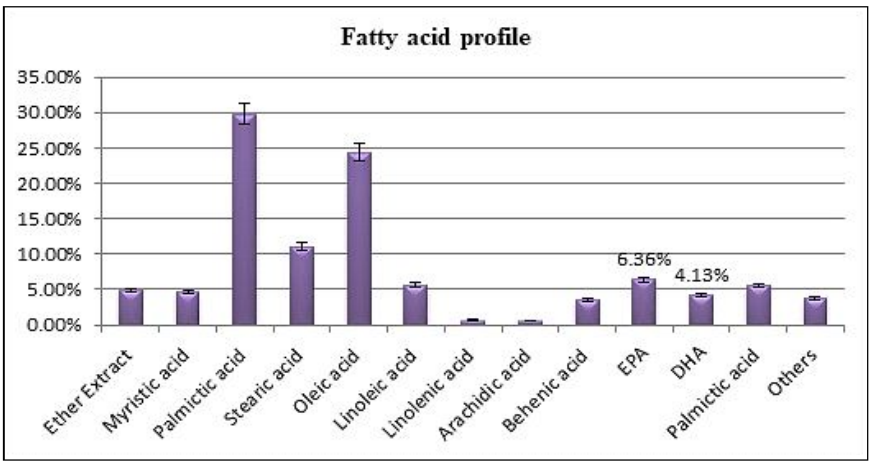

Fig 4: Fatty acid profile of animal muscle of pearlspot fingerlings fed graded level of methionine and lysine supplemented diets.

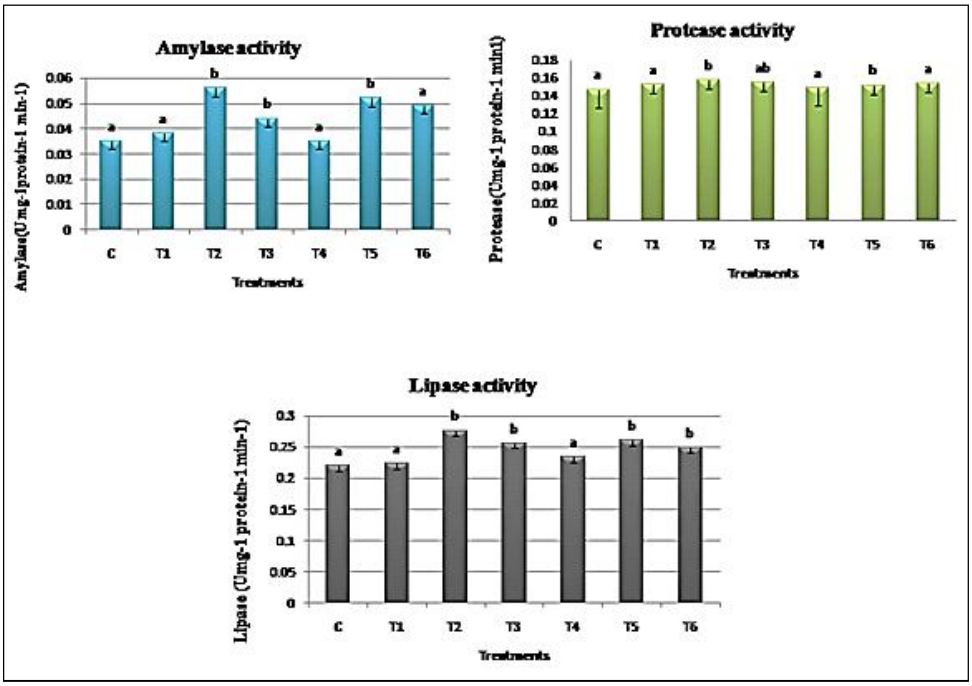

Fig 5: Digestive enzyme activity in the liver of *Etroplus suratensis* fingerlings fed graded level of methionine and lysine supplemented diets.

tilapia (Semra *et al*., 2013), Nile tilapia (De Azevedo *et al*., 2015), Red tilapia (Malik *et al*., 2017). In the present study, the Hb, RBC, MCV, MCH and MCHC were also affected by dietary methionine and lysine supplementation and were found significantly ($p<0.05$) higher in fish fed methionine at 2.2% and lysine at 0.9% of diet, indicating that dietary methionine and lysine supplementation activates immune response and promotes fish health. The reason behind an increase in RBCs and hemoglobin might be the adaptiveness towards saline conditions. Similarly, fishes reared in lower salinity showed the fastest movement and were found to be highly active and this may be one of the possible reason for higher growth rate at lower salinity (Malik *et al*., 2017). Results of haematological parameters of *Etroplus suratensis* fed with different inclusion levels of methionine and lysine supplemented diets were depicted in Fig 6, 7 and 8. Dietary methionine and lysine supplementation also had significant influence on the values of GLU, CHO and TG. Jeanette *et al*., (2007) reported that in *Oreochromis mosambicus* the blood glucose levels increases significantly with an increase in the environmental salinity and temperature. In the present study, the lowest blood glucose level was found in T2 and T5. Cholesterol is the main structural component of animal cell membrane which acts as a precursor for biosynthesis of vitamin D3, prostaglandins, steroids and bile acts (Steffens, 1989). High concentration of blood cholesterol may suggest that the dietary lipid imbalance resulting in poor growth performance (Wedemeyer *et al*., 1990). In the present study the maximum CHO and TG was found in T3 followed by T2. Xiao *et al*., (2019) observed no significant ($p>0.05$) changes in the haematological and biochemical responses of Nile tilapia fed graded levels of methionine, lysine and phenylalanine supplemented diets. Biochemical parameters of *Etroplus suratensis* fed with different inclusion levels of methionine and lysine supplemented diets were depicted in Fig 9 and 10.

During challenge test study, mortalities associated with *V. anguillarum* infection occurred only during days 4, 7, 11 and 14 post infection (with five, four, three and two dead fish,

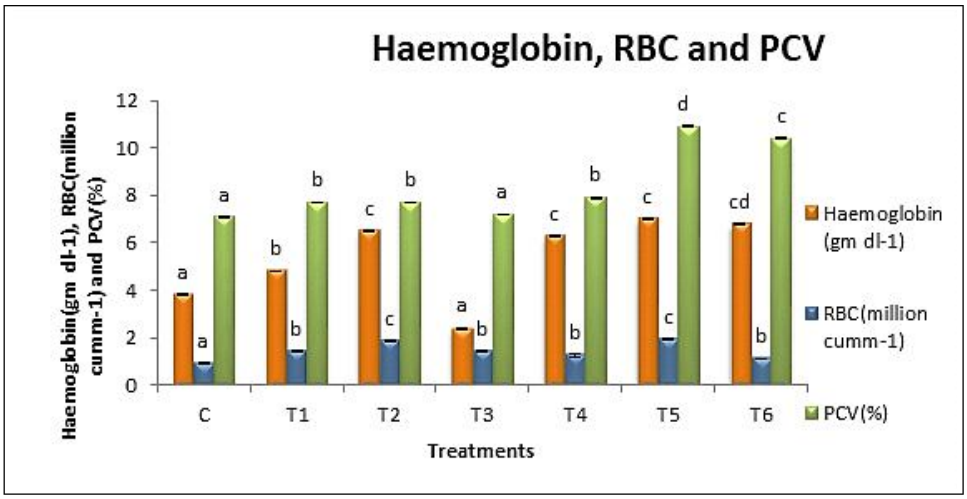

Fig 6: Haematological parameters of *Etroplus suratensis* fed with different inclusion levels of methionine and lysine supplemented diets. *PCV (%) - Packed cell volume, RBC - Red blood cell.

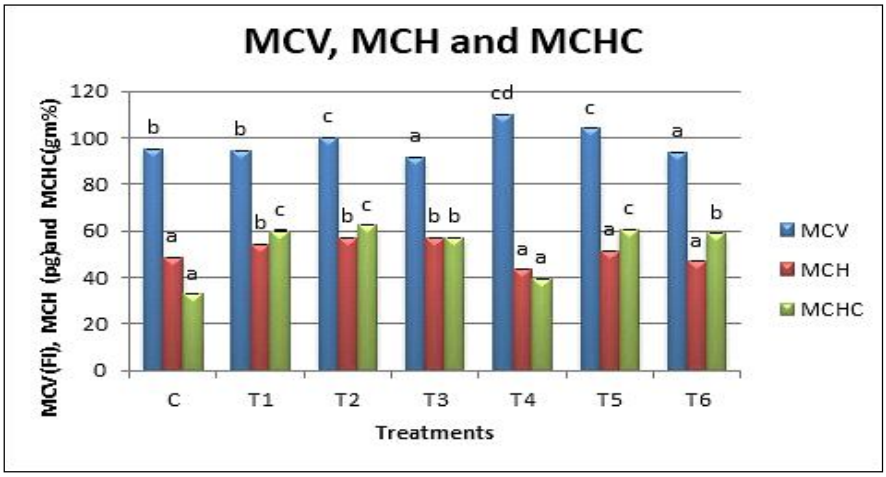

Fig 7: Haematological parameters of *Etroplus suratensis* fed with different inclusion levels of methionine and lysine supplemented diets. *MCV (Fl)- Mean corpuscular volume *MCH (pg)-Mean corpuscular haemoglobin *MCHC (gm%) Mean corpuscular haemoglobin concentration.

respectively) among fish injected with strain *V. anguillarum* API20E. The results showed the presence of lower degree levels of infection in the treatment T2 and T5. And there were no histopathological alterations in unchallenged groups. The results obtained from this experiments revealed that, methionine at 2.2% and lysine at 0.9% supplemented diets showed a decrease in their virulence in comparison to others. These results suggest that the resistant strains showed a significant decrease in their virulence. Based on the conventional microbiological methods and PCR using

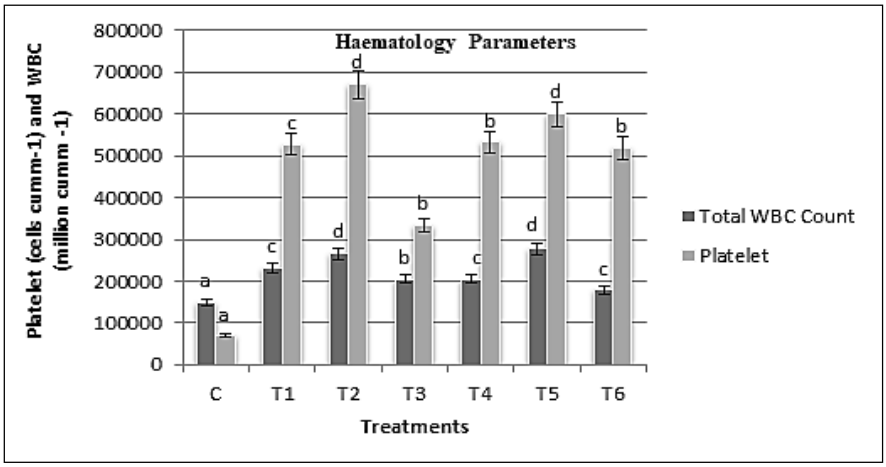

Fig 8: Haematological parameters of *Etroplus suratensis* fed with different inclusion levels of methionine and lysine supplemented diets.

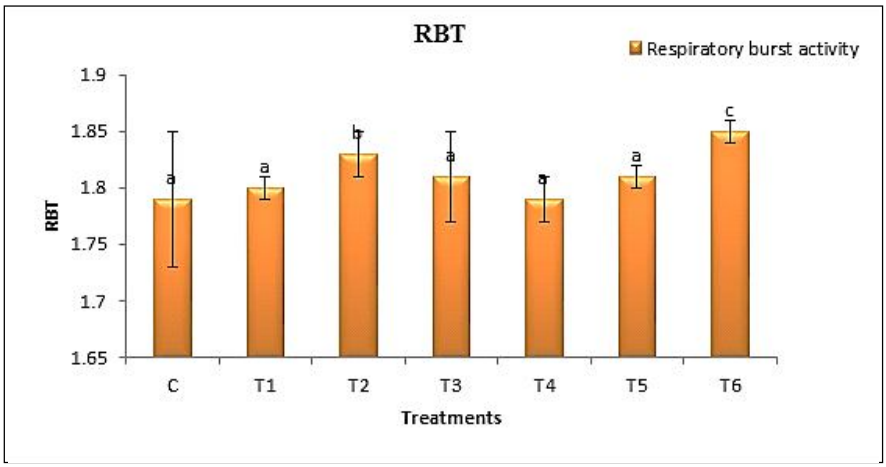

Fig 9: Respiratory burst activity of *Etroplus suratensis* fed with different inclusion levels of methionine and lysine supplemented diets.

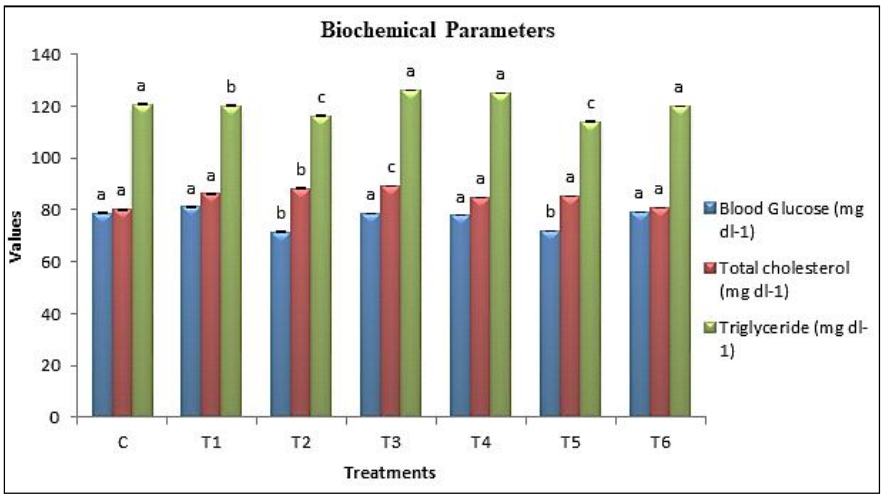

Fig 10: Biochemical parameters of *Etroplus suratensis* fed with different inclusion levels of methionine and lysine supplemented diets.

species specific primers, the infective bacteria were identified as *Vibro anguillarum.*

In the present study the fishes were exposed to freshwater. There were hyperplastic changes in gill with mild to extensive. Additionally, hepatic parenchyma revealed fatty changes, vascular engorgement due to active congestion, degeneration and necrotic areas among perivascular hepatocytes. Mild changes in gut muscularis and ileum lamina was observed in intestine. Morphological variations in the gill and liver tissue such as degeneration of gill arch and vacuolization and no major/severe physiological changes were observed were depicted in Fig 11,12 and 13.

Vidhya *et al*., 2019 reported that the histological alternations like necrosis and degeneration of hepatocytes were prominent changes observed in the liver of *E. suratensis* exposed to the lower concentration of lambda-cyhalothrin (0.005 ppm). Ologo *et al*., 2005 observed degeneration of the hepatocytes and focal necrosis in the liver of *Clarias gariepinus* exposed to lead. Makesh Marappan *et al*., 2019 reported that the susceptibility of the brackishwater cichlid, pearlspot, *Etroplus suratensis* to NNV the infected pearlspot brain cells and IEK cells showed cytopathic effect at second and third passage of the virus and they were positive for NNV by nested RT-PCR. The present results included many alterations produced pathological changes in the tissues such as macrobiotic changes in the liver, tubular damage of kidneys, gill and lamellar abnormalities. Histopathological techniques are rapid, sensitive, reliable and comparatively inexpensive tools for the assessment of stress-response to pathogen. These changes attributed to infective bacteria *V. angullarium.* Macroscopic lesions observed during experimental challenge were depicted in Fig 14. Results of histopathological observation during challenge test were given in Fig 15. PCR amplification of *V. anguillarum* virulence marker specific for fish virulent strain were given in Fig 16.

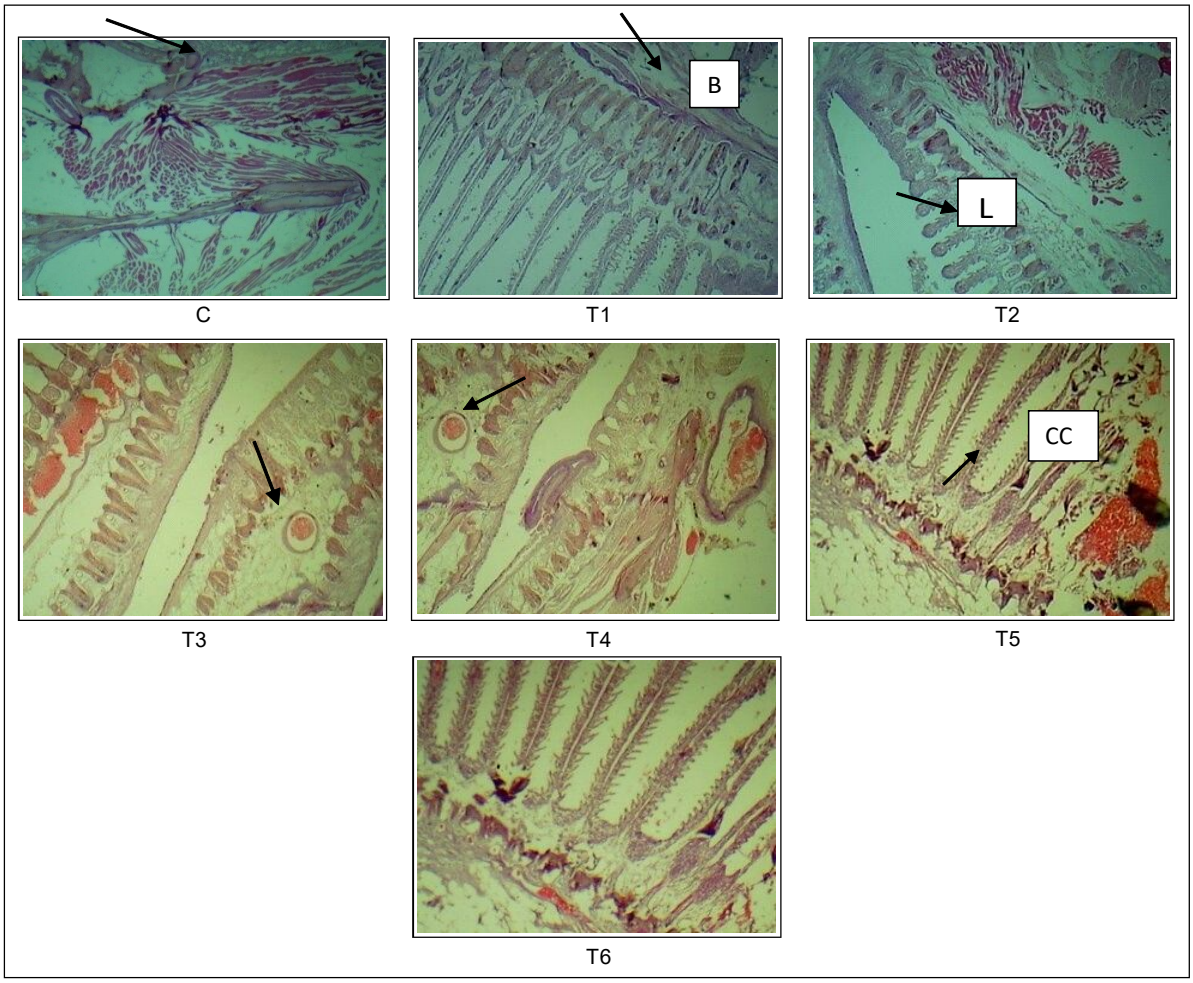

Fig 11: Gill structure of *Etroplus suratensis* exposed to 0ppt and fed with lysine and methionine aminoacid diet showed. C-Degeneration of fill filaments, T1-Normal gill filaments, T2-Normall fill filaments, T3-Vacuolization of gill, T4-Vacuolization of gill, T5 and T6-Degeneration of gill arch observed without any major/severe physiological changes. (L-Lamella, CC-Chloride cell and B-Blood channels).

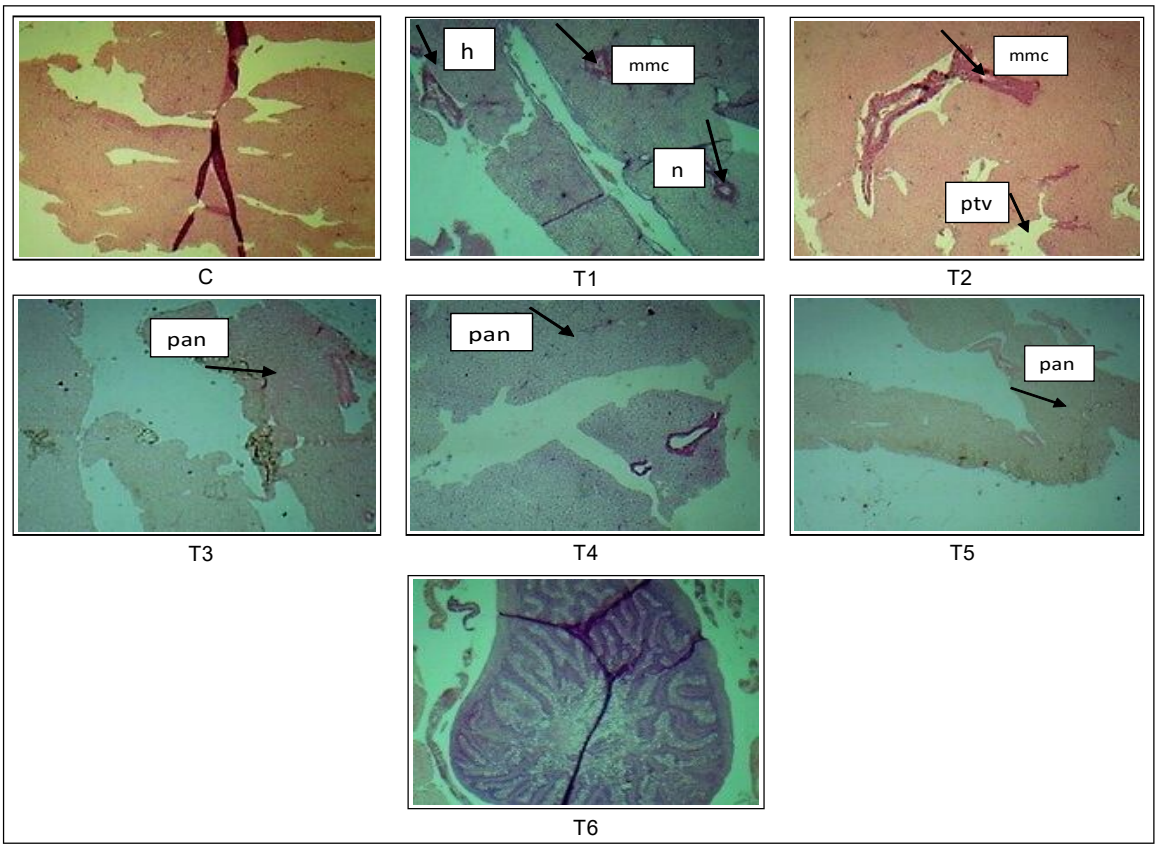

Fig 12: Liver structure of *Etroplus suratensis* exposed to 0 ppt and fed with lysine and methionine aminoacid diet showed. C-Normal liver filaments, T1-Normal nucleus hepatocyte, T2-Normal melanomacrophage centre, T3-Portal vein, melanomacrophage centre, T4-Exocrine pancreatic tissue, T5-Exocrine pancreatic tissue and T6-Normal liver tissue.

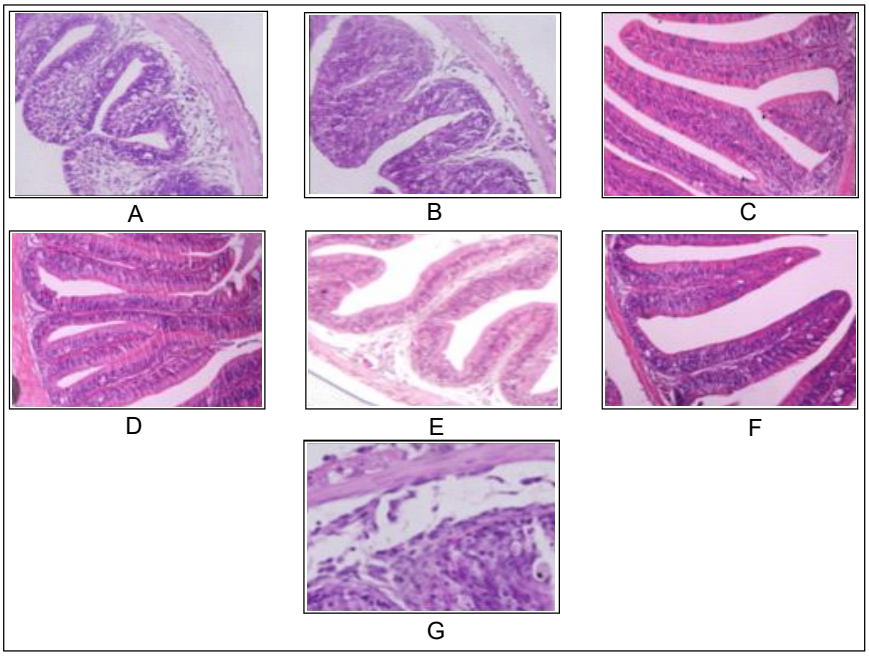

Fig 13: Intestine structure of *E. suratensis* exposed to 0 ppt and fed with lysine and methionine amino acid diet showed. a) Histological changes of the ileum (C) b) Histological changes of the ileumand lamina (T1) c) Normal intestinal epithelial cells (T2) d) Normal intestinal epithelial cells (T3) e) Normal intestinal epithelial cells (T4) f) Normal intestinal epithelial cells (T5) g) intestine muscularisis (T6).

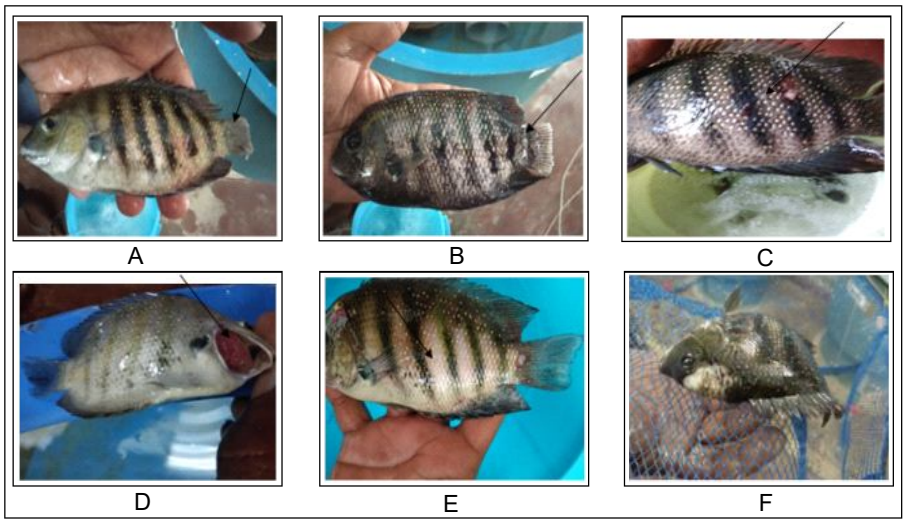

Fig 14: Macroscopic lesions observed during experimental challenge.
a) Hemorrhages on caudal fin. b) Petachiae in the mouth and skin c) Hemorrhagic skin d) Pale gills e) Bodydiscolouration f) Eroded skin.

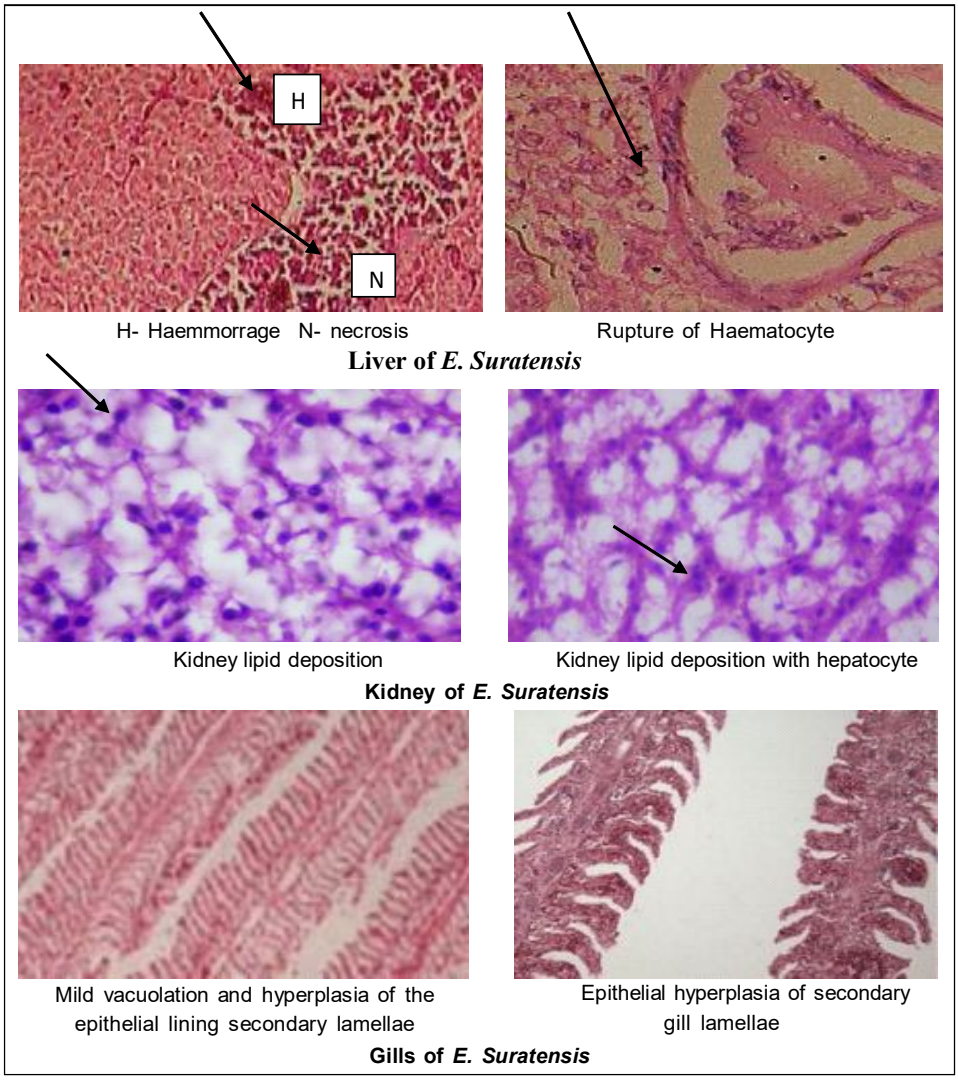

Fig 15: Histopathological observation during challenge test.

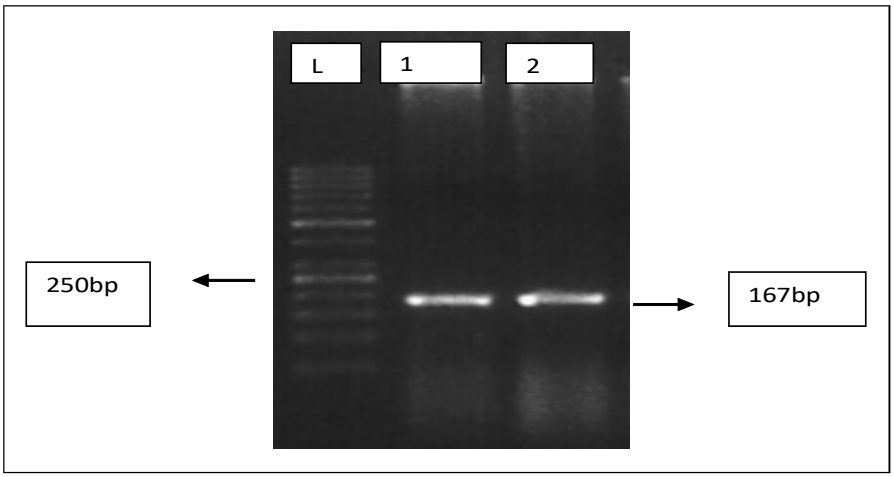

Fig 16: PCR amplification of *V. anguillarum* virulence marker specific for fish virulent strain species specific primer for *V. anguillarum* (Where, L- Marker DNA (100 bp ladder); Lane 1 and 2 -species specific prime, virulent sequences by PCR and the specific amplification was confirmed by sequencing of the representative amplicon.

CONCLUSION

The present study concluded that, dietary supplementation of methionine and lysine was efficiently utilized by pearlspot fingerlings, which was evident from the improved growth performances. In this study, dietary methinine and lysine requirement for pearlspot fingerlings was estimated to be 2.2 g/100 g (T2) and Lysine at 0.9 g/g (T5) based on second-order polynomial regression analysis. Results of challenge tests confirmed that *V. anguillarum* could act as a primary pathogen for pearlspot reared in freshwater. Hence, the present study recommended the optimum requirement of methionine at 2.2 g/100 g (T2) and Lysine at 0.9 g/g (T5) in the diet of pearlspot for better growth.

Conflict of interest: None.

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