Acta Scientific Veterinary Sciences (ISSN: 2582-3183)

Volume 2 Issue 8 August 2020

Research Article

Partial Fishmeal Replacement by *Azolla* Meal on GIFT Tilapia (*Oreochromis niloticus*) Diet: Effect on Growth Performance, Antioxidant Enzymes, Immunology and Stress Response

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Received: June 20, 2020

Published: July 27, 2020

Abstract

A 60 days growth trial was carried out to evaluate the effect of partial replacement of fishmeal with *Azolla* meal on growth performance, antioxidant enzymes, immunology, muscle quality and stress response in GIFT tilapia (Oreochromis niloticus). One hundred and twenty fingerlings (3.3 ± 0.32g) were randomly stocked in plastic containers (70 L) in triplicates. Four isonitrogenous (32%) and isocaloric (17 KJ Kg-1 DM) diets; control (0%), T1 (15%), T2 (30%) and T3 (45%) *Azolla* meal inclusion levels were fed at 5% body weight for the whole trial. The optimum water quality parameters were maintained in all the groups. The growth performance, serum biochemistry and immunological parameters, muscle quality and stress response parameters of GIFT tilapia were higher in T1 compared to control and other treatments. Catalase (CAT), Superoxide dismutase (SOD) and Glutathione peroxidase (GPx) activity in liver did not significantly differ between the treatments however were slightly higher in T1. Thus, the study results recommend 15% inclusion level of *Azolla* meal as a feed ingredient in fish diets.

Keywords: *Azolla* Meal; GIFT Tilapia; Antioxidant Enzymes; Fish Immunity

Introduction

Fish supply from aquaculture sector plays a significantly important role in contributing to food security, poverty alleviation and economic development of the poor [1]. In India, the sector dominated by carps with a production of 5,863,263 tonnes in 2017 [2]. It is estimated that, in 2030, per capita protein intake from fish is expected to be 18% in the country [3]. However, the sector is constrained by various other factors, including lack of technical innovation, absence of suitable cost effective feeds and inappropriate feed management practices [1].

Due to these, some initiatives including diversification of different fish species have been implemented. Among fish species, Tilapia strains, GIFT Tilapia (*Oreochromis niloticus*) have been cultured and represents approximately 80% of Tilapia production worldwide [4]. It has good growth rate, high-market value and reported to convert raw protein from the aquatic plant materials such as *Azolla* species into the best digestible protein, thus reduces the higher cost of fish feeds [5]. However, its culture is constrained by various other factors, including several disease outbreaks, lack of technical innovations, absence of feed formulation and processing knowledge, and adoption of inappropriate feed management practices [1]. These lead researchers to find ways to improve the production by implementing various strategies including use of biofloc technology, RAS and non-conventional plants origin ingredients to replace fishmeal.

Among non-conventional plant ingredients, a floating freshwater fern, *Azolla* is proven to be used as an alternative ingredient for protein source in fish diet [6]. It has gained its prominence in tilapia culture due to higher content in crude protein (13-30%) and essential amino acid (EAA) composition (rich in lysine) than most forage crops and other aquatic macrophytes [7]. Additionally, several species of *Azolla* including *A. microphylla*, *A. pinnata*, and *A. fiiculoides* have been well documented in Nile tilapia, *O. niloticus* [8] and *Tilapia mossambica* [9]. Despite its high nutritional qualities

and relatively ease to be produced in an open water bodies, the nutritional properties and digestibility of this *Azolla* species has not been explored in correlating with the overall physiological changes of GIFT tilapia. Therefore, the present study was conducted to assess the effect of partial replacement of fishmeal with *Azolla* meal on growth performance, antioxidant enzymes, immunology, muscle quality and stress response in GIFT tilapia (*O. niloticus*).

Materials and Methods

Azolla culture

Azolla was cultured in five pits lined with HDPE sheet, having the size of 6 x 1 x 0.2m each. Twenty-five kilogram of red soil were uniformly spread in each pit and filled with water to a height of 20 cm. Then, 2 kg of fresh cow dung slurry and 30g of Single Super Phosphate were added. Each pit was inoculated with 200g fresh culture of *Azolla* and left for 3 weeks. All pits were re-fertilized with the same amount of fresh cow dung slurry and Single Super Phosphate after every 6 days. Each pit was harvested after three weeks with a scoop net, washed and dried under sunlight for three days, milled and packed in a polythene bag, and stored at room temperature prior to use.

Proximate composition analysis of ingredients and formulated diets

All ingredients and diets formulated were subjected to proximate composition analysis. Crude protein (Kjeldahl digestion method), ether extract (Soxhlet extraction method), moisture (Oven drying method), crude fibre (Moisture free defatted and Fibertec System method), total ash (Muffle furnace method) and gross energy (Bomb Calorimeter method) were analyzed according to AOAC [10]. The results were shown in table 1 and 2.

Ingredients	Proximate composition (%)				
	Crude protein	Ether Extract	Crude fibre	Total ash	Moisture
Fish meal	60.28	13.76	<1.0	14.83	10.62
Azolla meal	23.99	3.37	13.84	18.10	10
Soybean meal	47.17	1.52	5.30	7.46	5.39
Corn flour	7.87	4.31	1.40	1.69	6.67
Cassava starch	9	-	-	-	-
Groundnut Oil Cake	39.21	10.11	5.35	3.77	17.65

Table 1: Proximate composition of experimental feed ingredients (% dry matter).

Ingredients (g kg^{-1})	Experimental Diets			
	Control (0% AZ)	T1 (15% AZ)	T2 (30% AZ)	T3 (45% AZ)
Fish meal	200	120	60	0
Azolla meal	0	150	300	450
Soybean meal	220	220	260	280
Corn flour	340	270	140	30
Cassava starch	50	50	50	50
Groundnut Oil Cake	172.5	172.5	172.5	172.5
Methionine	2.5	2.5	2.5	2.5
Vitamin premix	5	5	5	5
Mineral premix	5	5	5	5
Chromium oxide (Cr_2O_3)	5	5	5	5
Proximate composition (g kg^{-1})				
Crude Protein	321.0	318.9	318.0	318.5
Ether extract	31.2	30.7	30.9	30.6
Total ash	78.7	93.6	103	124.6
Moisture	74.6	46.2	63.4	67.6
Gross Energy (KJ kg^{-1})	17.17	17.29	16.83	16.96

Table 2: Dietary formulation and proximate composition of experimental diets in dry weight basis.
T: Treatment; AZ: *Azolla* meal.

Experimental design and feeding

Based on available information from the literatures on different plant materials supplementation in fish diets, four isonitrogenous (32%) and isocaloric (17 KJ kg^{-1} DM) experimental diets were formulated and used to replace fish meal with *Azolla* meal at 0, 150, 300 and 450 g kg^{-1} (Table 2). 120 healthy GIFT tilapia fingerlings (body weight 3.3 ± 0.32g) were randomly stocked at a rate of 10 fishes per container in 12 plastic containers (70L capacity) for 60 days in triplicates. Experimental animals were fed with formulated diets at 5% of their body weight in two rations for all the treatments throughout the trial.

Water quality parameters

Water quality parameters were monitored throughout the experimental period. Temperature (Pro 20, YSI-USA), dissolved oxygen (Pro 20, YSI-USA) and pH (model: LT-10) were monitored daily. Nitrite (NO_2-N) and ammonia (NH_4-N^+) were measured weekly once by titration method using spectrophotometer (Systronics, Ahmedabad, India) [11].

Growth performance and survival

The growth parameters and survival rate were evaluated at the end of 60 days feeding trial according to Olvera-Novoa., *et al*. [12].

Antioxidant enzymes activity

At the end of feeding trial, three fish from each treatment (1 fish $replicate^{-1}$) were anesthetized using clove oil (100 ppm L^{-1}) and dissected. Liver sample weighing 0.1 g was collected into a screw tube and crushed with a glass rod. The tissues were homogenized (10% w/v) in ice-cold 50 mM Tris buffer (pH 7.4) and centrifuged (REMI, Vesai, India) at 10,000 rpm for 20 min at 4°C and the supernatant was used to assay the enzyme activities. CAT activity was measured according to Takahara., *et al*. [13]. SOD and GPx activities were determined according to the method of Mishra and Fridovich [14], and Paglia and Valentine [15] respectively.

Blood serum biochemistry

Blood serum biochemistry contents such as total serum protein, albumin and globulin were measured at the end of 90 days feeding trial. Prior to blood sample collection, the fish were starved for 24 hours. Three fish from each treatment (1 fish $replicate^{-1}$) were anesthetized using clove oil (100 ppm L^{-1}). The blood samples (0.5 Units) were collected from the caudal vein using heparinized syringe and left to clot for 30 minutes at room temperature. The clotted blood was transferred into 1.5 mL of micro tube and centrifuged (2,500 × g, 15 minutes, 27°C) to obtain serum which used for the analysis of blood serum biochemistry contents. The serum total protein was estimated by Lowry's method [16]. Albumin and globulin contents were assayed following the method of Doumas., *et al*. [17].

Immunological indices

Respiratory burst activity (RBT) in the blood plasma was measured using the modified method of Anderson and Siwiki [18]. Myeloperoxidase activity (MPO) was assayed according to Quade and Roth [19]. Plasma lysozyme activity was determined according to Shugar [20], and glucose content was estimated by using a standard kit method (Beacon Diagnostics Pvt. LTD, India).

Muscle quality index

The total carotenoid concentration (TCC) in fish muscle tissues were analyzed immediately after the completion of the experiment following the pigment extraction method as described by Olson [21].

Bacterial challenge study

Prior to the challenge, a pure culture of *Aeromonas hydrophila* strain obtained from Advanced Research Farm Facility, Biotechnology Laboratory was centrifuged at 10,000 rpm at 4°C for 10 min. The cells were washed with PBS and diluted to 1×10^7 CFU/mL. One unit of the strain was injected intracellular to five fish from each treatment and reared for 7 days. The mortalities were recorded daily for the whole challenge trial and calculated according to Olvera-Novoa., *et al*. [12]. At the end of seven days challenge trial, Respiratory burst activity (RBT) and Myeloperoxidase activity (MPO) from blood samples were determined according to Anderson and Siwiki [18] and Quade and Roth [19] respectively. Glucose content was assayed by using a standard kit method (Beacon Diagnostics PVT. LTD, India).

Statistical analysis

Statistical analysis for all the parameters in the study was performed using Analysis of Variance (one-way ANOVA) followed by Turkey's multiple range test at 5% ($p < 0.05$) level to compare mean between the treatments. The results obtained were presented as mean ± SE (standard error of mean). All calculations were performed using IBM SPSS Statistics V21 (IBM Cop. Armonk, New York, USA).

Results and Discussion

Water quality parameters

Water temperature is expressed as mean values ranged from 29.60 to 30.20°C, Dissolved oxygen (5.50 to 6.00 mg L^{-1}), pH (7.10 to 7.37), Total ammonia (0.01 to 0.03 mg L^{-1}) and Nitrite (0.001 to 0.002 ppm). The values shown that, the experimental diets have no negative effect on the culture water. Therefore, the values are in consistence with the recommended values for tilapia growth [22].

Growth performance and survival

The overall growth performance results showed that, the experimental diets incorporated with *Azolla* meal up to 30% did not

negatively affect the overall fish growth (Table 3). The fish fed with T1 (15% *Azolla* inclusion level) showed significant ($p < 0.05$) final weight (FW), average daily gain (ADG) and specific growth rate (SGR) compared to other treatments (Table 3). Similar results have been reported by Abou., *et al*. [23] in tilapia, *O. niloticus* when fed with diet contained *Azolla* up to 15% inclusion level.

The significantly lowest ($p < 0.05$) FCR value was recorded in T1 (15% *Azolla* inclusion level) compared to T3 (45% *Azolla* inclusion level), suggesting the effective utilization of the nutrients by the experimental fishes. The highest fishmeal replacing levels (T3) significantly ($p < 0.05$) reduced ADG for the different nutrients (CP and GE) and negatively affected the growth parameters (FW and SGR). This might be due to high fiber contents in the diet when plant materials exceed 30% inclusion level [6]. The similar results were observed by El-Sayed [24] when *Azolla* incorporated diets were fed with *O. niloticus*.

The protein efficiency ratio (PER) was decreasing when *Azolla* inclusion level increases and therefore PER was highest in T1 (15% *Azolla* inclusion level) and lowest in T3 (45% *Azolla* inclusion level). Therefore, the result implies that, T1 diet was economically viable since the PER is a considerable economic indicator in aquaculture feed practices [25].

Higher survival rate was found in T1 and control, and was low in T2 and T3. This might be due to the poor feed intake of the fishes. The results are in agreement with the findings of Yousif., *et al*. [26] in tilapia (*Oreochromis aureus*).

Parameters	Experimental Diets			
	Control (0% AZ)	T1 (15% AZ)	T2 (30% AZ)	T3 (45% AZ)
Initial Weight (IW, g)	3.39 ± 0.09^{a}	3.42 ± 0.12^{a}	3.39 ± 0.15^{a}	3.39 ± 0.17^{a}
Final weight (FW, g)	29.97 ± 0.37^{a}	38.47 ± 0.21^{b}	30.16 ± 0.53^{a}	24.83 ± 0.61^{c}
Average daily gain (ADG, g)	0.44 ± 0.01^{a}	0.58 ± 0.03^{b}	0.45 ± 0.01^{a}	0.36 ± 0.01^{c}
Feed conversion ratio (FCR)	1.08 ± 0.02^{a}	0.97 ± 0.07^{ab}	1.04 ± 0.01^{a}	1.22 ± 0.14^{ac}
Protein efficiency ratio (PER)	3.70 ± 0.05^{a}	4.17 ± 0.03^{ab}	3.82 ± 0.05^{a}	3.28 ± 0.04^{ac}
Specific growth rate (SGR)	3.63 ± 0.02^{a}	4.03 ± 0.06^{b}	3.64 ± 0.03^{a}	3.31 ± 0.04^{c}
Survival rate (SR)	93.33 ± 0.33^{a}	94.00 ± 0.57^{a}	85.67 ± 0.33^{b}	83.66 ± 0.33^{c}

Table 3: Growth parameters of GIFT tilapia fed with experimental diets for 60 days.
Values are means ± standard error ($n = 30$). Means with different superscripts letters within each row are statistically significant ($P < 0.05$). (T, Treatment; AZ, *Azolla* meal).

Antioxidant enzyme activities

No significant ($p > 0.05$) differences were seen on catalase (CAT), Superoxide dismutase (SOD) and Glutathione peroxidase (GPx) levels in fish fed with *Azolla* incorporated diets and the control (Table 4). However, these enzymes activity were slightly higher in T1, possibly due to high nutrients uptake and digestibility noted in this study which might influenced by high levels of digestive enzymes activity. This resulted in boosting immunity of the fish and high activation of the antioxidant enzymes, which detoxify and counteract the deleterious reactive oxygen species (ROS) effects [27]. Since, it has been reported that, the oxidative stress in fishes is more profound during nutritional deficiency, elevated temperature, hypoxia and exposure to xenobiotics [28]. Therefore, higher levels of antioxidant enzymes in fish diminishes intracellular ROS levels which in turn protect the liver damage, cell death and improving survival [29].

In addition, *Azolla* biomass is rich in protein, vitamins, phenolics and essential amino acids profile compared to soy [30]. These nutrients particularly vitamins and phenolics play role as antioxidants and can scavenge free radicals which help to regulate and stimulate the immune function and reduce morbidity of infectious diseases [31]. The results of the present study are in accordance with Souza., *et al*. [32], who reported high levels of CAT and SOD in Nile tilapia *Oreochromis niloticus* as indication of capacity to convert H_2O_2 to O_2^- and protection of the cell against oxidative damage in liver. These findings support our results in terms of improving fish antioxidant enzymes in liver at a limited level of *Azolla* inclusion diet (15%), as it has been noted that, liver is site of multiple oxidative reactions and maximal free radical generation [33].

Parameters	Experimental Diets			
	Control (0% AZ)	T1 (15% AZ)	T2 (30% AZ)	T3 (45% AZ)
CAT (Mol mg protein^{-1})	1.48 ± 0.26^{a}	1.75 ± 0.17^{a}	1.46 ± 0.51^{a}	1.42 ± 0.33^{a}
SOD (U mg protein^{-1})	44.83 ± 0.06^{a}	44.94 ± 0.02^{a}	44.92 ± 0.03^{a}	44.85 ± 0.07^{a}
GPx (U mg protein^{-1})	9.48 ± 0.82^{a}	10.77 ± 2.23^{a}	10.48 ± 2.29^{a}	10.34 ± 2.70^{a}

Table 4: Antioxidant enzyme activities from liver samples of GIFT tilapia fed with experimental diets for 60 days. Values are expressed as means ± standard error mean (n = 30). Means with different superscripts letters within each row are statistically significant ($p < 0.05$) (CAT, Catalase; SOD, Superoxide dismutase; GPx, Glutathione peroxidase activity; AZ, *Azolla* meal).

Blood serum biochemistry

Serum biochemistry analyses are used as valuable tools to analyze the health status of fish as these indices provide reliable information on disorders, deficiencies and stress status prior to the appearance of clinical symptoms [50]. In this study, total serum protein, albumin and globulin were significantly higher ($p < 0.05$) in T1 group (Figure 1). The increment of these parameters in fishes fed with *Azolla* diets at 15% inclusion level might be due to high protein synthesis and other metabolic processes, which play a significant role in the overall fish performance and immune response [34]. However, the combined values of globulin and albumin were higher than the total protein in T1 and T2 compared to other groups. This might be explained as several researchers reported that, the high levels of albumin and globulin compared to the total protein are thought to be associated with strong innate response in fish [35], hence improves the ability of fish to fight against pathogenic microorganisms. The results of the present study agree with the findings reported in *O. niloticus* [36] and Grass Carp, *Ctenopharyngodon idella* [37].

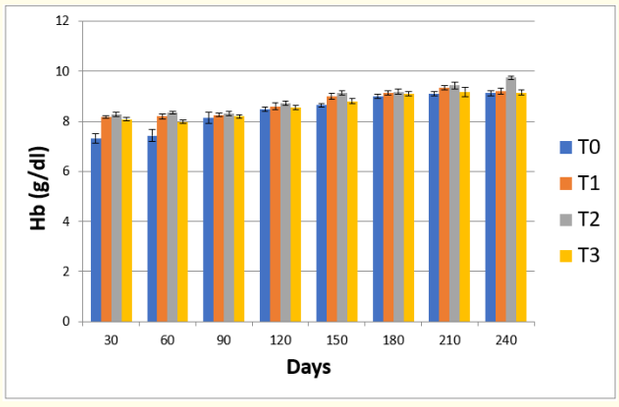

Figure 1: Graph showing effect of germinated maize on Hb (g/dl) in Kadaknath.

Immunological indices

The blood immunological parameters, such as Respiratory burst activity (RBT), Lysozymes and Myeloperoxidase activity (MPO) were significantly higher ($p < 0.05$) in T1 compared to other treatment groups (Table 5). The higher values might be due to the presence of various phyto-constituents molecules such as phenol contents, flavonoids, carotenoids and tannins [38] which forms part of fish body's defense mechanisms. Amutha., *et al*. [39] reported that, *Azolla* contains 80 - 85% flavonoids and nearly 50-55% carotenoids, which play an important role in the antioxidant activities. Significant concentrations of these compounds in *Azolla* supplemented diets, indeed boost fish immune system compared to the control diet.

In addition, Saurabh and Sahoo [40] reported that, the increase in lysozyme and MPO activity stimulate the immune response of fish and may contribute to host resistance against infectious pathogens. The better immunity status in fish fed with T1 compared to other groups is suggestive of concordance of this study with above. Similar immune response has also been observed in *O. niloticus* [41] when fed with diets containing plants materials.

Muscle quality index

Carotenoid compounds such as astaxanthin and canthaxanthin are commonly used as pigmentation sources in aquaculture [42]. Recently, the consumers are using these compounds as an important quality criterion [43]. In the present study, total carotenoid concentration (TCC) was significantly higher ($p < 0.05$) in T1 compared to other treatments and control (Figure 2). The presence of chemical compounds such as anthocyanins and flavonoids, and important minerals (zinc and phosphorus) in *Azolla* [44] could explain high levels of total carotenoid concentration as quality indicator in treatment diets compared to the control. Carotenoid compounds promoting fat burning within the fat cells in white adipose tissue by increasing the expression of thermogenin, which play important role in the rigidity of cell muscles, and maintaining the quality of fish muscles [45].

Stress parameters

The results of stress parameters are shown in table 6. Respiratory burst activity (RBT), Myeloperoxidase activity (MPO) and survival rate were significantly higher ($p < 0.05$) in T1 compared

Parameters	Experimental Diets			
	Control (0% AZ)	T1 (15% AZ)	T2 (30% AZ)	T3 (45% AZ)
RBT (U mL^{-1}enzyme)	1.33 ± 0.19^{b}	1.45 ± 0.20^{b}	1.10 ± 0.08^{c}	1.06 ± 0.12^{a}
Lysozyme (U mL^{-1}enzyme)	35.33 ± 0.29^{b}	39.88 ± 0.17^{d}	36.87 ± 0.38^{c}	33.10 ± 0.43^{a}
MPO (U mg^{-1} protein)	10.09 ± 1.19^{c}	12.65 ± 3.29^{d}	8.33 ± 4.12^{b}	5.94 ± 0.73^{a}

Table 5: Blood immunological indices of GIFT tilapia fed with experimental diets for 60 days.

Values are expressed as means ± standard error mean (n = 30). Means with different superscripts letters within each row are statistically significant ($p < 0.05$) (RBT, Respiratory burst activity; MPO, Myeloperoxidase activity; AZ, *Azolla* meal).

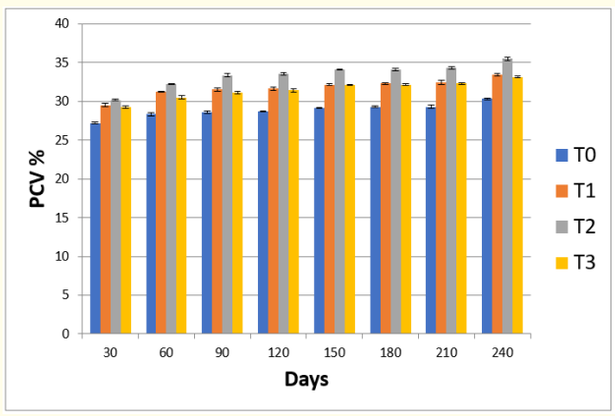

Figure 2: Graph showing effect of germinated maize on PCV (%) in Kadaknath.

to other treatment groups, while glucose content shown a reverse trend after fish challenged with *A. hydrophila*. The higher values in the present study could be due to the presence of antimicrobial properties (proline and fucoxanthin) in *Azolla*, which reported to have antioxidant activity and their protective effects on animal's health [39,46]. These biomolecules are related to different bioactivities and many enzymatic reactions, resulting in a decrease of platelet activation and aggregation, against bacterial diseases and anti-inflammatory activity [47]. Indeed, as protective proteins increase due to antioxidant activities, a decrease in bacterial infection as well as an enhanced serum bactericidal activity occurs [48]. The increased respiratory burst activity, myeloperoxidase activity and survival rate in *A. hydrophila* challenge of this study is reflective of high immune response and immunomodulatory effects of *Azolla* in fish. The findings of this experiment is in concordance with Cao.,

Parameters	Experimental Diets			
	Control (0% AZ)	T1 (15% AZ)	T2 (30% AZ)	T3 (45% AZ)
RBT (U mL^{-1}enzyme)	0.34 ± 0.06^{c}	0.49 ± 0.01^{d}	$0.28 \pm 0.04b^{e}$	0.26 ± 0.03^{ae}
MPO (U mg^{-1} protein)	22.15 ± 0.83^{a}	42.68 ± 1.15^{c}	31.07 ± 0.58^{b}	23.99 ± 0.97^{a}
Glucose (mg dL^{-1})	21.21 ± 0.61^{b}	15.83 ± 0.57^{a}	22.23 ± 1.02^{b}	33.13 ± 0.89^{c}
Survival rate (%)	80.00 ± 0.11^{b}	93.33 ± 0.33^{c}	80.00 ± 0.44^{b}	66.67 ± 0.33^{a}

Table 5: Stress parameters of GIFT tilapia fed with experimental diets for 60 days.

Values are expressed as means ± standard error mean (n = 15). Means with different superscripts letters within each row are statistically significant ($p < 0.05$) (RBT, Respiratory burst activity; MPO, Myeloperoxidase activity; AZ, *Azolla* meal).

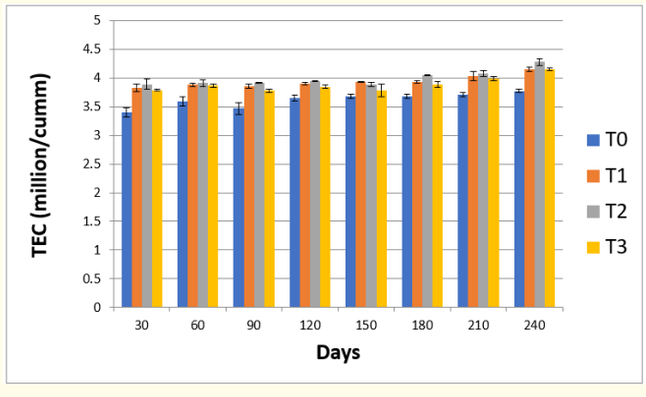

Figure 3: Graph showing effect of germinated maize on TEC (million/cumm) in Kadaknath.

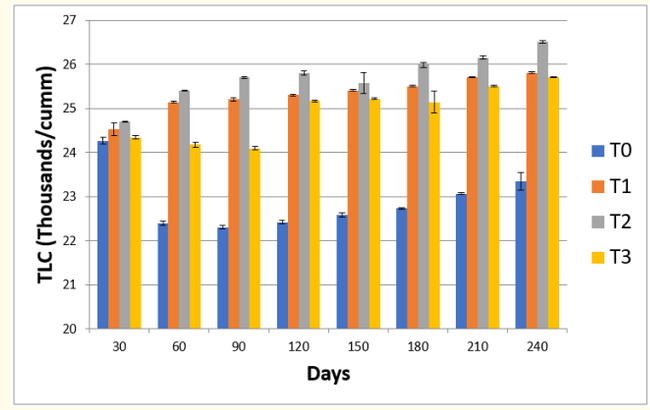

Figure 4: Graph showing effect of germinated maize on TLC (Thousand/cumm) in Kadaknath.

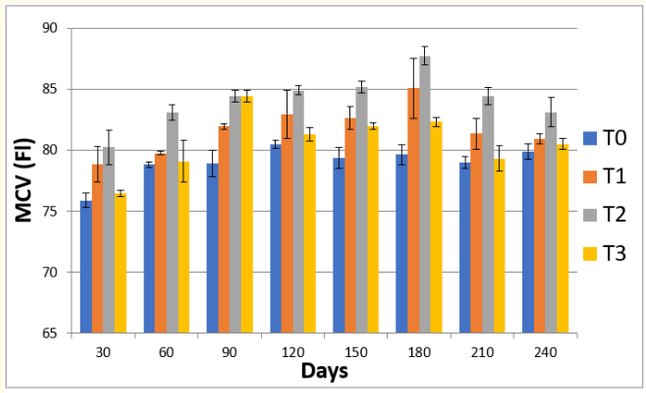

Figure 5: Graph showing effect of germinated maize on MCV (fl) in Kadaknath.

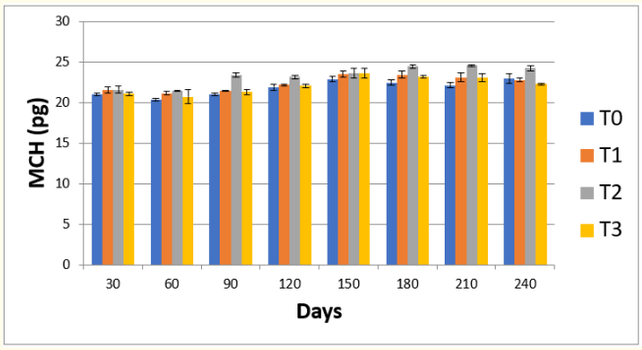

Figure 6: Graph showing effect of germinated maize on MCH (pg) in Kadaknath.

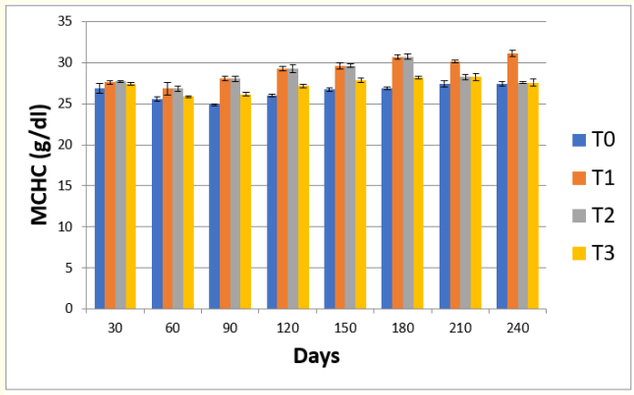

Figure 7: Graph showing effect of germinated maize on MCHC (g/dl) in Kadaknath.

et al. [49] who reported a significant increase of health condition parameters and survival rate even in carnivorous snakehead fish, *Ophiocephalus argus* when fed with plant materials (*Bacteriovorax sp*) and exposed to *A. veronii.*

Significance statement

This study discovered the improved overall fish performance at 15% *Azolla* inclusion level that can be beneficial for cost reduction in feed formulation. Therefore, this study will help the researchers to uncover the critical areas of fish nutrition by using *Azolla* meal as an alternative protein source in the diets that many researchers were not able to explore. Thus, a new theory on limited amount of *Azolla* meal (15%) may be arrived at.

Conclusion

The present study revealed the effective incorporation of *Azolla* meal as a fish feed ingredient in the GIFT tilapia diet for the first time. The venture for using this *Azolla* meal have brought the positive impact in the growth of fishes at 15% inclusion level with enhanced blood serum biochemistry performance thereby reducing the cost of fish feed in the tilapia culture. The study also envisioned the impact of non-conventional ingredient in the immunological performance, muscle quality and stress response in fishes. Nevertheless, efforts need to be continued to satisfy the complete replacement of fishmeal to improve the digestibility of ingredients of plants origin in obtaining better growth performance and feed utilization in fish.

Acknowledgements

The authors are very grateful to the Government of India through Indian Council of Agricultural Research (ICAR) and African Union (AU) for providing the financial and material support in the form of Research Fellowship during the period of doctoral study for the first author.

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