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Publication and Conferences

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1. Muchahary, S. & Khangembam B.K. (2024). Evaluation of the effects of *Lemna minor* supplementation on growth, digestive enzyme activity, and carcass composition of *Heteropneustes fossilis*. *Iranian Journal of Ichthyology*, 11(1): 56-70.
2. Muchahary, S., Narzary, B. & Khangembam B.K. (2023). Effect of Partial Replacement of Fish Meal by *Lemna minor* on the Growth and Immune Response of *Heteropneustes fossilis*. *International Journal of Bioscience*, 2(5): 21-32.
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4. Sanraja Mushahary and Bronson Kumar Khangembam. (2021). Current status of utilising plant proteins as replacement for animal protein in fish feed formulation in '*Recent Trends in Basic Researches in Northeast India*' eds: Dr. Subha Gaurab Roy, Dr. Rupam Sen, Dr. Taj Uddin Khan, Dr. Golab Chandra Nandi, Dr. Pinak Pani Nath Choudhury, Dr. Ramya Brata Chakraborty, Dr. Ahmed Hussain Barbhuiya, Dr. Munmun Nath, Mr. Subrata Das. Assam Science Society, Hailakandi Branch. pp. 86-96. ISBN: 978-81-953496-5-6.
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ORIGINAL ARTICLE

Evaluation of dietary *Lemna minor* supplementation on growth, digestive enzyme activity, and carcass composition of *Heteropneustes fossilis* (Bloch, 1794)

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Abstract
Finding affordable protein-rich replacements for fishmeal in fish feeds is important for sustainable aquaculture. *Lemna minor* is emerging as a promising high-protein yet low-cost alternative to traditional fishmeal. *Heteropneustes fossilis* is a valuable and highly nutritious food fish, but its production in India is low due to the high cost of feed production. In this study, the potential of incorporating *L. minor* into the diet of *H. fossilis* was assessed for its impact on growth, digestive enzyme activity, biochemical parameters, and carcass composition. Five isonitrogenous diets LM0 (0%), LM5 (5%), LM10 (10%), LM15 (15%) and LM20 (20%) with varying percentage inclusion of *L. minor* were fed to *H. fossilis* fries (0.51±0.01g, 4.1±0.03cm) for 60 days. LM15 diet-fed fish showed better Feed Conversion Ratio (0.93±0.05), Specific Growth Rate (2.60±0.06% day^{-1}), and Protein Efficiency Ratio (2.68±0.14) among all the groups. The inclusion of *L. minor* increased amylase, lipase, and pepsin activities, whereas chymotrypsin, trypsin and, total protease activities did not differ significantly (P>0.05). Arginine, histidine, methionine, valine and polyunsaturated fatty acids were significantly (P<0.05) elevated in LM15 diets fed fish. Biochemical parameters (thiobarbituric acid reactive substances, superoxide dismutase, and aminotranferases) showed no adverse effect of *L. minor* on the fish. Our results indicated that *L. minor* can be added to the fish diet up to 15% for optimum growth without adversely affecting fish health. The results of this study may be useful for the development of a cost-efficient and sustainable plant-based nutrient-rich feed for fish using freely available local resources.

Keywords: Aquafeed, Plant protein, Stinging catfish, Sustainable feed.

INTRODUCTION

With the rise in global population, the consumption of aquatic food has increased, escalating from 9.9kg per capita during the 1960s to 20.2kg per capita by 2020 (FAO 2022). Aquaculture holds the promise of fulfilling the nutritional needs of this expanding population. However, the advancement of the aquaculture sector hinges on accessible, economically viable, and nutritionally well-rounded diets. Moreover, the dependence on wild fish for aquafeed raises concerns regarding excessive harvesting and sustainability concerns (Naseem et al. 2021). This issue stems from the conventional employment of fishmeal, a key component in fish feed, which not only incurs high costs but is also environmentally unsustainable (Ali & Kaviraj 2021). Consequently, numerous investigations have been conducted to assess the potential of local feed resources to achieve both cost-effectiveness and sustainability in aquaculture (Dorothy et al. 2018).

In fish farming, fish feed becomes a prime factor in determining the cost and quality of fish (Mukherjee et al. 2010), as it constitutes 50-70% of the production cost of farmed fish (Iskandar et al. 2019). Therefore, several studies have evaluated alternative protein sources, especially plant proteins, by partial and complete substitution of fishmeal with plant protein to decrease the feed cost so that farmers can grow fish more economically on a large scale (Naseem et al. 2021). Aquatic macrophytes are regarded as a potential alternative plant protein for replacing fishmeal, among which *L. minor* is highly valued due to its rich content of protein, vitamins, carotenoids, essential amino acids, and availability (Kabir et al. 2009; Chakrabarti et al. 2018). Various studies have highlighted the potential benefits of *L. minor* diet in

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Table 1. Analysis of experimental diet composition and proximate components (% dry matter).

Ingredients (%)	LM0	LM5	LM10	LM15	LM20
Wheat flour*	51.33	47.11	42.9	38.68	34.46
L. minor	0	5	10	15	20
Dry Fish Powder*	47.27	46.49	45.7	44.92	44.14
Vitamin-mineral premix$^{\phi}$	0.4	0.4	0.4	0.4	0.4
Cod Liver Oil$^{\Psi}$	1	1	1	1	1
Proximate analysis (%)					
Protein	38.43	38.93	37.47	38.99	39.76
Moisture	5.26	5.21	5.42	5.50	5.75
Ash	6.79	7.38	8.00	8.96	9.46
Fibre	1.94	1.77	2.43	3.58	3.73
Lipid	4.90	4.48	3.95	5.06	4.99
Carbohydrate	42.68	42.23	42.73	37.91	36.31
Energy (Kcal $100g^{-1}$)	368.54	364.96	356.35	353.14	349.19

*Local Market, Kokrajhar, Assam.
$^{\phi}$Supradyn, Bayer Consumer Care AG, Basel, Switzerland. Vitamins: 5000 IU Vitamin A, 500mcg Methylcobalamin, 400 IU Vitamin D3, 150mcg D – Biotin USP, 75mg Ascorbic acid, 50mg Vitamin B3, 25mg Tocopheryl Acetate, 10mg Calcium D-Pantothenate, 5mg Vitamin B2, 5mg Vitamin B1, 1.5mg Folic Acid, and 1.5mg Vitamin B6. Trace Elements: 2mg Copper Sulphate, 250mcg Chromium Picolinate, 70mcg Selenium, 25mcg Sodium Molybdate, 5mg Manganese Sulfate Monohydrate. Amino acid: 50mg L- glutamic acid
$^{\Psi}$SEACOD, Cod Liver Oil (Type B) BP Universal Medicare, Mumbai, India.

several fish species (Noor et al. 2000; Yilmaz et al. 2004; Devi et al. 2022; Goswami et al. 2022).

Heteropneustes fossilis (Bloch, 1794) is a high-value fish species widely distributed in India and Bangladesh (Rahman et al. 2019) that has high market value because of its low fat content, high flesh quality, high nutrient content, and medicinal value (Nushy et al. 2020). Although it has gained market popularity, the dependency of fish farmers on fishmeal for its production has resulted in a rise in the production cost of the fish (Hossain et al. 2023). Nevertheless, few studies have reported replacing fishmeal with locally available plant protein sources up to certain levels. Ali & Kaviraj (2021) successfully included fermented *Ipomea aquatica* in the diet of *H. fossilis*, replacing fishmeal up to 25-50% with better growth rates at 50% inclusion. Although, the optimal inclusion level for fermented mulberry leaf meal was reported to be 52.28% (Ali et al. 2019), sunflower meal was observed to replace up to 14.3% fishmeal in *H. fossilis* optimally (Hossain et al. 2023). Other plant proteins, such as soybean meal, have also been reported as potential replacements for fishmeal in the feed of the species without affecting growth, feed efficiency, and health status (Howlader et al. 2023). Nandi et al. (2023) observed better growth when fermented *Ipomoea aquatica* replaced fishmeal in *H. fossilis*. Additionally, the growth and feed conversion efficiency of *H. fossilis* were unaffected by adding up to 15% soybean meal to their diet (Siddiqui et al. 2013). These studies suggest that plant proteins may be incorporated up to a certain level into the diet of the species, and hence, the aquatic macrophyte *L. minor* may be a good option for inclusion in the aquafeed of the species. Thus, the current study aimed to assess the effects of *L. minor* incorporated diets at varying substitution levels on the growth, digestive enzymes, nutritional profile, and biochemical parameters of *H. fossilis*.

MATERIALS AND METHODS

Experimental diet: *L. minor* samples were collected from Kokrajhar, Assam, India. The plant samples were air-dried in a shed until the moisture level dropped to below 50%, then oven-dried at 50°C, crushed into a fine powder, and sieved through 1mm wire mesh. Five isonitrogenous diets (40% crude protein) with increasing percentages of *L. minor* inclusion were prepared and labelled LM0 (0%), LM5 (5%), LM10 (10%), LM15 (15%), and LM20 (20%),

respectively (Table 1). The incorporation levels of *Lemna minor* in the diet were determined based on studies showing positive growth effects in various fish species, including *Barbodes gonionotus* Bleeker (Noor et al. 2000), *Cyprinus carpio* (Yilmaz et al. 2004; Goswami et al. 2022), and *Oncorhynchus mykiss* (Fiordelmondo et al. 2022). All feed ingredients were gently blended with water to form a paste and passed through a 1mm mesh. The strands were manually cut and left to oven-dry at 50°C. For 60 consecutive days, the fish were fed satiation at 9:00 a.m. and 4:00 p.m. each day. Uneaten feeds were removed after one hour of feeding and oven-dried at 50°C to determine feed intake.

Feeding experimental unit: The trial was conducted at Bodoland University's wet laboratory facility in Kokrajhar, Assam, India for 60 days. A batch of 750 *H. fossilis* juveniles (average weight: 0.51±0.01g, average length: 4.1±0.03cm) was procured from the Bijni fish farm in Chirang, Assam. These were randomly distributed among 15 aquaria, each with a 50-litre capacity. For each treatment, 50 fish were stocked in triplicate in each aquarium. An inlet and outlet system were established in each aquarium to facilitate water aeration and renewal. Regular assessments of pH, dissolved oxygen, and water temperature were conducted, following the standardised procedures outlined in the APHA (2017) guidelines. Throughout the study period, the recorded values for temperature, dissolved oxygen and pH ranged from 25.2°C to 27.4°C, 6.40 to 7.36mg L^{-1} and 6.97 to 7.09, respectively.

Sampling and growth parameters: Fish length and weight were consistently measured weekly throughout the experimental period. After the completion of the 60-day trial, the fish were fasted for 24 hours. Subsequently, phenoxyethanol (0.5mL L^{-1}) was used for anesthetisation, and the final length and weight were noted. Several parameters, including Body Mass Gain (BMG), Specific Growth Rate (SGR), Feed Conversion Ratio (FCR), Survival Rate (SR), Feed Efficiency (FE), and Protein Efficiency Ratio (PER), were assessed using the following formulae:

BMG (%) = [(Final body mass in g – initial body mass in g)/Initial body mass in g] × 100

SGR (% day^{-1}) = [(ln final body mass in g – ln initial body mass in g)/number of trial days] × 100

FCR = Dry feed fed (g)/body mass gain (g)

Survival rate (%) = (Final number of fish/Initial number of fish) × 100

FE (%) = 100 × (total final body weight – total initial body weight)/total dry feed intake

PER = BMG (g)/protein intake (g)

Proximate composition: Proximate composition analysis of the diet and dry muscle tissue of all the different treatment groups was carried out following the standard method specified in AOAC (2000). The Micro Kjeldahl technique was employed to assess the total nitrogen concentration and to obtain the crude protein percentage by multiplying the concentration of nitrogen by 6.25. Moisture content was determined by heating at 135°C for 2 hours in a hot-air oven. Crude lipids were analysed using petroleum ether extraction followed by Soxhlet extraction. Ash combustion in a muffle furnace for 16 hours at 550°C. Crude fibre was measured gravimetrically following chemical digestion and solubilisation of other components.

Digestive enzyme activity analysis: After a 60-day trial period, the whole digestive tract was collected from fish that underwent a 24-hour fast and anaesthesia. A total of 15 fish per diet were included by randomly selecting 5 fish from each aquarium. Dissections were performed in a chilled environment, followed by sample homogenisation (1:10 w/v, tissue: distilled water) using a mechanical tissue homogeniser. After centrifugation (10,000×g) at 4°C, the supernatants were stored at -20°C.

The amylase activity was assessed using starch as a substrate, following Bernfeld's (1955) method. To determine the lipase enzyme activity, the Winkler and Stuckman (1979) method was employed using *p*-nitrophenyl palmitate as a substrate. Pepsin activity was measured following Anson's (1938) method, using haemoglobin as a substrate. Total protease enzyme activity was determined using an azocaesin substrate according to the method described by

Garcia-Carreno (1992). Trypsin and chymotrypsin enzyme activities were determined spectrophotometrically using N-α-Benzoyl-L-arginine ethyl ester and N-Benzoyl-L-tyrosine ethyl ester substrates, respectively (Bergmeyer 1974). Total protein content was determined following Bradford (1976) method using Bovine Serum Albumin (BSA) as the standard.

Biochemical parameters: To examine the biochemical parameters, wet muscle tissue samples were obtained from all treatments in triplicate. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined using buffered aspartate-alpha-ketoglutarate and buffered alanine-alpha-ketoglutarate substrates, respectively (Reitman & Frankel, 1957). Superoxide dismutase (SOD) activity was tested using Xanthine oxidase following Roy et al. (2020). The thiobarbituric acid reactive substances (TBARS) assay was performed following Ohkawa et al. (1979) method using 1,1,3,3-tetramethoxy propane as the standard.

Fatty acid analysis: Fatty acid analysis was performed in a Perkin Elmer (USA) GC-MS, Clarus 680 GC and Clarus 600C MS, controlled by Turbo Mass Ver. 6.4.2 software. A 60m x 0.25mm film Elite-5MS (0.25µm) capillary column containing 5% diphenyl 95% dimethyl polysiloxane was employed with a helium injection volume of 1µL (carrier gas, 1mL min^{-1}). EI+ mode at 70eV was employed for mass spectra, covering the m/z 50-600amu mass range. Compound identification was based on the NIST-2014 database comparison. Peaks were analysed using the data analysis software NIST-2014 to obtain insights into the names, molecular weights, and empirical formulas of components.

Amino acid analysis: Amino acid profiling procedure using LC-MS involves two extraction methods (Nimbalkar et al. 2012): one for free amino acids and another for bound amino acids via acid hydrolysis. Sample homogenisation with formic acid (0.1%) was done in methanol (20%) followed by centrifugation and filtration, to extract free amino acids. For bound amino acids, samples are hydrolysed with 6M hydrochloric acid, processed, and reconstituted before injection. LC-MS conditions included gradient composition, temperature control, and a PDA detector for amino acid monitoring. The mobile phases for the analysis included water with formic acid (0.1%) and a mixture of water and methanol (50:50) with formic acid (0.1%), utilising the Waters Acquity UPLC H (TQD MS/MS, USA) system.

Statistical analysis: The data is presented as mean±standard deviation. Prior to analysis, the normality (determined by the Shapiro-Wilk test) and homogeneity (determined by Levene's test) of the data were examined. Tukey post hoc analyses along with one-way ANOVA to compare group means. $P<0.05$ was used to define the significance level. To determine the optimal dietary level of *L. minor* incorporation, quadratic polynomial regression analysis was performed on FCR and SGR.

RESULTS

Growth performance: The growth performance of fish is presented in Table 2. No mortality was recorded for any of the treatments throughout the experiment. The LM15 diet-fed fish showed higher FW, BMG and SGR values. Additionally, they exhibited enhanced FE and PER, and a lower FCR compared to the control (LM0) group. According to the FCR and SGR regression analysis, the optimal dietary inclusion level of *L. minor* for *H. fossilis* was within the range of 11.89-12.30% (Fig. 1a, b).

Proximate composition: The proximate composition of the fish muscle samples is presented in Table 3. Ash content, lipid, and crude protein content in *H. fossilis* fed the diet LM15 were significantly higher ($P<0.05$), whereas moisture content was higher in the LM0 group than in the other diet groups. The fibre content in the fish fed different diets did not significantly differ ($P>0.05$).

Digestive enzyme activity: The digestive enzyme activities in the fish fed with different levels of *L. minor* are shown in Fig. 2 (a-f). Fish fed the LM15 diet exhibited significantly higher amylase activity ($P<0.05$) than those fed the LM0 diet. However, there was no notable difference ($P>0.05$) in amylase activity

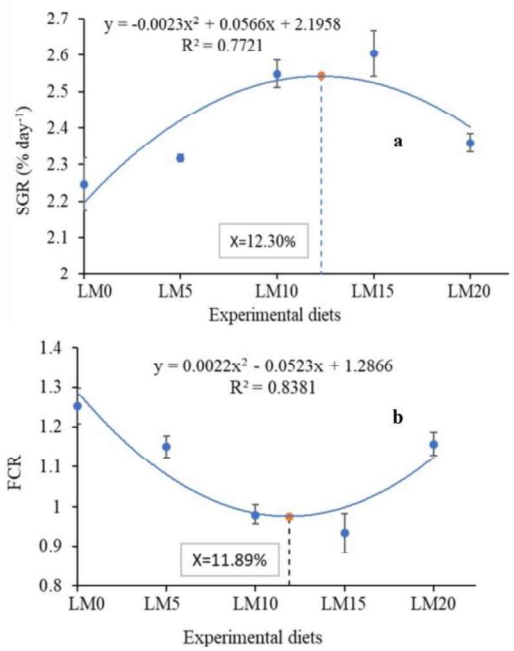

Fig.1. Optimizing *Lemna minor* dietary intake via polynomial regression analysis based on (a) SGR and (b) FCR.

between the LM10 and LM15 groups. Lipase activity was markedly increased in both LM10 and LM15 groups compared with the other treatments. Trypsin, total protease, and chymotrypsin activities did not differ significantly (P>0.05) between the treatments. Pepsin activity was highest in the plant-fed group, LM15 (P<0.05), while LM10, LM15 and LM20 did not vary significantly (P>0.05).

Fatty acid composition: The fatty acid composition of fish fed different levels of *L. minor* is presented in Table 4. In all treatments, C17:0 was the predominant saturated fatty acid (SFA), while C18:0 was consistently the least abundant. Monounsaturated fatty acids (MUFA) exhibited a decreasing trend, whereas polyunsaturated fatty acids (PUFA) increased with increasing *L. minor* in the diet. Increased levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were found with increasing *L. minor* inclusion levels in the study. Notably, the LM15 group exhibited the highest PUFA, EPA+DHA, and PUFA/SFA ratio (P<0.05).

Amino acid composition: Among essential amino acids (EAA), arginine, valine, histidine and methionine were highest in LM15, whereas leucine and phenylalanine were highest in LM0 (Table 5). Lysine levels were highest in LM5, whereas the highest threonine and tryptophan content were observed in LM10. Alanine, glycine, and citrulline were among the non-essential amino acids (NEAA) found to be highest in LM20, whereas cysteine and serine were maximum in LM5. Additionally, proline, asparagine and tyrosine were recorded highest in the control among all the groups.

Biochemical parameters: SOD activity did not vary significantly (P>0.05) between any of the treatment groups (Table 6).

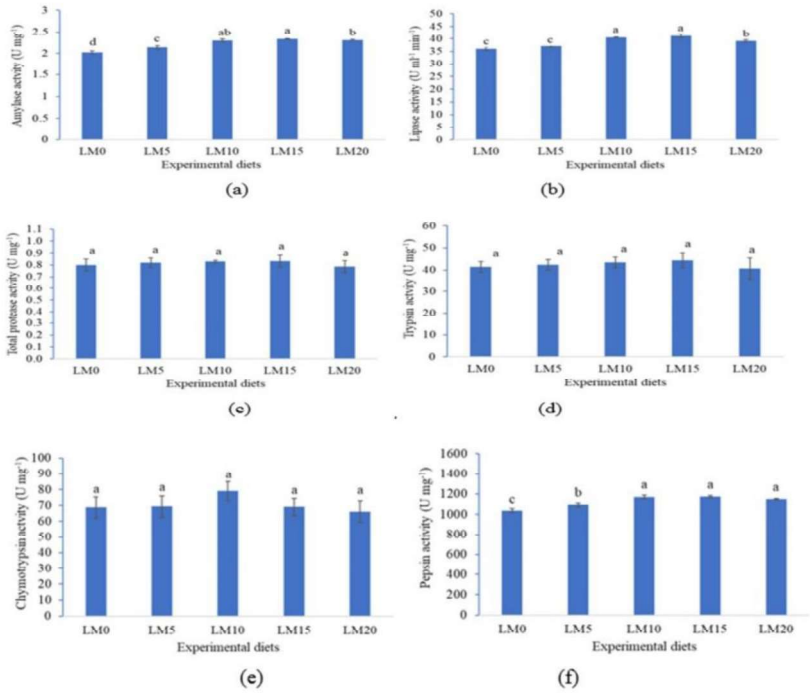

Fig.2. Digestive enzyme activity of *Heteropneustes fossilis* fed with different levels of *Lemna minor* in the diet for 60 days. Bars with different lower cases indicate significant differences (n= 3, P<0.05). (a) Amylase, (b) Lipase, (c) Total Protease, (d) Trypsin, (e) Chymotrypsin, and (f) Pepsin activity.

Lower AST and ALT activities were observed in LM10 compared with the other groups (P 0.05). TBARS levels decreased non-significantly among all plant-fed groups of fish compared with the control group.

DISCUSSION

Most studies on plant proteins as a replacement for fishmeal in aquafeed are restricted to fish such as cyprinids, salmonids, trout, etc., and limited studies are available on catfish and air-breathing species (Dorothy et al. 2018; Naseem et al. 2021). However, some studies have demonstrated the positive impacts of aquatic macrophyte incorporated diets on the growth performance of catfish (Kari et al. 2020; Naseem et al. 2021; Nandi et al. 2023). *L. minor*, known for its rich nutrient content (Chakrabarti et al. 2018), has been extensively tested in various fish species (Noor et al. 2000; Raj et al. 2001; Herawati et al. 2020; Fiordelmondo et al. 2022; Goswami et al. 2022; Irabor et al. 2022). This present study was conducted to assess the potential of *L. minor* as an alternative protein source for *H. fossilis* by examining its effects on growth performance, digestive enzyme activities, biochemical parameters, and the carcass composition of the fish. Our results showed better growth efficiency and nutrient utilisation in *H. fossilis*, when *L. minor* was incorporated in the diet up to a certain level, beyond which there seems to be no effect. This is evident from the increased FW, BMG, and SGR in the groups fed with dietary *L. minor* incorporated diet.

Table 2. Growth and nutrient efficiency of *Heteropneustes fossilis* fed varying levels of *Lemna minor* incorporated diet for 60 days.

Parameters	LM0	LM5	LM10	LM15	LM20	*P* value
IW (g)	0.51±0.01	0.51±0.00	0.51±0.01	0.51±0.01	0.50±0.01	0.068
FW (g)	1.95±0.10^{b}	2.05±0.02^{b}	2.34±0.05^{a}	2.44±0.08^{a}	2.06±0.03^{b}	<0.001
BMG (%)	284.76±16.69^{b}	301.58±2.34^{b}	361.50±10.13^{a}	377.16±18.07^{a}	311.94±6.29^{b}	<0.001
SGR (% day^{-1})	2.24±0.07^{b}	2.32±0.01^{b}	2.55±0.04^{a}	2.60±0.06^{a}	2.36±0.03^{b}	<0.001
FCR	1.25±0.05^{a}	1.15±0.03^{b}	0.98±0.02^{c}	0.93±0.05^{c}	1.16±0.03^{ab}	<0.001
Survival (%)	100	100	100	100	100	
FE (%)	79.93±2.88^{b}	86.96±2.08^{b}	102.03±2.52^{a}	107.29±5.61^{a}	86.47±2.15^{b}	<0.001
PER	2.00±0.07^{b}	2.17±0.05^{b}	2.55±0.06^{a}	2.68±0.14^{a}	2.16±0.05^{b}	<0.001

Table 3. Proximate analysis of *Heteropneustes fossilis* (% dry weight basis) fed varying levels of *Lemna minor* incorporated diet for 60 days.

Parameters	LM0	LM5	LM10	LM15	LM20	*P* value
Moisture	17.57±0.01^{a}	17.45±0.01^{c}	17.18±0.01^{d}	16.76±0.01^{e}	17.53±0.01^{b}	<0.001
Protein	63.54±0.02^{e}	63.66±0.03^{d}	63.84±0.03^{b}	63.95±0.05^{a}	63.69±0.02^{c}	<0.001
Lipid	7.97±0.05^{e}	8.07±0.02^{d}	8.27±0.05^{b}	8.44±0.06^{a}	8.13±0.01^{c}	<0.001
Ash	9.38±0.02^{d}	9.44±0.01^{c}	9.51±0.03^{b}	9.60±0.05^{a}	9.50±0.06^{b}	<0.001
Fibre	0.10±0.01^{a}	0.10±0.01^{a}	0.11±0.01^{a}	0.10±0.01^{a}	0.11±0.01^{a}	0.499
Carbohydrate	1.44±0.03^{a}	1.28±0.03^{b}	1.09±0.05^{cd}	1.15±0.05^{c}	1.04±0.04^{d}	<0.001

Note. Superscript letters indicate significant differences in a shared row (n= 3, P<0.05).

Table 4. Fatty acid composition (% of total fatty acid) in *Heteropneustes fossilis* fed varying levels of *Lemna minor* incorporated diet for 60 days.

Fatty acid	LM0	LM5	LM10	LM15	LM20	P value
C13:0	1.72±0.01^{b}	2.88±0.01^{a}	2.87±0.02^{a}	2.70±0.02^{c}	2.47±0.02^{d}	<0.001
C14:0	2.45±0.01^{a}	2.39±0.01^{b}	2.38±0.01^{b}	2.31±0.01^{c}	2.30±0.02^{c}	<0.001
C17:0	15.85±0.01^{b}	15.69±0.01^{c}	15.56±0.02^{d}	15.96±0.03^{a}	15.69±0.02^{c}	<0.001
C18:0	0.14±0.01^{a}	0.08±0.00^{b}	0.09±0.01^{b}	0.09±0.01^{b}	0.08±0.01^{b}	<0.001
C20:0	1.01±0.01^{b}	0.37±0.01^{e}	0.57±0.02^{c}	0.53±0.02^{d}	1.06±0.01^{a}	<0.001
C27:0	2.77±0.02^{a}	2.58±0.01^{bc}	2.55±0.02^{c}	2.55±0.02^{c}	2.62±0.01^{b}	<0.001
C34:0	2.07±0.01^{a}	1.97±0.01^{b}	1.96±0.01^{b}	1.72±0.01^{d}	1.86±0.02^{c}	<0.001
Σ SFA	26.01±0.05^{ab}	25.96±0.06^{ab}	25.96±0.03^{ab}	25.84±0.09^{b}	26.08±0.04^{a}	0.009
C16:1n-5	12.39±0.01^{a}	12.26±0.01^{b}	12.10±0.04^{c}	11.95±0.03^{e}	12.03±0.03^{d}	<0.001
C16:1n-7	3.07±0.01^{a}	2.75±0.01^{b}	2.65±0.01^{c}	2.63±0.02^{c}	2.67±0.02^{c}	<0.001
C18:1n-9	1.53±0.01^{a}	1.48±0.02^{b}	1.42±0.01^{c}	1.36±0.01^{d}	1.28±0.02^{e}	<0.001
C18:1n-16	14.45±0.02^{a}	14.32±0.02^{b}	14.26±0.02^{c}	14.18±0.02^{d}	14.25±0.01^{c}	<0.001
C20:1n-9	3.96±0.02^{a}	3.90±0.02^{d}	3.85±0.02^{e}	3.81±0.01^{a}	3.82±0.01^{b}	<0.001
C18:1n-5	13.30±0.01^{a}	13.29±0.01^{a}	13.24±0.03^{b}	13.30±0.01^{a}	13.27±0.01^{ab}	0.004
Σ MUFA	48.71±0.06^{a}	48.01±0.08^{b}	47.51±0.08^{c}	47.23±0.12^{d}	47.32±0.10^{cd}	<0.001
C18:3n-3	1.29±0.02^{c}	1.35±0.01^{b}	1.37±0.01^{b}	1.41±0.02^{a}	1.27±0.01^{c}	<0.001
C20:5n-3	2.56±0.01^{c}	2.62±0.02^{c}	2.77±0.02^{a}	2.72±0.01^{b}	2.70±0.02^{b}	<0.001
C20:3n-3	2.13±0.02^{c}	2.16±0.02^{c}	2.22±0.01^{b}	2.24±0.03^{b}	2.29±0.02^{a}	<0.001
C22:6n-3	1.40±0.02^{d}	1.46±0.01^{c}	1.55±0.02^{b}	1.64±0.03^{a}	1.64±0.03^{a}	<0.001
C20:4n-6	3.91±0.01^{d}	3.97±0.02^{c}	4.05±0.02^{b}	4.07±0.02^{ab}	4.11±0.02^{a}	<0.001
C20:2n-6	3.72±0.01^{d}	3.93±0.03^{c}	3.95±0.02^{bc}	3.99±0.02^{ab}	4.00±0.01^{a}	<0.001
C18:2n-6	8.24±0.02^{c}	8.38±0.02^{b}	8.45±0.02^{a}	8.45±0.02^{a}	8.46±0.01^{a}	<0.001
C22:4n-6	1.92±0.02^{c}	2.03±0.02^{b}	2.05±0.01^{b}	2.16±0.01^{a}	2.12±0.01^{a}	<0.001
Σ PUFA	25.17±0.01^{d}	25.90±0.13^{c}	26.41±0.09^{b}	26.69±0.11^{a}	26.59±0.06^{ab}	<0.001
PUFA/SFA	0.97±0.00^{d}	1.00±0.00 ^{c}	1.02±0.00^{b}	1.03±0.01^{a}	1.02±0.00^{b}	<0.001
ω6/ω3	2.41±0.02^{a}	2.41±0.01^{a}	2.34±0.01^{b}	2.33±0.02^{b}	2.36±0.02^{b}	<0.001
EPA+DHA	3.96±0.01^{c}	4.08±0.03^{b}	4.32±0.03^{a}	4.36±0.04^{a}	4.35±0.05^{a}	<0.001

Note. Superscript letters indicate significant differences in a shared row (n= 3, P<0.05).
SFA: Saturated fatty acid
MUFA: Monounsaturated fatty acid
PUFA: Polyunsaturated fatty acid
EPA: Eicosapentaenoic acid
DHA: Docosahexaenoic acid

Table 5. Amino acid composition of *Heteropneustes fossilis* fed varying levels of *Lemna minor* incorporated diet for 60 days.

Amino acids (mg/g)	LM0	LM5	LM10	LM15	LM20	*P* value
EAA						
Arginine	24.94±0.26^{e}	27.79±0.10^{d}	28.81±0.19^{c}	32.31±0.01^{a}	31.54±0.10^{b}	<0.001
Histidine	14.99±0.41^{d}	17.33±0.07^{c}	18.03±0.23^{b}	20.25±0.13^{a}	14.92±0.10^{d}	<0.001
Lysine	73.12±0.12^{d}	90.83±0.21^{a}	82.15±0.20^{b}	81.61±0.01^{c}	70.39±0.07^{e}	<0.001
Leucine	114.61±0.50^{a}	78.58±0.26^{c}	59.68±0.04^{e}	106.04±0.12^{b}	61.37±0.27^{d}	<0.001
Methionine	51.82±0.11^{b}	33.19±0.28^{c}	25.91±0.06^{e}	63.50±0.58^{a}	30.64±0.12^{d}	<0.001
Phenylalanine	70.86±0.93^{a}	57.27±0.66^{c}	35.14±0.11^{d}	59.70±0.25^{b}	27.69±0.40^{e}	<0.001
Threonine	25.60±0.28^{d}	27.67±0.09^{c}	46.66±0.10^{a}	27.69±0.36^{c}	44.86±0.40^{b}	<0.001
Tryptophan	5.51±0.04^{b}	5.34±0.03^{c}	6.60±0.01^{a}	3.85±0.01^{e}	4.85±0.01^{d}	<0.001
Valine	22.29±0.08^{ab}	16.20±0.70^{d}	20.58±0.07c	22.36±0.11^{a}	21.45±0.06^{b}	<0.001
Total	403.75±0.53^{b}	354.21±1.03^{c}	323.56±0.53^{d}	417.30±1.52^{a}	307.70±0.46^{e}	<0.001
NEAA						
Alanine	43.84±0.18^{d}	50.04±0.80^{c}	73.47±0.05^{b}	44.40±1.00^{d}	79.44±1.07^{a}	<0.001
Aspartic acid	73.10±1.13^{c}	59.25±1.29^{e}	92.89±0.82^{a}	64.57±0.76^{d}	89.54±0.47^{b}	<0.001
Cysteine	0.66±0.01^{a}	0.72±0.05^{a}	0.60±0.01^{b}	0.57±0.02^{b}	0.56±0.01^{b}	<0.001
Glycine	0.55±0.02^{b}	0.39±0.02^{c}	0.58±0.01^{b}	0.34±0.02^{d}	0.64±0.00^{a}	<0.001
Glutamic acid	45.56±0.11^{e}	74.73±0.46^{c}	110.46±0.87^{a}	47.43±0.69^{d}	99.60±0.81^{b}	<0.001
Proline	90.39±0.15^{a}	53.71±0.15^{e}	63.01±0.04^{d}	74.64±0.18^{c}	81.42±1.31^{b}	<0.001
Serine	52.20±0.23^{d}	96.91±2.01^{a}	93.57±1.12^{a}	57.00±1.57^{c}	86.50±1.58^{b}	<0.001
Tyrosine	22.64±0.05^{a}	11.29±0.11^{d}	13.67±0.03^{c}	18.14±0.24^{b}	8.40±0.02^{e}	<0.001
Citrulline	1.30±0.05^{d}	1.56±0.03^{c}	1.29±0.03^{d}	2.57±0.01^{b}	2.93±0.05^{a}	<0.001
Asparagine	1.40±0.01^{a}	0.80±0.00^{b}	0.08±0.00^{e}	0.58±0.00^{c}	0.45±0.00^{d}	<0.001
Beta 3-4 dihydroxy phenylalanine	0.07±0.01^{a}	0.03±0.01^{b}	0.01±0.00^{c}	0.03±0.00^{b}	0.02±0.00^{cb}	<0.001
Total	331.70±1.42^{c}	349.42±0.59^{b}	449.61±2.90^{a}	310.26±4.45^{d}	449.49±1.21^{a}	<0.001
Total amino acids	735.45±1.96^{c}	703.63±1.62^{d}	773.18±3.43^{a}	727.56±5.96^{c}	757.19±0.75^{b}	0.001

Note. Superscript letters indicate significant differences in a shared row (n= 3, P<0.05).
EAA: Essential Amino Acids
NEAA: Non-Essential Amino Acids

Table 6. Biochemical parameters of *Heteropneustes fossilis* fed with different levels of *Lemna minor* incorporated diet for 60 days.

Parameters	LM0	LM5	LM10	LM15	LM20	*P* value
SOD (U mg^{-1})	371.78±92.38^{a}	370.77±66.59^{a}	371.97±92.64^{a}	370.62±35.12^{a}	371.63±68.07^{a}	0.780
TBARS (U mg^{-1})	2.78±0.07^{ab}	2.74±0.09^{b}	2.77±0.12^{a}	2.75±0.03^{ab}	2.76±0.22^{a}	<0.001
AST (U mg^{-1})	2.20±0.09^{a}	2.13±0.05^{a}	2.11±0.08^{b}	2.16±0.15^{ab}	2.14±0.05^{b}	<0.001
ALT (U mg^{-1})	2.16±0.35^{ac}	2.07±0.09^{c}	2.06±0.14^{b}	2.15±0.13^{a}	2.14±0.08^{ab}	0.002

Note. Superscript letters indicate significant differences in a shared row (n= 3, P<0.05).
SOD: Superoxide dismutase, TBARS: Thiobarbituric acid reactive substances, AST: Aspartate aminotransferase (AST), ALT: Alanine aminotransferase

Higher BMG and SGR in the *L. minor* incorporated diet fed fish indicated the acceptance, and subsequent digestion of the *L. minor* incorporated diet, which led to the improved growth performance. This may also indicate the improvement in feed utilisation due to proper digestion of the plant-incorporated diets. Our results align with Siddiqui et al. (2013), where the growth and feed conversion efficiency of *H. fossilis* were unaffected by a diet containing up to 15% soybean meal instead of fishmeal. Similar positive results in growth performance were also observed in various fish species when *L. minor* was included upto 10% in *Barbodes gonionotus* (Noor et al. 2000), 50% in *Channa striatus* (Raj et al. 2001), 20% in *Oncorhynchus mykiss* (Fiordelmondo et al. 2022), 20% in *Cyprinus carpio* (Goswami et al. 2022), and 40% in *Clarias gariepinus* (Irabor et al. 2022). Growth performance, however, did not exhibit a noteworthy distinction from the control group at the 20% incorporation level in this study. This implies that a higher proportion of plant protein might affect digestion and assimilation processes. Contrary to our findings, however, a higher percentage inclusion of some plant proteins gave better results in the same species. For instance, a complete replacement with soybean meal resulted in improved growth and feed

conversion efficiencies (Shukla et al. 2018). Similarly, water spinach meal replacing up to 50% fishmeal resulted in improved growth and health of *H. fossilis* (Nandi et al. 2023). Additionally, soybean meal also improved the health and growth performance of *H. fossilis* (Howlader et al. 2023).

The use of regression analysis is an effective technique for determining the optimal dose of feed additives (Yossa & Verdegem 2015). Based on SGR and FCR regression analysis, *L. minor* concentration is recommended at 11.89-12.30% in *H. fossilis* diets. This technique was used to find the optimum sunflower meal inclusion level (14.3%) for *H. fossilis* (Hossain et al. 2023). The ash, lipid and protein content of the fish were notably influenced by the increasing dietary inclusion of *L. minor*. Similar trends were reported in *Channa striatus* fed with *L. minor* (Raj et al. 2001; Fiordelmondo et al. 2022). This indicated that the fish can digest and successfully assimilate the nutrients present in the plant. However, few studies have also reported decreased carcass protein content, such as in *Clarias gariepinus* (Irabor et al. 2022) and in *Barbodes gonionotus,* Bleeker (Noor et al. 2000).

Assessing the activity of digestive enzymes yields insights into the overall digestive capacity and utilisation of nutrients of the fish through hydrolysis of protein, lipids and carbohydrates (Gawlicka et al. 2000; Johnston et al. 2004; Devi et al. 2022). Higher amylase activities may be correlated with the increase in plant content in the diet. Fish fed the LM10 and LM15 diets exhibited significantly elevated amylase and lipase activities, indicating efficient utilisation of carbohydrates and lipids in the diet incorporating *L. minor*. This finding aligns with earlier reports on *H. fossilis* fed 50% inclusion of mulberry leaf (Ali et al. 2019), and soybean meal (Khanom et al. 2022). Protein digestion in *H. fossilis* fed with *L. minor* improved as pepsin activity was significantly higher in LM10, LM15, and LM20. The chymotrypsin, trypsin and total protease activities were not significantly affected, although there was an increasing trend. Similar results were also observed in *Centropmus viridis* fed soybean meal (Arriaga-Hernández et al. 2021). Similarly, an increase in enzyme activity was observed in *Cyprinus carpio* when fed with *L. minor* (Goswami et al. 2022) and *Spirodela polyrhiza* supplemented feed (Shrivastav et al. 2022). The elevated enzymatic activities found in our study indicated that the plant-based diet did not negatively affect the digestion of protein, lipids and carbohydrates up to 15% *L. minor* incorporation. This led to efficient utilisation of the ingested feed, thereby, resulting in increased PER, decreased FCR and improved growth.

Fatty acids are pivotal in determining the nutritional quality of fish (Zhang et al. 2020). The fish nutritional content is markedly influenced by the composition of its diet (Ackman 1989). In this study, the fatty acid composition of *H. fossilis* was significantly affected by *L. minor* in its diet, leading to increased PUFA and decreased SFA in the fish with higher percentage of *L. minor*. Similar findings were reported in *Oreochromis niloticus* fed with fermented *L. minor* (Herawati et al. 2020). The ratios of n6/n3 (Simopoulos 2002) and PUFA/SFA (HMSO 1994) are important indicators of fat quality. Our study suggests that the observed ranges of these indices hold good for human health. This may be due to the enhanced fatty acid composition of *H. fossilis* when fed with *L. minor*, suggesting potential advantages for human health.

The efficient utilisation of amino acids in animal feed is essential for sustainable protein production (Kaushik et al. 2010). *L. minor* exhibits a rich source of amino acids, making it suitable for aquafeed (Chakrabarti et al. 2018). *H. fossilis* fed with LM15 diets exhibited significantly elevated levels of EAA, particularly arginine, histidine, methionine, and valine. This agrees with the results of Goswami et al. (2022), where *Cyprinus carpio* fed with *L. minor* exhibited elevated levels of EAA, including arginine, isoleucine, methionine, tryptophan, threonine and valine. Thus, feeding *H. fossilis* with *L. minor* incorporated diet may help enhance the composition of amino acids in the fish carcass.

The capacity of the fish to withstand environmental stress relies on the health of their immune systems (Adel et al. 2015). Plant-based proteins added to the fish diet impact fish development and immunological state (Dossou et al. 2018). Antioxidant defences and reactive oxygen species (ROS) in animal cells were highlighted by Tocher et al. (2002), where an imbalance in ROS causes oxidative stress. Sheikhzadeh et al. (2012) established a connection between fish antioxidant defences and nutrition. In response to toxicants, the generation of ROS increases, which is counteracted by an antioxidant enzyme system (Lushchak 2011). The first line of protection against ROS oxidative damage is provided by the antioxidant enzyme SOD (Fridovich 1995). This study found no significant difference in SOD activity in the plant-fed fish compared to the control. Similar results were also reported in *Ctenopharyngodon idella* and *Hypophthalmichthys molitrix* fed with *L. minor* (Aslam et al. 2021). However, Wang et al. (2017) observed a noteworthy diminishing trend of SOD in *Larimichthys crocea* when fed with soy protein concentrate. The TBARS assay evaluates the peroxidative damage to lipids by generating free radicals to quantify oxidative stress (Oakes et al. 2003). In this study, the TBARS in *L. minor* fed fish was comparable with the control. However, *Ctenopharyngodon idella* and *Hypophthalmichthys molitrix* fed *L. minor* showed no significant difference (Aslam et al. 2021). AST and ALT are found in various tissues of the skeletal muscles, liver, erythrocytes, etc. (Hadi et al. 2009), and these are tested as biomarkers for liver dysfunction and fish health status. These enzymes provide specific information about organ dysfunction when present in blood serum or plasma; for instance, as an elevated ALT activity reflects liver disease (Shahsavani et al 2010). Lower activities of both the enzymes in *L. minor* fed groups than control in the present study indicates no negative impact on the liver. Similar results were also reported in *Lates calcarifer* fed with *L. minor* incorporated diet (Mustofa et al. 2022). However, increased activity of both these enzymes was also observed in the plasma of African catfish fed with fermented soy pulp (Kari et al. 2020).

In conclusion, the results of our study indicated that *L. minor* may be incorporated in the diet of *H. fossilis* up to 15% for better growth performance, with the optimal results observed at 11.89-12.35% *L. minor* supplementation in the diet. This dietary supplementation also increased digestive enzyme activity, improving feed utilisation and nutrient digestibility. Moreover, including *L. minor* enriched the fish nutritional profile of the fish by increasing protein, fatty acid, and amino acid content. The results of this study may be useful in the development of a cost effective and sustainable feed based on aquatic macrophytes, *L. minor* for enhanced production of *H. fossilis*.

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Effect of partial replacement of Fish meal by *Lemna minor* on the growth and immune response of *Heteropneustes fossilis*

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Key words: *Heteropneustes fossilis*, *Lemna minor*, Growth, Immune response

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Abstract

Aquaculture research in recent times has been focused on finding more affordable sources of plant protein for inclusion in the fish diet. *Lemna minor* is a widely reported alternative protein source in fish feed but its effect on the immune system of fish especially catfish is not yet fully understood. This study, therefore, evaluated the effect of dietary inclusion of *L. minor* on the growth, immune response and catalase activity of *Heteropneustes fossilis*. The fry of *H. fossilis* was fed five iso-nitrogenous diets containing graded percentage inclusion levels of *L. minor* as 0% (Control), 5% (T1), 10% (T2), 15% (T3) and 20% (T4) for 60 days. The final weight, body mass gain and specific growth rate were significantly higher in T3 diet-fed fish than in others. The feed conversion ratio was lowest in the T3 group. Total muscle protein, mucus protein and total immunoglobulin content did not differ significantly between the control group and plant-fed fish. The lysozyme and alkaline phosphatase activity was significantly higher in T1. Antioxidant enzyme catalase activity did not differ significantly in all the treatments. This study shows that *L. minor* can be incorporated up to 20% in the feed of *H. fossilis* without a negative effect on its growth and immune response of *H. fossilis*. *L. minor* may be a potential protein source in fish feed for sustainable aquaculture.

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Introduction

With the growing population, global consumption of aquatic foods has increased from 9.9kg in 1960 to 20.2kg in 2020 (FAO, 2022). Aquaculture has the potential to meet the nutrition required for the increasing human population. To meet the increasing demand for fish production globally, there is a need for alternative low-cost noble feedstuff for fish production, which will replace high-cost and unsustainable traditional fish meals. Therefore, several studies have evaluated the local feed resources for producing cost-efficient and sustainable feed for aquaculture (Awad and Awaad, 2017; Dorothy *et al.,* 2018; Sonta *et al.,* 2019). Freshwater aquatic weeds especially macrophytes have been regarded as a potential replacement for animal proteins in fish diets, and their effect on various fish species has been the subject of numerous studies (Naseem *et al.,* 2021). *L. minor* of the family Lemnaceae is one such floating aquatic plant widely available in India. Due to its high protein, amino acid and fatty acid content and low fibre (Chakrabarti *et al.,* 2018), the plant has been widely tested in fish for the replacement of traditional fish meal (Sonta *et al.,* 2019; Irabor *et al.,* 2022; Goswami *et al.,* 2022, Devi *et al.,* 2022).

H. fossilis is a highly preferred food fish because of its high nutritive value, low fat and medicinal value (Banerjee *et al.,* 2018). Because of its ability to utilise both plant and animal-origin feedstuff with medicinal value and market potential, it has gained importance for intensive culture (Pillay, 1990). Some information on aspects of the nutrient requirement of *H. fossilis* is available (Mohamed, 2001; Usmani *et al.,* 2003). Siddiqui *et al.* (2009) reported that the inclusion of 40-43% dietary protein is optimum for the growth and efficient feed utilization of protein for the growth of young *H. fossilis*. Very less studies are available on the dietary inclusion of alternative protein sources for the *H. fossilis* diet. *Mondal et. al.* (2011) reported the use of mulberry leaf meal along with fish offal meal, Bag *et al.* (2012) reported the use of Mulberry leaf meal and Ali *et al.* (2021) reported the use of fermented *Ipomoea aquatica* leaf meal for *H. fossilis*. However, other potential alternative protein sources can be evaluated for dietary inclusion in the intensive culture of this species.

One of the undesirable effects of the dietary inclusion of plant ingredients in the feed is related to the presence of antinutritional factors, antiproteases and implications in the immune health of the fish. Reports of the effect on the immune system are varying according to the species of fish and/or plants used. Some previous studies on the dietary inclusion of plant proteins have reported a decrease in the immunity of fish especially carnivorous fish (Maita *et al.* 2006; Daniel, 2018), whereas, several studies have also reported a positive effect of plant-based dietary protein inclusion on the growth and immune parameters of fish (Dossou *et al.,* 2018; Zhang *et al.,* 2020). Hence, it is essential to understand the effect of commonly used plant proteins on the immune system of target fish before its utilisation as an alternative source of protein in its feed. Therefore, the aim of the present study is to evaluate the effect of dietary inclusion of *L. minor* as a partial replacement of fish meal for *H. fossilis* on the growth and immune parameters of the fish.

Materials and methods

Experimental fish and design

H. fossilis were obtained from a local fish farm in Bijni, Assam, India. The fry of *H. fossilis* obtained by induced breeding was collected and acclimatized to laboratory conditions for one week prior to the experiment, during which period, they were fed a control diet containing 40% protein. Initially, a total of 750 fries with average size (0.51 ± 0.01g, 4.1 ± 0.03cm) were distributed randomly among 15 aquaria of 50 L capacity each (3 replicates for each treatment), and the stocking density was maintained at 50 fish fry per aquarium. Each tank was connected with an inlet, outlet and continuous aeration. About one-third of the water in each aquarium was changed every alternate day. The water quality parameters like dissolved oxygen, temperature and pH were regularly checked using standard procedures (APHA, 2017). Temperature, pH and dissolved oxygen ranged from 25.2 to 27.4 °C, 6.97 to 7.09 and 6.40 to 7.36mg L^{-1}, respectively throughout the experimental period.

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Experimental diet and feeding

Five isonitrogenous (40% crude protein) experimental diets were prepared with an increase in percentage inclusion of *L. minor* in the diet, Control (0%), Treatment 1 (T1) (5%), T2 (10%), T3 (15%) and T4 (20%) presented in table 1. Fish were fed one of the five different feeds prepared with three replicate aquaria per diet. Fish were fed twice every day at 9.30 am and 4.30 pm @ 5% body weight. Uneaten feed was collected after 1 hour of feeding for the calculation of the actual feed consumption rate.

Table 1. Feed composition and proximate analysis of the experimental diets (% dry matter basis).

Ingredients (%)	T1	T2	T3	T4	T5
Fishmeal	47.27	46.49	45.7	44.92	44.14
L. minor	0	5	10	15	20
Wheat flour	51.33	47.11	42.9	38.68	34.46
Vitamin premix	0.4	0.4	0.4	0.4	0.4
Oil	1	1	1	1	1
Proximate analysis (%)					
Moisture	5.26	5.21	5.42	5.50	5.75
Ash	6.79	7.38	8	8.96	9.46
Fat	6.01	4.48	3.95	5.06	4.99
Fibre	1.94	1.77	2.43	3.58	3.73
Protein	38.43	38.93	37.47	38.99	39.76
Carbohydrate	43.51	44	45.16	41.49	40.04
Energy (Kcal $100g^{-1}$)	381.85	370.04	366.07	367.46	364.11

Proximate analysis of experimental feeds

Proximate analysis of feed ingredients was determined following the method of the Association of Official Analytical Chemists (AOAC, 2000). Micro Kjeldahl method was used for the determination of total nitrogen (N) content and then crude protein (%) was calculated as N × 6.25. Moisture and ash content were determined by weight differences. Fat content was determined by using petroleum ether as a solvent. Fibre content was determined gravimetrically.

Sampling and growth parameters

After the end of the feeding trial for 60 days, the fish were starved for 24 hours. After this, the fish were anaesthetized with phenoxyethanol (0.5mL L^{-1}) and the final weight and length of individual fish were measured. Specific growth rate (SGR), body mass gain (BMG), feed conversion ratio (FCR) and survival rate (SR) were calculated as follows following standard protocols:

BMG (%) = [(Final body mass in g – initial body mass in g)/Initial body mass in g] x 100

SGR (% day^{-1}) = [(ln final body mass in g – ln initial body mass in g)/number of trial days] x 100

FCR = Dry feed fed (g)/body mass gain (g)

Survival rate (%) = (Final number of fish/Initial number of fish) × 100

Immune parameters and antioxidant enzyme activity

For immune parameters, mucus and wet muscle tissue were collected from each treatment in triplicates. Total protein, total immunoglobulin, alkaline phosphatase and lysozyme activity were determined in the mucus sample, whereas the muscle tissue samples were used for the determination of total protein and catalase activities. Mucus was collected following the method of Ross *et al.* (2000) with slight modifications. Briefly, fish were starved for 24 hours prior to mucus collection and anesthetised with phenoxyethanol (0.5mL L^{-1}). Thereafter, 10 individual fish from each tank were collected on a polyethene bag containing 10mL of 50mM NaCl followed by a gentle shake. The sample thus collected was centrifuged at 1500×g for 10 minutes at 4°C and the supernatant was stored at -80°C for further use.

Total protein concentration

The total protein concentrations of the mucus and muscle tissue samples were determined according to the Bradford method (Bradford, 1976).

Total immunoglobulin concentration

The total immunoglobulin concentration was determined by the method of Siwicki & Anderson (1993). 100µL mucus sample was mixed with 100µL of polyethylene glycol solution (12%) followed by agitating down the immunoglobulin molecules and then centrifuged at 10000 × g for 10 minutes. Total immunoglobulin was calculated using the following formula:

Total immunoglobulin (mg mL^{-1}): Total protein in mucus sample – Total protein in the supernatant

Lysozyme activity

For the lysozyme activity, turbidometric assay (Ross *et al.*, 2000) was followed. Briefly equal volume of

mucus sample and 40mM Sodium phosphate buffer of pH 6.5 incubated at 30 °C for 15 minutes. Based on the lysis of lyophilised *Micrococcus lysodeikticus* cells, (0.3mg mL^{-1} in 40 mM sodium phosphate buffer, pH 6.5), one unit of activity was defined as the amount of enzyme that catalysed a decrease in absorbance at 450 nm of 0.001 min^{-1}.

Alkaline phosphatase activity

Alkaline phosphatase activity was determined by the method of Ross *et al.* (2000). The absorbance at 405nm was measured over a period of 30 minutes at 30°C using 50µL of mucus that had been reconstituted in 100mM ammonium bicarbonate buffer containing 1 mM $MgCl_2$, pH 7.8, at 30 °C for 15 minutes. The quantity of enzyme needed to release 1 µM of *p*-nitrophenol (PNP) product in a minute was used to define one unit of activity.

Catalase activity

Catalase activity was determined by measuring the decrease in absorbance at 240 nm (Aebi, 1983; Li and Schellhorn, 2007; Vinagre *et al.*, 2012) when the sample is added to H_2O_2. The muscle tissue sample was processed using a tissue grinder in cold sodium phosphate buffer solution (pH 7.4) for five minutes at 16,000 × g. Following the addition of the sample to hydrogen peroxide, absorbance was measured at 240 nm every 15 seconds. The activity was expressed as mM of H_2O_2 reduced per minute per milligram protein.

Statistical analysis

Data were represented as mean ± SD. One-way analysis of variance (ANOVA) and Tukey's post hoc test was used to test the significant difference between the means using SPSS 23.0. The level of statistical significance was accepted at $P < 0.05$.

Results

Growth performance

The growth performance and survival (%) were recorded after 60 days of culture. No mortality was recorded in all the treatments and therefore the survival was 100% in all the groups. The growth parameters recorded are represented in Table 2. The final weight of *H. fossilis* fed with the T3 diet (2.44 ± 0.08g) and T2 (2.34 ± 0.05g) were significantly ($P < 0.05$) higher than other treatments *viz.* T4 (2.06 ± 0.03g), T1 (2.05 ± 0.02g) and control (1.95 ± 0.10g). No significant difference ($P > 0.05$) in the final weight was observed between the control, T1 and T4 groups. A similar trend was observed in the BMG and FCR. Significantly ($P < 0.05$) highest BMG and SGR were observed in T3 (377.16 ± 18.07% & 2.60 ± 0.06%, respectively) followed by T2 (361.50 ± 10.13% & 2.55 ± 0.04%, respectively) compared to other groups. Although the BMG and SGR were lowest in the control group, there was no significant difference ($P > 0.05$) between the control, T1 and T4 groups in the study. The feed conversion ratio was lowest in T3 (0.93 ± 0.05) compared to other treatments i.e., Control (1.25 ± 0.05), T1 (1.15 ± 0.03), T2 (0.98 ± 0.02) and T4 (1.16 ± 0.03). The FCR in T1, T2 and T3 did not differ significantly. No change in the total protein of the muscle tissue was observed in all the treatments except the T2 group where the value increased significantly ($P < 0.05$).

Table 2. Growth performance and immune indices of *H. fossilis* fed control and *L. minor* incorporated diets for 60 days.

Parameters	Control	T1	T2	T3	T4
IW (g)	0.51 ± 0.01^{a}	0.51 ± 0.00^{a}	0.51 ± 0.01^{a}	0.51 ± 0.01^{a}	0.50 ± 0.01^{a}
FW (g)	1.95 ± 0.10^{a}	2.05 ± 0.02^{a}	2.34 ± 0.05^{b}	2.44 ± 0.08^{b}	2.06 ± 0.03^{a}
BMG (%)	284.76 ± 16.69^{a}	301.58 ± 2.34^{a}	361.50 ± 10.13^{b}	377.16 ± 18.07^{b}	311.94 ± 6.29^{a}
SGR (% day^{-1})	2.24 ± 0.07^{a}	2.32 ± 0.01^{a}	2.55 ± 0.04^{b}	2.60 ± 0.06^{b}	2.36 ± 0.03^{a}
FCR	1.25 ± 0.05^{c}	1.15 ± 0.03^{b}	0.98 ± 0.02^{a}	0.93 ± 0.05^{a}	1.16 ± 0.03^{bc}
Survival (%)	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
TPt ($mgmL^{-1}$)	0.30 ± 0.03^{a}	0.33 ± 0.01^{ab}	0.34 ± 0.01^{b}	0.30 ± 0.01^{ab}	0.32 ± 0.01^{ab}
TPm ($mgmL^{-1}$)	0.32 ± 0.01^{a}	0.32 ± 0.02^{a}	0.33 ± 0.00^{a}	0.31 ± 0.01^{a}	0.33 ± 0.01^{a}
T Ig ($mgmL^{-1}$)	0.21 ± 0.00^{ab}	0.22 ± 0.01^{ab}	0.22 ± 0.00^{b}	0.21 ± 0.00^{a}	0.22 ± 0.00^{ab}
LYS (U mg $protein^{-1}$)	94.26 ± 8.52^{bc}	105.56 ± 11.11^{c}	80.37 ± 1.09^{a}	80.40 ± 0.65^{b}	81.95 ± 11.36^{b}
ALP (U mg $protein^{-1}$)	1.44 ± 0.04^{bc}	1.80 ± 0.10^{d}	1.67 ± 0.21^{cd}	1.15 ± 0.06^{a}	1.28 ± 0.06^{ab}
CAT (U mg $protein^{-1}$)	1.85 ± 0.44^{a}	1.39 ± 0.69^{a}	1.32 ± 0.34^{a}	1.86 ± 0.44^{a}	1.60 ± 0.36^{a}

T1 = 5% *L. minor* incorporated diet, T2 = 10% *L. minor* incorporated diet, T3 = 15% *L. minor* incorporated diet, T4 = 20% *L. minor* incorporated diet, Control = 0% *L. minor* incorporated diet.

IW: Initial Weight, FW: Final Weight, BMG: Body Mass Gain, SGR: Specific Growth Rate, FCR: Feed Conversion

Ratio, TPt: Total Protein (Tissue), TPm: Total Protein (Mucus), T Ig: Total Immunoglobulin, LYS: Lysozyme, ALP: Alkaline Phosphatase, CAT: Catalase.

Values are represented as mean values ± SD. Means within the same column having different superscripts are significantly different ($P < 0.05$).

Immune parameters and antioxidant enzyme activity

The immune parameters of *H. fossilis* evaluated in the study are presented in Table 2. The total protein content of the mucus was similar and showed no significant difference ($P > 0.05$) in all the treatments. The total mucus protein ranged from 0.31 ± 0.00mg mL^{-1} to 0.33 ± 0.01mg mL^{-1} in all the treatments. A similar trend was also observed in the total immunoglobulin content, where no significant difference ($P > 0.05$) was observed among all the treatments. However, it was marginally higher in the plant-fed group *viz.*, T1 (0.22 ± 0.01mg mL^{-1}), T2 (0.22 ± 0.00mg mL^{-1}) and T4 (0.22 ± 0.00mg mL^{-1}) than in other groups. Lysozyme activity ranged between 80.37 ± 1.09 U mg $protein^{-1}$ in T2 to 105.56 ± 11.11 U mg $protein^{-1}$ in T1. Alkaline phosphatase activity showed significantly ($P < 0.05$) higher activity in T1 (1.80 ± 0.10 U mg $protein^{-1}$) compared to the other diet-fed fish and the control group. A decrease in alkaline phosphatase activity was observed in the T3 group compared to the control fish, whereas T2 and T4 treatments showed no significant variation compared to the control. Catalase activity showed no significant ($P > 0.05$) variation among all the groups. A higher activity catalase activity was observed in the T3 group (1.86 ± 0.44 U mg $protein^{-1}$), whereas the activity was reduced in the T2 group (1.32 ± 0.34 U mg $protein^{-1}$). However, these variations were not significant ($P > 0.05$) as compared to the control group indicating no significant change in the catalase activity.

Discussion

Growth performance

The dietary inclusion of *L. minor* in the diet of *H. fossilis* was found to influence the growth performance in the present study. Significantly, higher BMG and SGR were found in the experimental diet-fed fish compared to the control diet indicating that *L. minor* may be positively accepted by the fish resulting in better growth performance. This may be due to the ability of the fish to digest this plant protein. However, at a higher 20% *L. minor* incorporated diet, the growth performance was not significantly different from that of the control. This indicated that higher replacement of animal protein may not be suitable for the fish as it hindered the growth performance. The FCR determines the suitability and acceptability of the formulated feed by fish, lower FCR value indicates better utilisation of feed into flesh (Jabeen *et al.*, 2004). FCR decreased with the increase in % of *L. minor* in the diet up to 15%. At 20% plant-incorporated diet FCR showed an increase with no significant difference between that of the control group. This indicated a higher food conversion rate in T2 and T3 compared to others. Similar results were also found in *Channa striata*, where higher SGR and lower FCR were reported in a 50% *L. minor* fed diet than in the conventional fishmeal diet (Raj *et al.*, 2001). In another study, *Clarias gariepinus* fed with up to 40% *L. minor* showed better weight gain and lower FCR than the control diet (Irabor *et al.*, 2022). Lower FCR in *L. minor* incorporated diet-fed fish than in the control diet in the present study shows better and more efficient utilisation of feed in these groups than in the control diet. Generally, higher inclusion of plant protein in the diet for replacement of fishmeal results in a decrease in feed intake and growth performance (Daniel, 2018). In the present study growth performance was not affected by up to 20% inclusion of *L. minor* in the diet for *H. fossilis*. Similar results were found in *H. fossilis* fed fermented mulberry leaf meal (Ali *et al.*, 2020) and *Ipomoea aquatica* (Ali *et al.*, 2021). Results of the present study indicated that *L. minor* may be successfully incorporated up to a maximum of 20% in the diet for *H. fossilis* without affecting the growth of the fish.

Immune parameters and antioxidant enzyme activity

The immune system of fish is crucial for ensuring their tolerance to environmental stress (Adel *et al.*, 2016). Various studies have been reported on the effect of the dietary inclusion of plant proteins on fish growth and immune status (Kokou *et al.*, 2015; Dossou *et al.*, 2018).

The innate immune system provides the first line of defence for fish against a variety of diseases and is most important for fish than mammals (Saurabh *et al.,* 2008). Fish skin mucus also provides protection as it contains various immune parameters like lectins, pentraxins, lysozyme, complement proteins, antibacterial peptides and IgM (Magnadottir, 2006). In the present study, no significant difference ($P < 0.05$) in the immune indices estimated in the skin mucus of *H. fossilis* was found due to the incorporation of *L. minor* in its diet. The total mucus protein content of all the treatments did not differ significantly indicating that *L. minor* did not adversely affect the immune system of the fish. An increase in total protein content was also found in *Rutilus frisii kutum* fed *Mentha piperita* (Adel *et al.,* 2015). The mucus protein content of fish is reported to vary with the temperature, mode of collection of mucus, and the environment they lived (Baba *et al.,* 2021).

Immunoglobulin acts as the primary antibody of fish and acts as a crucial part of adaptive immunity and has been studied to evaluate the health condition of fish (Wei *et al.,* 2014; Baba *et al.,* 2021). In the present study, no significant difference ($P < 0.05$) was found between all the treatments. However, slightly higher immunoglobulin content was found in *L. minor* incorporated diets than in the control diet. Similar results were reported in juvenile hybrid grouper fish with an increasing level of peanut meal (Ye *et al.,* 2020). In a similar study in *Cyprinus carpio,* fed with *Heracleum persicum,* an increase in immunoglobulin content was also observed by Hoseinifar *et al.* (2016).

The lysozyme activity of fish is an important indicator of the innate immunity of fish, with its lytic against both gram-positive and gram-negative bacteria (Saurabh *et al.,* 2008). In the present study, no significant difference ($P < 0.05$) was found in the lysozyme activity of plant-fed fish except in the T1 and T2 group. Previous studies have reported an increase in lysozyme activity when fed with soy protein concentrate (Hoseinifar *et al.,* 2016; Wang *et al.,* 2017). A significant increase in serum lysozyme activity was also found in gibal carp (*Carassius auratus gibelio*) fed with fermented *Moringa oleifera* leaf meal than fishmeal (Zhang *et al.,* 2020).

Alkaline phosphatase in the skin mucus displays antimicrobial activities, helps defend against water pathogens (Lalles, 2019), and is also an indicator of stress in fish (Guardiola *et al.,* 2016). In the present study, higher alkaline phosphatase was found in T1 and T2 groups among all groups. A similar increase in alkaline phosphatase activity was also reported in previous studies (Adel *et al.,* 2015; Zhang *et al.,* 2020; Zhang *et al.,* 2020). The specific mechanisms behind the enhancement of immune activities in fish-fed plant-based diets are poorly described (Reverter *et al.,* 2020), however presence of some phytochemicals like alkaloids, phenolic compounds and steroids are attributed to it (Awad and Awaad, 2017). Catalase is an antioxidant enzyme that helps in the antioxidant defence mechanism protecting the body from damage by reactive oxygen species (Machlin, 1987; Michiels, 1994). Variations were observed in catalase activity in plant-fed fish, although these were not significant compared to the control diet-fed fish. However, higher catalase activity was reported in juvenile grouper fed 33% soy protein concentrate than the control diet (Wang *et al.,* 2020), in juvenile gibal carp fed fermented *Moringa oleifera* Lam. leaves (Zhang *et al.,* 2020) and in *Pagrus major* fed fermented rapeseed meal (Dossou *et al.,* 2018). Results of all the immune parameters indicate that *L. minor* did not have an adverse negative effect on the skin and tissue immune parameters of *H. fossilis* when the plant replaces up to 20% of the animal protein in its diet. However, there are indications of negative affect at higher doses probably due to incompetent digestive metabolism for higher plant proteins in its diet. Hence, further studies may be suggested to evaluate the fish response at higher doses by investigating important parameters at different ages of the fish.

Conclusion

This study shows that *L. minor* may be incorporated up to 20% in the diet of *H. fossilis* to partially replace fish meal (animal based protein) without affecting the growth and immune response of the fish. *L. minor* was found to meet the nutritional requirement of fish at this level with better nutrient utilisation and growth performance.

No significant change in the immune response and antioxidant enzyme activity was induced by the plant-based diet in the fish. Further investigation may be recommended to evaluate other metabolic responses for maximal utilisation of the plant. This preliminary study has shown that *L. minor* may be an economical and sustainable source of alternative protein in aquafeed for the mass production of *H. fossilis*.

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THEME: EMERGING TRENDS IN AGRICULTURE, LIFE SCIENCE AND APPLIED COMPUTING
NOVEMBER 24 - 25, 2022; VENUE- NEHU, TURA CAMPUS, MEGHALAYA, INDIA

CERTIFICATE OF PARTICIPATION

This is to certify that Prof./Dr./Mr./Mrs./Ms. Sanraja Muchahary
of Dept. of Zoology, Bodoland University presented a paper titled Effect of Jeevamrit mixture on the growth and ... Heteropneustes fossilis and authored by Sanraja Muchahary and Bhanoram Kumar Khanglekhan in ORAL/POSTER mode during ICESD 2022 at NEHU, Tura Campus, Meghalaya, India conducted under the aegis of Biotech KISAN Hub, North-Eastern Hill University, Tura Campus, Meghalaya, India funded by DBT, New Delhi, India in collaboration with Department of Computer Application, North-Eastern Hill University, Tura Campus, Meghalaya, India.

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His / Her title of the paper was Current Status of Utilising Plant Proteins as Replacement for animal protein in Fish Feed Formulation

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(Debadutta Chakravarty) Coordinator, IQAC, S. S. College, Hailakandi
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Title of the paper: Role of plant proteins as an alternative source of protein in fish feed for sustainable aquaculture

Mode of Presentation: Poster

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