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

Sanraja Muchahary, Bichitra Narzary, Bronson Kumar Khangembam*

Department of Zoology, Bodoland University, Kokrajhar, Assam, India

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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.

* **Corresponding Author:** Bronson Kumar Khangembam ✉ kbronson173@gmail.com

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.

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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