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Introducing Mulberry Leaf Meal along with Fish Offal Meal in the Diet of Freshwater Catfish, *Heteropneustes fossilis*

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Abstract

Experiments were made to evaluate different combinations of fermented fish-offal meal (FOM) and mulberry leaf meal (MLM) as protein supplement in the formulation of diets for the freshwater catfish, *Heteropneustes fossilis*. One reference diet (20 % FM) and six experimental diets containing either mixture of fermented FOM (20-30 %) and MLM (24-35 %) or MLM alone (65-80 %) partially replacing FM were formulated. A ten days digestibility experiment and a sixty days growth experiment with these diets exhibited that the diet with 30 % FOM, 5 % FM and 24 % MLM, replacing 75 % of FM, rendered best growth of the *H. fossilis*. Deposition of crude protein and lipid in the whole body was also significantly higher in this diet as compared to the reference diet. It was concluded that effectiveness of fermented FOM in replacing FM could be substantially increased by limited inclusion of MLM in the formulation of diet for *H. fossilis*.

Keywords: Fishmeal, Fish-offal, Mulberry leaf meal, Diet, Catfish.

1. Introduction

In recent years there have been intensification of research in India towards utilization of animal and plant by-products as feed staff in fish diet to replace fishmeal, which is expensive and gradually becoming scarce [1-2]. Fermented fish-offal has been proved to be a viable source of protein for the diet of carps [3]. The freshwater catfish *H. fossilis* has also been found to accept a diet containing fermented fish offal [4]. However, only 50 % of fishmeal could be replaced when fish-offal was used as a source of protein. Mulberry leaves are rich in protein and mineral elements [5-6]. Incorporation of mulberry leaves in the diet of poultry [7] and rabbit [8] resulted in better egg production of poultry and growth of rabbit. However, there is no record of this plant resource being used in fish diets. Although plant proteins (PP) are cost effective, their use is limited by deficiencies in essential amino acids and minerals, and the presence of anti-nutritional factors (ANFs) and complex carbohydrates [9-10]. Fermentation is a simple and cheap method to decrease the anti-nutritional factors and crude fibre contained in the plant by-products [1].

The present study was therefore undertaken to investigate if mulberry leaf meal could be fermented and used along with fish-offal to increase efficiency of the diet and replace more amount of fishmeal without compromising growth and nutrient deposition of the fish.

2. Materials and Methods

2.1. Experimental diet formulation and preparation

Seven experimental diets were prepared with fermented fish-offal meal (FOM), sun dried mulberry leaf meal (MLM), mustard oil cake (MOC), rice bran (RB) and fishmeal (FM). The fish-offal, comprising viscera of cultured carps (Labeo rohita and Catla catla), was obtained from the local retail fish markets. The portion of guts contained in the offal was carefully uncoiled, cut open and the gut contents were removed using clean water. For fermentation, the FOM, mustard oil cake, rice bran and MLM were mixed at proportion mentioned in Table 1. The mixture was added to a solution of microbial suspension (108 cell mL–1) (*Lactobacillus* sp., *Rhodopseudomonas* sp., *Azotobacter* sp., *Streptomyces* sp. and *Saccharomyces* sp.). (the microbial suspension (EMTM) was obtained as a gift from M/S, Maple Orgtech Pvt. Ltd. Kolkata.), molasses and water (2.5 mL : 2.5 g : 100 mL) and was fermented anaerobically in an anaerobic fermentation chamber under ambient temperature (27–30°C) for 36 to 44 days, depending on the proportion of fish-offal meal and mulberry leaf meal. Initially the pH of the fermented mixtures was 7.6 to 8.2 but the pH value gradually decreased and at the end of the fermentation the range of the pH was 4.4 to 4.6. The Final fermented product was mixed with fish meal, vitamin and mineral mixture. The diets

were formulated in such a way that these contained not less than 30% crude protein. To test the apparent protein digestibility of the diets, 1% chromic oxide (Cr2O3) was included in each diet separately as non-absorbent reference substance and 0.5% carboxymethyl cellulose (CMC) as a binder. The mixtures were ground, blended, and pelleted using a hand pelletizer fitted with a 2-mm dia to prepare the final experimental diets (Table 1). Diets were sun dried before use.

2.2. Experimental design

Two experimental systems were used for the present study: one with indoor glass aquaria (50 L) to evaluate voluntary diet intake and apparent protein digestibility (APD) and other one with outdoor cement tanks (400 L) to evaluate growth and biochemical composition of the body. Deep tube-well water stored in an overhead tank was used in both trials. Fingerlings of *Heteropneustes fossilis* (mean initial length 7.26 ± 0.43 cm and mean initial whole body weight 6.11 ± 0.38 g) were obtained from a local fish farm and were acclimatized to the laboratory conditions for one week prior to start of the experiment. The fingerlings were fed to satiation (twice a day six days a week) with the reference diet (T1) during acclimatization. The acclimatized fingerlings were randomly distributed at the rate of 10 per aquarium and 40 pieces per tank. The aquaria or tanks were laid out in a completely randomized block design [11] with three replicates for each of the seven diet treatments.

The indoor feeding experiment in aquaria was continued for 10 days. The fish were fed a ration at 5% of their body weight. The ration was provided at 08:00 hours and the fish were allowed to eat for 6 h. Left over diets were collected by siphoning after 6 hours of feeding, oven-dried, weighed and stored at –20°C. The rate of leaching rate of the ingredients from the diet was estimated by placing weighed diets in aquaria without fish for 6 h and then recollecting, drying and re-weighing the diets. The average leaching rate was used to calibrate the amount of uneaten diets. Faecal samples were collected by siphoning from each aquarium continuously at a 3-4 h interval for a period of 17 h after the removal of uneaten diets. To minimize nutrient leaching, only fresh and intact faeces were collected and dried to a constant weight at 60°C in an oven and weighed before preserving at –20°C. Apparent protein digestibility (APD) of the diet was calculated from the proportion of Cr and protein in the diet and faeces following the methods described by Ellestad et al. [12]. Water temperature in the aquaria ranged from 21–24°C and aeration was provided to maintain a dissolved oxygen level of approximately 8.4–8.5 mg L–1.

In the outdoor growth experiment the fish were fed twice daily at 10:00 h and 16:00 h at a fixed feeding rate of 5% of body weight for the entire experimental period of 60 days. The quantity of the diet given was readjusted every 15 days after weighing the fish. Samples of water were collected every week to determine selected parameters like dissolved oxygen, free carbon dioxide, total ammonia, alkalinity, hardness, and pH following the standard procedures of APHA [13]. Water quality parameters (temperature 27.1-28.0ºC, pH 6.9-7.9, dissolved oxygen 8.6-9.6 mg L-1, free carbon dioxide 3.8-4.9 mg L-1, total alkalinity 187-205 mg L-1 as CaCO3, total hardness 204-215 mg L-1 as CaCO3 and ammonia nitrogen 0.13 -0.43 mg L-1) recorded during the growth study were within the optimum ranges required for rearing catfish fingerlings.

At the end of the growth trial,fish were sampled from each tank and were used for determination of biochemical parameters and growth. Five fish were randomly selected from each tank and frozen at -20°C for determination of biochemical parameters (crude protein, crude lipid, and ash content of the carcass). Determinations were made on pooled samples of fish from each tank thereby giving a total of three replicates for each diet. Rest of the sampled fish was used to determine increase in weight, specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), and apparent net protein utilization (ANPU) using standard methods of Castell & Tiews [14].

2.3. Analytical methods

Proximate analyses of the experimental diets, carcass and faecal samples were performed following the AOAC [15] procedures as follows: moisture was determined by oven drying at 105°C for 24 h; crude protein (nitrogen × 6.25) was determined by Kjeldahl method, after acid hydrolysis; lipid was extracted by petroleum ether (boiling point 40–60°C) for 7–8 h in a Soxhlet apparatus followed by determination of lipid gravimetrically, crude fibre was determined as loss on ignition of dried lipid-free residues after digestion with 1.25% H2SO4 and 1.25% NaOH and ash was determined by combustion at 550°C, in a muffle furnace, till a constant weight. Gross energy was calculated on the basis of methodology of Brafield [16].

Tannin content in both fermented and raw mulberry leaf was determined using Folin–Denis reagent [17]. Phytic acid content was determined according to Wheeler & Ferrel [18]. Chromium was determined in the diets and faecal samples by Atomic Absorption Spectrophotometer (AAS). Samples of diet (500 mg) or of lyophilized faeces (250 mg) were ashed in a muffle furnace at 450°C. Then 15 mL of nitric acid (70 %) and 5 mL of perchloric acid (60 %) were added to the ashed sample. The samples were then digested at 100°C for ~6 h until the green color changed to orange. The concentration of chromium in the sample was determined by atomic absorption spectrophotometer (Varian Spectra AA 240) at 425.4 nm using air

acetylene flame. The detailed methodology has been followed from Saha & Gilbreath [19].

Table 1. Ingredients and proximate composition of diets.

Diets	MOC	RB	FOM	MLM	FM	VM	Proximate composition (* % dry matter)					
							CP*	CL*	CF*	Ash*	GE(kJg$^{-1)}$	P:E
T1	390	390	-	-	200	20	30.94	11.63	3.81	11.42	18.65	16.59
T2	250	140	300	240	50	20	30.22	18.56	2.82	12.22	19.87	15.21
T3	250	140	250	240	100	20	30.42	17.90	3.42	14.12	19.74	15.41
T4	250	140	200	350	40	20	30.05	15.40	4.42	15.52	19.13	15.70
T5	140	90	-	650	100	20	30.62	12.50	5.62	22.82	18.45	16.59
T6	140	40	-	750	50	20	30.12	14.63	8.23	24.62	18.54	16.25
T7	100	40	-	800	40	20	30.02	14.55	8.63	25.22	18.52	16.21

MOC = Mustard oilcake; RB= Rice bran; FOM = Fish offal meal; FM = Fishmeal; VM = Mixture of vitamin and mineral (10+10); CP= Crude protein; CL- Crude lipid; CF= Crude fibre; GE = Gross energy; P:E = protein : energy.

2.4. Statistical analyses

The nature of distribution of the observations of each response variable from both the trials was verified by Kolmogorov-Smirnov (K-S) and Shapiro-Wilks (S-W) tests to ensure a Gaussian distribution. Since all data were found normally distributed they were subjected to single factor ANOVA, without any further transformation, followed by least significant difference (LSD) test to compare mean between the treatments [11, 20].

3. Results

Proximate compositions of the seven diets formulated for the feeding trial were presented in Table 1. Fermentation of fish-offal meal (FOM) and / or mulberry leaf meal resulted in a significant decrease in the levels of crude fibre. Concentration of the anti-nutritional factors, tannin and phytic acid, in the ingredient mixture of the diets T2, T3, T4, T5, T6 and T7, were respectively 0.1, 0.2, 0.36, 0.26, 0.36, 0.4 and 0.12, 0.23, 0.39, 0.28, 0.39, 0.46 % before fermentation. None of these anti-nutritional factors could be detected in the fermented products.

Survival rate of Heteropneustes fossilis, irrespective of treatments, was 100 % during the short-term feeding trial, while it ranged from 90 to 96% during the growth trial, without showing any significant variation between the dietary groups. The diet intake rate, which ranged between 2.24-2.30 g 100g BW-1 d-1,did not vary significantly between the diet groups. Diets containing mixture of FOM and MLM (T2-T3) and diet containing low level of MLM without FOM (T5) showed significantly higher apparent protein digestibility (APD) (Table 2) and growth (in terms of weight gains and SGR) than the reference (T1) and other diets (T6-T7) (Table 3). Diets containing higher level of MLM without the incorporation of FOM (T6 and T7) showed APD and growth similar to reference diet. Protein efficiency ratio (PER) and apparent net protein utilization (ANPU) also increased in T2 and T3 over T1. The mixture diet with low FOM (20%) and high MLM (35%) such as T4 did not show any increase in growth over the reference diet (T1), although PER and ANPU increased in T4 as compared with the reference diet (T1).

Table 2. Apparent protein digestibility (APD) and voluntary intake rate of diet of *H. fossilis* fingerling fed experimental diets.

Diets	APD[1] (%)*	Diet intake (g 100g BW^{-1} d^{-1})
T1	89.17±1.69^{a}	2.30±0.15^{a}
T2	92.13±2.19^{b}	2.24±0.09^{a}
T3	89.27±1.90^{a}	2.27±0.05^{a}
T4	88.10±0.17^{c}	2.22±0.16^{a}
T5	89.93±1.33^{ac}	2.27±0.05^{a}
T6	79.93±0.58^{d}	2.29±0.16^{a}
T7	78.70±1.13^{d}	2.28±0.16^{a}

Means with dissimilar superscript indicate significant difference (LSD;P <0.05)) between two means

*APD =100 -100 × ((% Cr in diet / % Cr in faeces) × (% protein in faeces / % protein in diet)).

Table 3. Growth performance and diet efficiency of *H. fossilis* fingerlings fed experimental diets for 60 days.

Diets	PIW	FCR	SGR	PER	ANPU
T1	118±8^{a}	1.3±0.1^{a}	1.7±0.1^{a}	1.6±0.1^{a}	44.7±0.2^{a}
T2	141±16^{b}	1.5±0.1^{b}	1.4±0.2^{b}	2.1±0.3^{b}	48.3±0.3^{b}
T3	133±8^{bc}	1.4±0.1^{bc}	1.5±0.1^{bc}	2.2±0.1^{bc}	57.5±0.4^{c}
T4	130±10^{a}	1.4±0.1^{a}	1.5±0.1^{ac}	2.1±0.2^{d}	56.8±0.2^{d}
T5	132±8^{bc}	1.4±0.1^{bc}	1.5±0.1^{bc}	1.9±0.1^{de}	47.2±0.3^{e}
T6	112±14^{a}	1.3±0.1^{a}	1.8±0.2^{a}	1.7±0.2^{af}	48.7±0.3^{fb}
T7	104±3^{a}	1.2±0.0^{a}	1.9±0.1^{e}	1.6±0.1^{af}	48.5±0.7^{bg}

PIW=Percentage increase in weight
FCR = Dry wt. of diet given / increase in weight of the fish
SGR = {(ln final wt – ln initial wt)/days on trial} × 100
PER = Wet weight gain of fish / Protein consumed .
ANPU = (Net increase in carcass protein / Amount of protein consumed) ×100.

The whole body composition of the experimental fish determined before and after the experiment is given in Table 4. The content of crude protein (CP) in the whole body was significantly higher in fish fed diets containing both FOM and MLM (T2 to T3) as compared with those fed the reference diet (T1). Content of CP in fish fed T5 diet was similar to those fed T3 diet, but CP decreased in fish fed T6 and T7 diets as compared to T2 diet and became comparable to the reference diet (T1). The lipid content of the whole body was significantly higher in all the experimental diets (T2 to T7) as compared with the reference diet. Percentage of ash in the body of fish also showed significant difference between the dietary groups. Ash content was highest in T2 and T5 diets.

Table 4. Proximate composition of carcass (% wet weight).

		Crude protein	Crude lipid	Ash
Initial		15.30+0.36	3.53±0.31	3.20±0.10
Final	T1	17.19±0.12^{a}	4.37±0.25^{a}	3.80±0.10^{a}
	T2	18.80±0.10^{b}	4.73±0.12^{a}	4.17±0.06^{b}
	T3	18.47±0.12^{cb}	5.17±0.06^{b}	3.96±0.16^{ab}
	T4	17.97±0.81^{dc}	4.37±0.25^{a}	3.82±0.08^{a}
	T5	18.37±0.21^{ebc}	4.47±0.23^{a}	4.13±0.21^{cb}
	T6	17.53±0.12^{ad}	5.33±0.11^{cb}	3.93±0.15^{ab}
	T7	16.93±0.23^{a}	4.97±0.32^{dab}	3.73±0.15^{a}

4. Discussions and Conclusions

Results of the present study indicate that mulberry leaf meal (MLM) can be effectively used as an ingredient to replace fishmeal in the formulation of diet for the catfish Heteropneustes fossilis. Digestibility of the diet and growth of the fish can be maximized by planned inclusion of MLM alone or in combination with fish offal meal (FOM). Inclusion level of MLM up to 35 %, whether used alone or in combination with FOM, significantly increased the digestibility and growth of the fish as compared to the reference diet. Obviously, efficiency of the diet increased when FOM and MLM were combined with 30 % FOM and 24 % MLM. Such combination could replace 75 % of fishmeal with no compromise on diet intake, digestibility, nutrient deposition and growth of the fish.

Fermentation improves nutritional quality of the ingredients and efficiency of the diet. This was evident from the researches on fermented silkworm pupae [21, 2], silage [22] and fish-offal [3-4]. The results of the present study indicate that although fermentation completely removed phytic acid and tannin from the diets containing mulberry leaf meal, levels higher than 65% mulberry leaf meal reduced digestibility of the diets probably because of higher amount of crude fibre. Similar reduction of digestibility due to increasing level of fibre was observed in diet containing 40% raw Lemna leaf meal as compared to only fishmeal containing diet [1]. Higher level of fat in ingredients containing plant resources has also been found to decrease digestibility of protein in Nile tilapia [23]. But Bahurmiz & Ng [24] observed that dietary lipid from palm oil did not affect protein digestibility of red hybrid tilapia. Results of the present study also indicated that high level of lipid in T2 to T4 diets (mixture of FOM and MLM) did not affect protein digestibility in *H. fossilis*.

H. fossilis is known to consume and digest wide variety of foods [25]. Usmani et al. [26] observed APD of *H. fossilis* to vary widely between practical feed ingredients with rice bran showing the least value (61.1 %) and soybean meal the highest value (95.4 %). *H. fossilis* fed silk worm pupae showed 78.8 to 86.72 % APD [27]. The present study indicated that inclusion of fish-offal meal at 30 % and 25 % level in mixed diet (T2 and T3) increased the APD of the diet as compared to the reference diet. The diet containing only 65 % mulberry leaf meal also showed higher APD than the reference diet (T1) and the other two diets containing 75 % and 80 % mulberry leaf meal alone (T6 and T7), but APD of 65 % mulberry leaf meal containing diet is lower than the mixed diet containing both 25 % and 30 % fish offal meal. Diet intake rate of the supplementary (T2-T7) diets was not significantly different from the reference (T1) diet. Diet intake rate in fish is affected by dietary composition [28-29]. In carnivorous southern catfish *Silurus meridionalis* Chen, diet intake was found inversely related to dietary protein [30]. The results of the present study also exhibited similar trend in diet intake rate by freshwater catfish *H. fossilis*, but the differences were not significant (P>0.05). It was assumed that catfish could regulate diet intake and selectively use assimilated nutrients. From juvenile stage onwards *H. fossilis* shows a preference for detritus as diet [31].The fish has also been found to grow better on diet containing composted weed with very less amount of protein as compared to common practical diet ingredients such as rice bran and mustard oil cake together containing an average value of 15-20 % CP [32].

Crude protein levels of the formulated diets used in the present study ranged from 30.94% in T1 (reference) diet to 30.02% in the T7 diet. Interestingly, best growth was obtained in fish fed diets containing crude protein level lower than the reference diet (30.20%-T2, 30.40%-T3 and 30.60 %-T5). The present study indicated that diet containing fermented FOM was accepted well by *H. fossilis* and showed highest growth performance, in T2 diet. Diets containing fermented fish offal did not increase crude protein level of the diet; yet *H. fossilis* grew better on such diet. Increased level of

lipid in the diet containing FOM might influence sparing of protein. Lipid as a non protein energy source allows protein sparing by effectively reducing organic matter and nitrogen losses. Protein sparing effects of dietary lipids has been demonstrated for *H. fossilis* [4] salmonids [33], red drum [34], sea bass [35] and the Indian major carp rohu [3, 36]. It has been observed that fish can utilize lipid in maximization of protein usage as long as crude protein in the diet is not lowered to the minimum level required by the fish [37]. For *H. fossilis* 27 to 35 % crude protein is required in the diet for its optimum growth [38, 4]. The diet used in the present investigation contained 30.02-30.94 % crude protein resulting in a 104.04-141.13 % increase in weight. Although the supplementary diets used in the present study contained less amount of protein as compared to the reference diet it is assumed that the CP level was adequate to meet the minimum requirement of protein by the fish, which could effectively utilize the energy from the lipid for growth. The present study indicated that the diet T2 resulted in best performance by *H.fossilis* with regard to FCR, gain in weight, SGR, PER and ANPU indicating that a mixture of 30 % FOM and limited amount (24 %) of MLM could be used safely as protein source in the formulation of diet to maximize growth of *H. fossilis.* Nwanna [39] observed that the best profit margin in the formulation of diet for African catfish *Clarias gariepinus* could be realized by replacing fishmeal with 30% fermented shrimp head meal (FSHM). This study dictated that growth of *C. gariepinus* fed 30% FSHM remained unchanged as compared with those fed only the fishmeal diet. The profit was made due to replacement of fishmeal and subsequent reduction of cost of the diet. The results of the present study indicated that the mixture of FOM and MLM in the T2 diet could replace 75% of fishmeal, thereby substantially reducing the cost of the diet.

In summary, the present study reveals that fishmeal (FM) can be partially replaced by fish-offal meal (FOM) and mulberry leaf meal (MLM) in the formulation of diet for *Heteropneustes fossilis*. Based on performance of the fish fed these diets, in terms of growth and protein deposition, it is concluded that replacement of FM by mixture of FOM and MLM is possible up to 75 %.

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