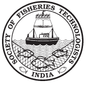

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Evaluation of Fermented Mixed Leaf Meal as an Alternate Protein Source in the Diet of *Labeo rohita* Fingerlings

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Abstract

The present study was designed to evaluate the potential of solid-state fermented mixed leaf meals (SFMLM), comprised of *Sesbania bispinosa*, *Glycine max*, *Leucaena leucocephala,* and *Eichhornia crassipes* leaves as an alternative protein source in the diet of *Labeo rohita* (Hamilton, 1822) fingerlings. Fermentation was carried out over 7 days using different microbes (*Bacillus subtilis*, *B. megaterium, Lactobacillus plantarum*, *Chaetomium globosum*, *Saccharomyces cerevisiae* and *Aspergillus niger*) either individually or in combinations. Nutritional quality of each combination was analysed at every 24 h interval. Fermentation significantly reduced the antinutritional factors such as phytic acid, tannins and saponins, making it superior than that of only mixed leaf meal (MLM). The best nutritional quality was obtained using a combination of *B. megaterium*, *L. plantarum* and *S. cerevisiae* fermented for 3 days. This SFMLM was used in a 60-day feeding trial. Five iso-nitrogenous (crude protein 30%) and iso-caloric diets (Digestible energy: 346 kcal/100 g) were formulated with 0% (C), 10% (T1), 20% (T2), 30% (T3), and 40% (T4) SFMLM. A total of 225 acclimated *L. rohita* fingerlings (5.2 ± 0.01 g) were randomly distributed across treatments in triplicate. Among the groups, T2 showed significantly higher ($p < 0.05$) weight gain, specific growth rate, and lower feed conversion ratio in *L. rohita* fingerlings. The activities of protease, lipase, and amylase were also significantly higher ($p < 0.05$) in this group. Hence, it can be concluded that the 20% solid-state fermented mix leaf meal (SFMLM) can effectively replace the traditionally used oil protein source in *L. rohita* diets without compromising the growth.

Keywords: *Bacillus subtilis, Bacillus megaterium, Lactobacillus plantarum, Aspergillus niger*, growth response, fermented leaf meal

Introduction

Aquaculture has emerged as the most efficient producer of edible protein and remains the fastest growing primary food production sector. Over the past few decades, the environmental impact of aquaculture has also gained increasing attention, highlighting the importance of applying high quality feed in aquaculture systems (Mannur et al., 2025). The aquafeed industry relies primarily on fishmeal due to its high protein content and the presence of highly digestible essential amino acids and fatty acids (Zinn et al., 2009; NRC, 2011). However, fishmeal is currently facing an impending crisis due to various environmental, economic, and social factors. At the same time, aquaculture is also experiencing a scarcity of other feed ingredients, such as the de-oiled rice bran, mustard oil cake, and soybean meal for various reasons (Naylor et al., 2021). Hence, it has become necessary for researchers around the globe to find substitutes for protein-based feed ingredients. In this context, leaf meals can be one of the potential alternatives to these ingredients in fish feed (Maiti et al., 2019). However, a proper strategy should be applied to overcome the issues of high crude fibre and antinutritional factors (ANFs) in leaf meals. The solid-state fermentation (SSF) process was chosen, as it is known to reduce the crude fibre and ANFs in the plant feed resources, thereby improving overall nutrient utilisation (Ranjan, Sahu, Deo, & Kumar, 2019). Jayant et al. (2020) observed a significant increase in crude protein and ether extract, along with a reduction in crude fibre, in sweet potato leaf meal (SPLM) after fermentation.

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The selected leaf meals for the study were *S. bispinosa* (dhaincha), *G. max* (soybean leaf), *L. leucocephala* (subabul leaf) and *E. crassipes* (water hyacinth) which are promising candidates for microbial fermentation due to their nutritional value and wide availability (Anand et al., 2020; Malik et al., 2024). Numerous studies have reported successful SSF of various leaf meals using different microbes, either alone or in combination. Bairagi, Ghosh, Sen, and Ray (2004) reported that levels of tannin, phytic acid, and mimosine in the *Leucaena* leaf meal decreased significantly after fermentation with *B. subtilis*. Similarly, Belewu and Sam (2010) observed a significant reduction in trypsin inhibitors, phytate, and tannins in the *A. niger* treated jatropha meal (*Jatropha curcas).*

Chaetomium globosum has been used for the successful fermentation of *Ipomea batatas* (sweet potato) leaf meal (Meshram, Deo, Kumar, Aklakur, & Sahu, 2018), *B. subtilis* for *S. bispinosa* leaf meal (Anand et al., 2020), *A. niger* for Moringa leaf meal (Manna et al., 2024), and *C. globosum* for *E. crassipis* leaf meal (Malik et al., 2024). Shi, Su, Chen, and Pian (2020) further reported that the nutritional contents of mixed leaf meals improved significantly when fermented with a mixture of microbes.

Considering these findings, the present study was planned to determine a combination of microbes (bacteria and fungi species) for efficient fermentation of the mixed leaf meal, and secondly, to evaluate the effects of the resulting solid state fermented mixed leaf meal on the growth and physiological performance of *L. rohita* fingerlings.

Materials and Methods

Sesbania bispinosa (dhaincha), *G. max* (soybean leaf), *L. leucocephala* (subabul leaf), *E. crassipes* (water hyacinth) leaves were collected fresh from different parts of Mumbai (India), cleaned and dried at 40 °C in a hot air oven for 2 days to reduce the moisture content. The dried leaves were weighed, pulverised, and sieved through a 50 μm mesh (procured from local market) to obtain a fine leaf meal powder, which was stored at 4 °C until use. The entire process was conducted in the Fish Nutrition Laboratories of ICAR-CIFE, Mumbai, India.

Microbial cultures were procured from the microbial-type culture collection (MTCC), Chandigarh, India. A total of three bacterial (*B. subtilis, B. megaterium* & *L. plantarum*) and three fungal species (*C. globosum, S. cerevisiae*, and *A. niger*) were used for fermentation. *B. subtilis* (MTCC 5981), *B. megaterium* (MTCC 2444) & *L. plantarum* (MTCC 6161) were cultured in nutrient broth, whereas *C. globosum* (MTCC 4179), *S. cerevisiae* (MTCC 170), and *A. niger* (MTCC 281) were grown in potato carrot broth at 40 °C. After 16-18 hours of incubation, bacteria-carrying nutrient broth were plated on nutrient agar, and fungi were plated on potato carrot agar media (HiMedia, Mumbai), and allowed to incubate for 20-24 hours in replicates. Bacterial and fungal isolates were then inoculated into sterilized test tubes containing 5 mL broth and incubated at 37 °C for 24 hours. Following incubation, 1 mL broth was centrifuged in 5 mL phosphate buffered saline (PBS) at 2000 rpm for 10 minutes. The supernatant was discarded, and the resulting pellet was rinsed with PBS, and optical density (OD) was measured at 600 nm. Based on the absorbance value, the microbes were serially diluted and streaked on agar plates. The appropriate dilution, determined based on the values of plate count, was selected for use in solid-state fermentation.

Solid-state fermentation of mixed leaf was carried out using different microbes for 7 days at the room temperature. Five hundred gram (500 g) of dried MLM (particle size <40 μ) was transferred into four separate 500 mL sterilized conical flasks, and autoclaved at 121 °C for 15 min at 1 kg/cm^2 pressure followed by cooling at room temperature. In each flask, 250 mL of water was added to maintain 50% moisture level. Subsequently, 40 μL of each fungal spore and bacterial cell suspension was inoculated into the substrate using a sterile pipette and mixed thoroughly with a sterilized glass rod. The microbial concentration used for the fermentation was 3×10^5 cfu/mL. After 7 days of fermentation, samples were collected, oven-dried at 50 °C for 24 hours, and stored in airtight containers at 4 °C until use. The mixed leaf meal fermented using *B. megaterium*, *L. Plantarum*, and *S. cerevisiae* was selected for inclusion in the experimental diets of *L. rohita* fingerlings due to its increased protein and reduced levels of ANFs and crude fibre.

A total of five isonitrogenous (crude protein : 30 g/ 100g) and isocaloric (digestible energy : 346 kcal/ 100g) diets viz., Control (0% SFMLM), SFLM10 (10% SFMLM), SFMLM20 (20% SFMLM), SFLM30, (30% SFMLM), and SFMLM40 (40% SFMLM) were prepared (Table 1). Soybean meal, mustard oil cake, and groundnut oil cake were used as protein

sources, while wheat flour and de-oiled rice bran served as carbohydrate sources. Cod liver oil and sunflower oil were used as lipid sources. All practical ingredients used in the experimental diet preparation were procured from the local market. Carboxymethyl cellulose (HiMedia, Mumbai) was added as a binder, butylated hydroxytoluene (HiMedia, Mumbai) as a preservative, choline chloride (HiMedia, Mumbai) as a chemoattractant, and vitamin-mineral mixture (Virbac, Mumbai) as a feed supplement in the feed.

The proximate composition of the diets was analysed using standard methods (AOAC, 1995). The moisture content was determined by oven-drying at 102 ± 2 ºC until constant weight was attained. Crude protein (CP), total ash (TA), and ether extract (EE) contents were determined by using the micro-Kjeldahl method (Kelplus, PELICAN, India), a muffle furnace (550 ºC for 5 h) and the Soxhlet extraction method (SOCS plus, PELICAN, India), respectively. Crude fibre (CF) of the defatted samples was determined using FibroTRON (Foss, India) apparatus, followed by oven drying at 102 ºC for 24 h and incineration in a muffle furnace at 550 ºC for 5 hours. The nitrogen-free extract (NFE) and digestible energy (DE) were calculated using the following formulae:

$$NFE (\%) = 100 - \{CP (\%) + EE (\%) + CF (\%) + TA (\%)\}$$

$$DE (kcal/100g) = (CP (\%) \times 4) + (EE (\%) \times 9) + (NFE (\%) \times 4)$$

The anti-nutritional factors of the feed were also determined using the standard methods. Tannin and phytic acid contents was assessed following the procedures of Makkar, Siddhuraju, and Becker (2007) & Gao et al. (2007), respectively. Saponin content was determined by the vanillin-H_2SO_4 method of Hiai, Oura, and Nakajima (1976), following methanolic extraction, and was quantified using the standard curve of saponin.

Based on the standard formula, WG, SGR, FCR and PER were calculated as follows:

$$WG = \text{Final weight} - \text{Initial weight}$$

$$SGR (\%/day) = \frac{(\ln \text{ of final weight} - \ln \text{ of initial weight}}{\text{Experimental period (days)}} \times 100$$

$$FCR = \frac{\text{Feed intake (g on dry matter basis)}}{\text{Body weight gain (g on wet weight basis)}}$$

$$PER = \frac{\text{Body weight gain (g on wet weight basis)}}{\text{Protein intake (g on dry matter basis)}}$$

The protein content of tissue homogenates was estimated using Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951), and was used for calculating the enzyme activities of the respective sample.

The intestinal protease activity was measured using Drapeau's method (Drapeau, 1976). The amount of enzyme required for releasing acid-soluble fragments equivalent to Δ0.001 at 280 nm per minute at 37 ºC and pH 7.8 is interpreted as one unit of protease activity. The activity was expressed as μmol tyrosine released/min/g protein.

Amylase activity was determined by the method described by Rick and Stegbauer (1974), which is based on the release of reducing sugars as products of carbohydrate hydrolysis by gluco-amylase and α-amylase in the presence of di-nitro salicylic-acid (DNS). Specific amylase activity was expressed as μmol maltose released/min/g protein.

Lipase activity was assessed using the titrimetric method described by Cherry and Crandall (1932), where triglyceride hydrolysis in a stabilized olive oil emulsion leads to the release of fatty acids, which were titrated against standard NaOH solution. The amount of NaOH consumed was considered as an index of lipase activity. Specific lipase activity was expressed as units/min/mg protein.

After checking the homogeneity of variance of the residual plots, the data were subjected to one-way analysis of variance (ANOVA) using IBM SPSS statistics (version 22) for Windows. Duncan's multiple range test (DMRT) with post hoc was used to determine differences among the analysed mean at a 5% probability level ($p < 0.05$). The analysed data were expressed as mean ± standard error.

Results and Discussion

The moisture, crude protein, ether extract, crude fibre, total ash, and NFE contents of the experimental feeds (Table 1) ranged from 6.20 to 6.91%, 30.20 to 30.35%, 6.01 to 6.06%, 7.03 to 7.33%, 7.15 to 7.19%, and 42.34 to 43.25%, respectively in C to T4 groups. The corresponding values of SFMLM were 5.45%, 30.40%, 5.17%, 7.9%, 7.05%, and 44.04%, respectively in C to T4 groups.

The ANFs such as tannin, phytic acid, and saponins were analysed for both the mixed leaf meal and fermented mixed leaf meal (Table 2). After fermentation, the levels of tannin, saponin, and phytic acid were reduced to 82.25%, 93.17%, and 86.43%, respectively

Dietary inclusion of SFMLM significantly ($p < 0.05$) enhanced the growth performance (Table 3) of *L. rohita* fingerlings. The group fed with 20% SFMLM (T2) exhibited significantly higher ($p < 0.05$) WG, SGR, and PER followed by the SFMLM10 group and the lowest growth ($p < 0.05$) was observed in the control. However, the T4 group had significantly ($p < 0.05$) lower growth compared to the control group. FCR was significantly ($p < 0.05$) lower in the SFMLM20 followed by SFMLM10.

Digestive enzyme activities were analysed at the end of 60-day experiment. Significant variation ($p < 0.05$) was observed in the current experiment, which are represented in Fig. 1, 2 and 3. Protease activity was significantly higher ($p < 0.05$) in the SFMLM10 and SFMLM20 groups compared to the control, whereas the lower protease activity was noted in the

Table 1. Composition of the different experimental diets

Ingredients	Control	T1	T2	T3	T4
Soybean meal	25	23	22	20	18.5
GNOC	20	17	14.5	12	8.5
MOC	10	9	7	5	4
SFLM[1]	0	10	20	30	40
Wheat flour	12.48	13.48	13.98	13.48	12.48
DORB	25	20	15	12	9
Veg oil	2	2	2	2	2
Fish oil	2	2	2	2	2
[2]Vitamin Mineral premix	2	2	2	2	2
BHT	0.02	0.02	0.02	0.02	0.02
Choline chloride	0.5	0.5	0.5	0.5	0.5
CMC	1	1	1	1	1
Proximate analysis of the experimental diets					
Moisture	6.41	6.20	6.85	6.91	6.62
CP	30.26	30.23	30.35	30.20	30.25
Ether extract	6.01	6.02	6.05	6.05	6.06
Crude fibre	7.03	7.15	7.24	7.25	7.33
Total ash	7.19	7.15	7.17	7.18	7.17
NFE	43.10	43.25	42.34	42.41	42.57
Digestible energy (Kcal/100g)	347.54	348.12	345.22	344.89	345.82

[1]SFLM, Solid state fermented mixed leaf meal

[2]Composition of vitamin mineral mix (PREMIX PLUS) (quantity/2.5 kg) Vitamin A, 55,00,000 IU; Vitamin D3, 11,00,000 IU; Vitamin B2, 2,000 mg; Vitamin E, 750 mg; Vitamin K, 1,000 mg; Vitamin B6, 1,000 mg; Vitamin B12, 6mcg; Calcium Pantothenate, 2,500 mg; Nicotinamide, 10 g; Choline Chloride, 150 g; Mn, 27,000 mg; I, 1,000 mg; Fe, 7,500 mg; Zn, 5,000 mg; Cu, 2,000 mg; Co, 450 mg; L- lysine, 10 g; DL- Methionine, 10 g; Selenium, 50 ppm; Satwari, 2500 mg; Stay C (Hoffman La Roche, Nutley, NJ, USA) 15% ascorbic acid activity. HiMedia laboratories, Mumbai, India. Alginic acid was supplied by Fisher scientific, Across, Mumbai branch of India

C (0% SFMLM), T1 (10% SFMLM), T2 (20% SFMLM), T3 (30% SFMLM), T4 (40% SFMLM) in the replacement of protein ingredients were prepared.

Table 2. The anti-nutritional factors of mixed leaf meal and fermented mixed leaf meals

Variables	MLM	SFMLM	% reduction
Phytic acid (mg $100g^{-1}$)	10.47±0.31	1.42±0.01	86.43
Tannin (mg $100g^{-1}$)	25.76±0.12	4.57±0.13	82.25
Saponin (mg g^{-1})	19.77±0.03	1.35±0.29	93.17

MLM: Mixed leaf meal; SFMLM: Solid-state fermented mixed leaf meal. Differences were considered significant at 95% level ($p < 0.05$).

Table 3. Growth and nutrient utilisation of *Labeo rohita* fingerlings fed with different experimental diets for a period of 60 days

Treatments	Initial Wt. (in gm)	Final Wt. (in gm)	WG (in gm)	SGR (%/day)	FCR	PER
C	5.22±0.001	11.86^{b}±0.032	6.64^{b}±0.032	1.37^{b}±0.004	1.81^{c}±0.009	0.22^{b}±0.001
T1	5.22±0.003	12.42^{c}±0.12	7.20^{c}±0.12	1.44^{c}±0.015	1.67^{b}±0.027	0.24^{c}±0.004
T2	5.22±0.007	13.07^{d}±0.27	7.85^{d}±0.27	1.53^{d}±0.033	1.53^{a}±0.051	0.26^{d}±0.01
T3	5.22±0.003	11.77^{b}±0.18	6.55^{b}±0.18	1.36^{b}±0.026	1.83^{c}±0.05	0.21^{b}±0.006
T4	5.22±0.001	10.53^{a}±0.02	5.30^{a}±0.02	1.17^{a}±0.003	2.26^{d}±0.007	0.17^{a}±0.0006

C (0% SFMLM), T1 (10% SFMLM), T2 (20% SFMLM), T3 (30% SFMLM), T4 (40% SFMLM) in the replacement of protein ingredients were prepared.

WG-Weight gain; SGR-Specific Growth Rate; FCR-Feed Conversion Ratio; PER-Protein Efficiency Ratio

Values with different superscript letters within the same column denotes significant difference ($p < 0.05$)

SFMLM40 group. The control and SFMLM30 groups showed similar activity of this enzyme. Amylase and lipase activities also varied significantly ($p < 0.05$) across different groups, following a similar trend to protease activity.

In recent years, the inclusion of plant leaves in carp feed production has been encouraged, with several researchers reporting the use of leaves in the feed as a replacement for either de-oiled rice bran, mustard oil cake, or soybean meal. Various studies have explored the use of *Eichhornia* leaf meal in *Cyprinus carpio* (Makkar et al., 2007) and *Pisum sativum* leaf meal in *L. rohita* (Nottanalan et al., 2021),

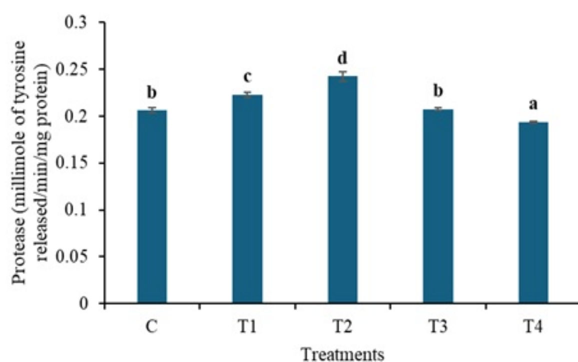

Fig. 1. Protease activity (millimole of tyrosine released/ min/mg protein) of *Labeo rohita* fingerlings fed with 30% protein diet for 60 day

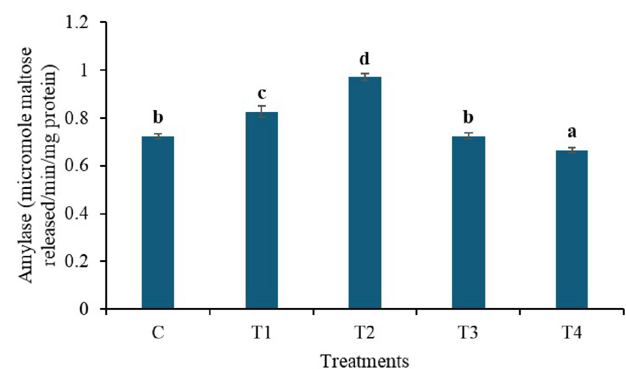

Fig. 2. Amylase activity (micromole maltose released/ min/mg protein) of *Labeo rohita* fingerlings fed with a 30% protein diet for 60 days.

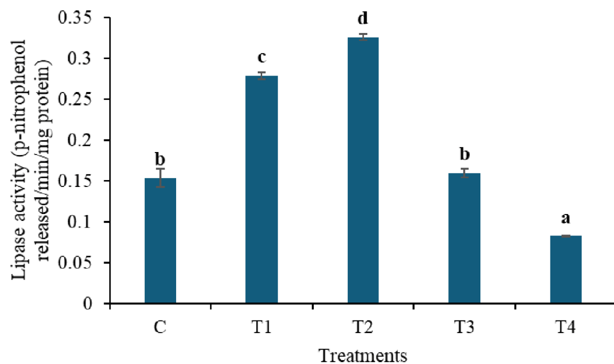

Fig. 3. Lipase activity (p-nitrophenol released/min/mg protein) of *Labeo rohita* fingerlings fed with a 30% protein diet for 60 days.

demonstrating that the inclusion of leaf meal in fish diet had accomplished a good outcome. Similarly, the *Hygrophilla spinosa* leaf meal (Maiti et al., 2019) and *S. aculeata* leaf meal (Anand et al., 2020) have been incorporated into the diets of *L. rohita.* Furthermore, sweet potato leaf meal (Meshram et al., 2018) and *Moringa oleifera* leaf meal (Manna et al., 2024) have also been used successfully in rohu diets. However, several studies have reported that the utilisation of leaf meals is often limited by the elevated levels of ANFs and crude fibres present in these materials. A potential strategy to overcome these limitations involves mixing and fermenting different leaf meals to achieve more balanced nutrient profile and reduced fibre content and specific ANFs (Shi et al., 2020; Mondal et al., 2022).

In the present study, the solid-state fermentation resulted in an increase in the crude protein level and a reduction in ANFs and crude fibre content in the leaf meal. These changes indicate that the microbial mixtures employed in the fermentation process may have utilised the crude fibre present in the mixed leaves, thereby increasing the protein content of the resulting SFMLM. Similar results have been reported in previous studies (Bairagi et al., 2004; Manna et al., 2024) that dealt with fermentation of mixed leaves. The phytate, tannin, and saponin contents present in the mixed leaf were reduced following fermentation due to microbial action, suggesting that the microbes utilised the fibre and breakdown ANFs (Manna et al., 2024).

The incorporation of the SFMLM into the diet had a notable impact on the growth performance of *L. rohita*, with the 20% fermented mixed leaf meal demonstrating superior growth compared to other treatments. This indicates that the increased protein level in SFMLM had supported the observed increased growth and nutrient utilisation in fish. Anand et al. (2020) reported that fermented *Sesbania* leaf meal (FSLM) with *B. subtilis* could substitute 50% DORB (15% FSLM group) in *C. carpio* diets. Similarly, Malik et al. (2024) observed a significantly improved growth rate in *C. carpio* when fed with 15% fermented *E. crassipes* leaf meal. The findings of this study are corroborated by the results of previous studies conducted by Anand et al. (2020) and Shi et al. (2020).

Feeding diets containing SFMLM also influenced digestive enzyme activities such as protease, amylase and lipase, which was highest in the T2 group indicating enhanced nutrient digestion at an inclusion level of 20% SFMLM. However, further enhancement in the inclusion of SFMLM resulted in a decline in enzyme activities. These results indicates that *L. rohita* can utilise fermented meal up to 20% level. At higher levels, leaf meals may prove toxic to fish (Mukhopadhyay & Ray, 1999).

In conclusion, the use of fermented mixed leaf meal in *L. rohita* diets offers a promising approach to reducing feed costs and improving fish performance. However, further studies are required to optimise inclusion levels and to evaluate the long-term effects of SFMLM on fish health and productivity.

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Ethical Statement

The feeding trial was conducted as per the ethical guidelines of ICAR-Central Institute of Fisheries Education (ICAR-CIFE), Mumbai, India. The harm caused to the fish during the experiment was intentional and exclusively for research purposes.

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