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Mixed leaf meal supplemented with exogenous enzyme and limiting amino acids can completely replace DORB (de-oiled rice bran) in the diet of *Labeo rohita*

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Abstract – Various single leaf meal can substitute costly de-oiled rice bran (DORB) in farm made feed for carps. However, the use of mixed leaf meal (LM) in fish feed is not reported yet. Therefore, *Vigna mungo, Ipomoea aquatica* and *Hygrophila spinosa* leaf meals were blended in an equal proportion to prepare LM. DORB was the major energy source in control diet (C) and LM substituted 50% and 100% of DORB in LM20 and LM40 experimental diets, respectively. In addition, control diet (C), LM20 and LM40 diets were supplemented with limiting amino acids and exogenous carbohydrases and denoted as CE, LM20E and LM40E, respectively. *Labeo rohita* fingerlings were fed till satiation for 60 days. Fish fed with LM20E diet exhibited maximum growth rates and feed conversion ($p < 0.05$) however these were similar in C and LM40 ($p > 0.05$). Amylase and aminotransferase activities were positively related with growth indices ($p < 0.05$). Difference in hematological indices was negligible (except total leukocyte count), and lactate dehydrogenase activities in DORB and LM-based fed groups ($p > 0.05$) while lower superoxide dismutase activities was observed in LM fed groups ($p < 0.05$). The present study concluded that LM up to 400 g kg^{-1} could be incorporated as complete DORB replacer in *L. rohita* diet, however, 200 g kg^{-1} LM supplemented with 0.98 g L-lysine kg^{-1} and 1 g exogenous enzyme kg^{-1} registered the best growth, nutrient utilization, feed conversion ratio, physio-metabolic responses and hematological status.

Keywords: De-oiled rice bran / mixed leaf meal / exogenous enzyme / growth / digestive and metabolic enzyme activities / hematological status

1 Introduction

Asia contributes a significant share in global aquaculture production, where major production comes from Indian major carps (IMCs). Among IMCs, *Labeo rohita* (rohu), is the leading cultivated species and its culture is mostly reliant on the farm-made feed is prepared by mixing either one of different oil cakes (mustard, groundnut, cotton seed, copra, soybean etc.) with de-oiled rice bran (DORB) in different proportions depending upon the availability in local market (Ramakrishna et al., 2013). DORB is considered as the easily available and cheapest ingredient in India, therefore mostly used in fish feed as an energy source (Tsvetanov and Duneva, 1990). Around 87% fish farmer in India use DORB as major feed ingredient (>70% inclusion) in farm-made feed (Ramakrishna et al., 2013). However, recent studies proved that 330-400 g kg^{-1} DORB inclusions exhibited better growth, nutrient utilization concomitant with up-regulated expression of growth genes (insulin like growth factor I, IGF-I and IGF-II) (Kumar et al., 2017, 2018a). Whereas, higher DORB inclusion (500-600 g kg^{-1}) lowered the growth and feed conversion in *L. rohita* (Kumar et al., 2018b). Moreover, intervention to improve the quality of DORB also attempted and found that supplementation of exogenous enzymes (phytase and xylanase) and deficient amino acids (L-lysine and DL/L-methionine) improved the growth indices in *L. rohita* (Ranjan et al., 2017). It has been noted that DORB is not only locally available but also well accepted by carps and thus final feed cost can be lowered by incorporating the optimal DORB inclusion (Meshram et al., 2018; Ramakrishna et al., 2013). However

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in recent years, the cost of DORB is increasing gradually due to its high demand in livestock and fish feed. In addition, the rice production across the Asian region is stagnating. Hence, an alternate to DORB is of paramount importance to ensure sustainable carp production. However, plant-based low cost ingredients will be a potential replacer for DORB.

Leaf meals are gaining a serious attention these days due to its abundant availability throughout the year in the tropics and many have higher protein content than the rice bran. Though seed proteins were considered as a potential ingredients in aquafeed, in comparison to seed proteins, harvest of leaf proteins from appropriate plant species are almost four times higher (Santamaría-Fernández and Lübeck, 2020). Thus, the leaf meal can be utilized as replacer for DORB in carp diet. Currently, most of the agriculture plants after harvest are dumped in the field, burnt or used for manuring. So, if meal is prepared out of such leaves, can reduce the high cost of fish feed as it is comparatively cheaper sources of protein. Some recent studies reported that *Hygrophila spinosa,* a terrestrial weed with common name *Gokulkanta* (Maiti et al., 2019), sweet potato (Meshram et al., 2018; Ahmad et al., 2019), black gram, *Vigna mungo* (Sahoo et al., 2020) and green pea, *Pisum sativum* (Nottanalan et al., 2021) leaf meal could completely replace DORB with 30% inclusion level in the carp (*L. rohita*) feed without compromising the growth performances of fish. But according to the Anand et al. (2020), fermented (*Bacillus subtilis*) sesbania leaf meal can replace only 50% DORB with 15% incorporation level in common carp diet without compromising the growth, IGF-I expression and physiological responses of fish probably due to presence of high level of antinutritional factors (ANFs) like tannin, saponin and phytic acid. ANFs affect the digestion and metabolism process and hinders the nutrient bioavailability to the fish and thus retards the growth (Acamovic and Brooker, 2005; Glencross et al., 2007; Hansen and Hemre, 2013).

Hence, suitable processing methods are prerequisite criteria for detoxification of plant-based ingredients prior to use in fish feed (Krogdahl et al., 2010). Simultaneously, the availability of a single leaf may not possible in a specific area over a time; but option of using mixed leaves is definitely a better option to ensure their year-round availability. Moreover, it offers the advantage of balancing the limiting amino acids and diluting the ANFs due to mixing of different leaves instead of an individual one.

Another major constraint in using leaf meals is because of higher amount of indigestible fibre present that can hinder the digestion processes in fishes. Therefore, there is a requisite to incorporate the fibre digesting enzyme in aquafeed. Exogenous cellulase and xylanase supplementation in plant-based diet can improve the nutrient utilization and growth in carps (Ranjan et al., 2017, 2018b). Similarly, plant-based ingredients have deficiency of essential amino acids (EAAs) like lysine and methionine thus needs to be supplemented (Sardar et al., 2007; Ranjan et al., 2017). Mixing of leaves to some extant may ensure balancing of amino acids and compensating deficient amino acid in one meal with the other. Hence in the present study, three leaves, viz., *V. mungo* (mung bean), *Ipomoea aquatica* (water spinach) and *H. spinosa* (gokulkanta) were mixed in equal proportion.

V. mungo (mung bean) is an important protein rich (lysine) pulse crop of Fabaceae family and annual production was 1.93–2.03 million tonnes (2020) in India (Jansen, 2006; Achakzai et al., 2012; DARE, 2021). *I. aquatica* (water spinach) is an emergent aquatic plant of Convolvulaceae family and estimated annual production was 0.08 million tonnes (2020) in India (Prasad et al., 2008; DARE, 2021). *H. spinosa* (gokulkanta) is herbaceous, medicinal plant in the Acanthaceae family with estimated annual production was 0.01 million tonnes (2020) in India (Shakya, 2020; DARE, 2021). These leaves are generally used as fodder for ruminants or treated as waste after harvesting. In this context, utilization of this large waste biomass for aquafeed formulation by replacing costly ingredients can be an alternative waste to wealth approach. Earlier studies indicated that the optimum inclusion levels of *V. mungo* (black gram or mung bean), *I. aquatica* (water spinach), and *H. spinosa* (gokulkanta) in *L. rohita* fingerlings diet are 300, 200 and 200 g kg^{-1}, respectively (Sahoo et al., 2020; Gurung, 2018; Maiti et al., 2019). However, no information is available regarding the use of mixed leaf meals in aquafeed. Therefore, the study was intended to substitute DORB with optimum level of mixed leaf meal, with or without supplementation of exogenous enzyme and synthetic amino acids in *L. rohita* diet in terms of nutrient utilization, growth and physio-metabolic responses.

2 2 Materials and methods

2.1 Collection of selected leaves and mixed leaf meal (LM) preparation

The selected leaves were collected from Kharagpur, West Bengal, India (22.346° N, 87.232° E), washed thoroughly in water, dried in sunlight and oven (42 °C for overnight). The leaves were milled, sieved with 250 μm mesh and mixed thoroughly at 1:1:1 ratio to obtain mixed leaf meal (LM).

2.2 Experimental design and preparation of experimental diets

Six iso-nitrogenous (300 g kg^{-1} crude protein), and iso-caloric (15.8 MJ digestible energy kg^{-1}) experimental diets were prepared (Table 1). The diets were C (400 g kg^{-1} DORB and 0 g kg^{-1} LM), LM20 (200 g kg^{-1} DORB and 200 g kg^{-1} LM), LM40 (0 g kg^{-1} DORB and 400 g kg^{-1} LM), CE (C with 1 g kg^{-1} cocktail enzyme mixture), LM20E (LM20 with 1 g kg^{-1} cocktail enzyme mixture) and LM40E (LM40 with 1 g kg^{-1} cocktail enzyme mixture). The CE diet was supplemented with 1.4 g kg^{-1} DL-methionine and 1.4 g kg^{-1} L-lysine while LM20E and LM40E were supplemented with 0.98 g kg^{-1} L-lysine and 1.5 g kg^{-1} L-lysine, respectively to fulfill the requirement of *L. rohita* (FAO, 2017). According to the formula, the dried ingredients were accurately weighed and homogenously mixed, steam cooked, cooled, mixed with oils and additives and then pelletized with mechanical pelletizer. The prepared pellets (1.5 mm diameter) were oven dried at 40 °C, packed airtight in a zipper bag with proper labeling and stored in a clean dry place until use.

2.3 Proximate analysis

Proximate analysis was done according to AOAC (1995). The sample was oven dried at 100 ± 2 °C until achieving a persistent weight. Crude protein (CP) was estimated using kjeltech (Pelican, India) method, where total nitrogen (TN) was estimated followed by multiplication of TN value with 6.25 to get the value of CP. Crude lipid was estimated by using

Table 1. Formulation, proximate composition and anti-nutritional factors of experimental diets

Ingredients (g kg^{-1})	[1]Experimental diets					
	C	LM20	LM40	CE*	LM20E*	LM40E*
De-fatted soybean meal	195.00	157.50	120.00	200.00	161.00	125.00
Groundnut oil cake	300.00	300.00	300.00	300.00	300.00	300.00
Wheat flour	23.80	61.30	98.80	15.00	55.82	91.30
De-oiled rice bran	400.00	200.00	0.00	400.00	200.00	0.00
Mixed leaf meal	0.00	200.00	400.00	0.00	200.00	400.00
Sunflower oil	20.00	20.00	20.00	20.00	20.00	20.00
Fish oil	20.00	20.00	20.00	20.00	20.00	20.00
Vitamin-mineral mix[2]	20.00	20.00	20.00	20.00	20.00	20.00
Betaine	5.00	5.00	5.00	5.00	5.00	5.00
Choline chloride	1.00	1.00	1.00	1.00	1.00	1.00
Carboxymethyl cellulose	15.00	15.00	15.00	15.00	15.00	15.00
Butylated hydroxytoluene	0.20	0.20	0.20	0.20	0.20	0.20
Exogenous enzyme[3]	0.00	0.00	0.00	1.00	1.00	1.00
DL-Methionine	0.00	0.00	0.00	1.40	0.00	0.00
L-Lysine	0.00	0.00	0.00	1.40	0.98	1.50
Total	1,000	1,000	1,000	1,000	1,000	1,000
Chromium oxide (Cr_2O_3)	5.00	5.00	5.00	5.00	5.00	5.00
Proximate composition (g kg^{-1} dry matter basis)						
Dry matter	911.8	911.5	913.0	909.0	908.0	905.7
Crude protein	301.0	301.0	301.2	302.1	301.7	301.30
Ether extract	58.00	59.00	59.60	58.30	61.00	59.20
Crude fiber	51.00	58.00	64.00	33.0	41.2	53.10
Nitrogen free extract	515.3	504.6	497.3	530.2	517.9	511.5
Total ash	74.70	77.40	77.90	76.40	78.20	75.00
GE[4] (MJ kg^{-1})	17.21	17.42	17.62	17.00	17.24	17.44
DE[5] (MJ kg^{-1})	15.83	15.68	15.59	16.10	15.99	15.81
P: E[6] (g protein MJ DE^{-1})	18.95	19.17	19.31	18.75	18.85	19.03
Anti-nutritional factors						
Total tannins (g 100g^{-1})	0.07	0.22	0.32	0.06	0.23	0.31
Alkaloid (g 100g^{-1})	0.11	0.24	0.32	0.11	0.25	0.33
Total oxalate (g 100 g^{-1})	0.06	0.13	0.19	0.07	0.13	0.20
Phytic acid (mg 100 g^{-1})	0.05	1.83	2.81	0.05	1.82	2.80

[1] Data (proximate composition and anti-nutritional factors) are expressed as mean values of triplicate. Please shift this 1 to next line like 1 C, Control with 0 g kg–1 mixed leave meal (LM) and……

C, Control with 0 g kg^{-1} mixed leave meal (LM) and 400 g kg^{-1} de-oiled rice bran (DORB); LM20, 200 g kg^{-1} LM in replacement of 50% DORB; LM40, 400 g kg^{-1} LM in replacement of 100% DORB; CE, Control diet supplemented with 1 g kg^{-1} xylanase and cellulase enzyme mixture; LM20E, LM20 diet supplemented with 1 g kg^{-1} xylanase and cellulase enzyme mixture; LM40E, LM40 diet supplemented with 1 g kg^{-1} xylanase and cellulase enzyme mixture.

* CE, LM20E and LM40E were supplemented with 1.4 g kg^{-1} DL-methionine and 1.4 g kg^{-1} L-lysine, 0.98 g kg^{-1} L-lysine and 1.5 g kg^{-1} L-lysine, respectively to fulfill the essential amino acid requirements of *L. rohita* (FAO, 2017).

[2] Composition of vitamin-mineral mix (AGRIMIN FORTE) (quantity kg^{-1}): vitamin A, 7,00,000 IU; vitamin D_3, 70,000 IU; vitamin E, 250 mg; nicotinamide, 1000 mg; cobalt, 150 mg; copper, 1200 mg; iodine, 325 mg; iron, 1500 mg; magnesium, 6000 mg; manganese, 1500 mg; potassium, 100 mg; sodium, 5.9 mg; selenium, 7200 mg; zinc, 9600 mg; calcium, 255 g; phosphorus, 127.5 g.

[3] Exogenous enzyme from *Trichoderma reesei* (Cellulase-10000 ECU/g; Xylanase- 350000 BXU/g), AB Vista, Wilshire, UK.

[5] GE, gross energy; [5]DE, digestible energy; [6]P: E, protein to energy ratio.

soxhlet's unit (Pelican, India). Total ash content was analysed by burning the samples in muffle furnace (Wilser & Tetlew, India) at 550 ° C for 5h. Crude fibre (CF) content was analyzed in Fibertech (Pelican, India) followed by oven drying at 100 ± 2 ° C and burning at 550 ° C for 5h. Nitrogen free extract (NFE) of ingredients, leaves and diets wascalculated by the following formula.

$$\text{NFE}\,(\text{g kg}^{-1}) = [1000 - (\text{g CP kg}^{-1} + \text{g EE kg}^{-1} + \text{g CF kg}^{-1} + \text{g TA kg}^{-1})]$$

As per manufacture's protocol, gross energy (GE) of ingredients and the experimental diets was estimated by a Bomb calorimeter (Changhsu Instruments, China). Digestible energy (DE) and protein to energy ratio (P: E) were calculated according to Halver (1976).

2.4 Anti-nutritional factors (ANFs)

The standard methods were employed for documentation of anti-nutritional factors (ANFs) of the leaf meals and experimental diets. Phytic acid and tannin were estimated spectro-photometrically (Shimadzu, UV1800, Kyoto, Japan) as per the procedure of Vaintraub and Lapteva (1988) and Makkar et al. (2007), respectively. Titrimetric method of Day and Underwood (1986) was used for the determination of oxalate content and calculation was done by considering 2.20 mg of oxalate corresponding to 1 ml 0.05 M $KMnO_4$ (Chinma and Igyor, 2007). The alkaloid content of the samples was determined gravimetrically (Harborne and Green, 1980).

2.5 Amino acid analysis

The diets and mixed leaf meal (LM) samples were hydrolyzed (6 M HCl) for 24 h maintaining a temperature 110 °C, and HPLC with fluorescence detector (Ishida et al., 1981) was used to determine the amino acid composition. Before acid hydrolysis, the samples were subjected to formic acid for oxidizing the sulphur-containing amino acid.

2.6 In vitro protein digestibility

Protein digestibility of leaf meal (LM) was determined following the pH drop method (Ali et al., 2009).

2.7 Experimental set-up and feeding trial

One thousand Rohu, *L. rohita* fingerlings (mean weight 2.84 ± 0.07 g) were transported in well-oxygenated bags from fish seed unit, Ankleshwar, to fish nutrition laboratory of ICAR-CIFE, India and reared in 2000 L rectangular fiber tanks with adequate aeration facility for three weeks and fed with control diet during acclimatization. Two hundred and sixteen fingerlings of *L. rohita* (mean weight 3.41 ± 0.01 g) were distributed in 18 rectangular tubs of 180 L (six treatments with triplicate) following completely randomized design. Fish were fed twice daily (09:30 and 16:30 h) to satiation level for 60 days. The siphoning of faecal matter was done in two days interval and equal volume of water was added. The fish body weight measurement was done at 0 days, 15 days intervals and after finishing of trial following overnight feed restriction to record the body weight.

Water quality parameters were routinely monitored by APHA (2005) methods. Water quality parameters viz. temperature, pH, dissolved oxygen (DO), total hardness, ammonia-N and nitrite-Nin the different experimental tanks were found to be within the range of 28.5–32.9°C, 7.2–8.2, 6.0–7.9 mg L^{-1}, 231–242 mg L^{-1}, 0.04–0.1 mg L^{-1} and 0.04–0.09 mg L^{-1}, respectively. Free carbon dioxide (CO_2) was not detectable in any tub during trial period.

2.8 Growth performance, body indices and survival

Fish growth parameters like WG%, SGR, FCR, PER and body indices HSI, ISI and survival were evaluated according to Talukdar et al. (2021) (supplementary file).

2.9 Digestibility studies

A 20 days trial for digestibility was conducted with the inclusion of 5 g kg^{-1} chromic oxide (Cr_2O_3) as external marker in experimental diets (Sardar et al., 2007). The experimental fishes were fed to adlibitum level daily at once at 10:00 h. Faecal matters were collected through siphoning from the bottom of each tub after a gap of 6 h from the time of feeding and then oven dried. The dried faecal matter sample was pooled for each treatment and both faecal and diet samples were analysed for crude protein and ether extract according AOAC (1995). The method of Furukawa (1966) was used to quantify Cr_2O_3 content of diets and faecal matter. The calculations of various co-efficients were done as per the following formulae given by Cho (1979) (supplementary file).

2.10 Collection and processing of samples

Three fish were randomly selected from each tub, anaesthetized with clove oil (50 mL/L) (Jana et al., 2018) and dissected to collect the tissue samples (gill, liver, intestine and muscle). The tissues were homogenized (MICCRA D-9, Germany) in 0.25M sucrose solution at ice-cold conditions to make 5% tissue homogenate. We centrifuged the homogenate at 5000 rpm for 10 min at 4 °C, collected the supernatant carefully, and stored at -80 °C until used for enzyme assays (Jana et al., 2021a).

Another three fishes were randomly collected and anaesthetized and blood was collected by puncturing the caudal vein with the help of EDTA (2.7 g 100 mL^{-1} solution) rinsed hypodermic medical syringe and poured into EDTA coated vials. It was stirred well to avoid blood hemolysis and used immediately for hematological analysis. Similarly, blood was collected and poured into eppendorf tubes (without EDTA coating) and kept for 45 min for blood coagulation. It was centrifuged at 6000 rpm for 15 min at 4 °C followed by collection and storage of yellow straw color serum at –80 °C until analysis. Standard method described by Lowry (Lowry et al., 1951) was used for the protein quantification of the tissue samples.

2.11 Enzyme assays

2.11.1 Digestive enzyme assay

Casein digestion method of Drapeau (1974) was employed for the assay of protease activity and amylase activity was estimated according to Rick and Stegbauer (1974) (di-nitrosalicylic-acid method) using maltose as standard. Lipase activity was assessed using titrimetric method (Cherry and Crandall, 1932).

2.11.2 Metabolic enzyme assay

Protein metabolic enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were assayed according to Wooten (1964). The activity of carbohydrate metabolic enzyme, lactate dehydrogenase (LDH) was determined by method of Wróblewski and Ladue (1955).

Table 2. Chemical composition and anti-nutritional factors of different leaf meal and mixed leaf meal (LM).

Chemical composition	*Vigna mungo*	*Ipomoea aquatica*	*Hygrophila spinosa*	Mixed leaf meal	DORB
Proximate composition (g kg^{-1} dry matter basis)					
Dry matter	917.1 ± 0.08	937.9 ± 0.16	923.8 ± 0.08	942.6 ± 0.17	938.2 ± 0.10
Crude protein	238.5 ± 0.17	231.2 ± 0.12	221.9 ± 0.15	235.4 ± 0.17	160.0 ± 0.20
Ether extract	24.3 ± 0.03	21.4 ± 0.09	20.9 ± 0.12	23.0 ± 0.07	12.0 ± 0.90
Crude fibre	160.2 ± 0.05	162.1 ± 0.11	166.0 ± 0.08	162.7 ± 0.29	137.3 ± 0.26
Nitrogen free extract	456.2 ± 0.06	454.1 ± 0.06	449.2 ± 0.04	441.5 ± 0.22	500.2 ± 0.28
Total ash	120.8 ± 0.04	131.2 ± 0.13	142.0 ± ± 0.04	137.4 ± 0.09	122.0 ± 0.27
Anti-nutritional factors and IVCPD[1]					
Total tannins (g $100g^{-1}$)	0.36	0.94	0.49	0.52	0.34
Alkaloid (g $100g^{-1}$)	0.31	0.73	1.82	0.68	0.23
Total oxalate (g $100g^{-1}$)	0.005	0.83	0.41	0.39	0.008
Phytic acid (mg $100g^{-1}$)	0.06	4.63	5.81	3.43	2.69
IVCPD[1] (%)	–	–	–	28.42	29.64

Data are expressed as mean ± SEM (standard error of mean).
[1] IVCPD, *in vitro* crude protein digestibility.

2.11.3 Activities of antioxidant or oxidative stress enzymes

For assay of catalase (CAT) and superoxide dismutase (SOD), standard methods of Takahara et al. (1960) and Misra and Fridovich (1972) were employed.

2.12 Haemato-biochemical and immunological parameters

2.12.1. Haemato-biochemical indices

Serum total protein (g dL^{-1}) and albumin (g dL^{-1}) levels were analyzed by using respective test kit (ERBA Diagnostic, India). Serum globulin and albumin to globulin ratio (A: G) were calculated by the standard equations (supplementary file).

2.12.2 Haematological assay

Haemoglobin (Hb) content of blood was measured by cyanmethemoglobin method (Van Kampen and Zijlstra, 1961). While, total erythrocytes count (TEC) and total leucocyte count (TLC) were determined using methods given by Hendricks (1952) and Shaw (1930), respectively.

2.12.3 NBT assay

The assessment of nitrobluetetrazolium (NBT) or the respiratory burst activity was done using standard method (Stasiak and Baumann, 1996).

2.13 Statistical analysis

Data was subjected to one-way ANOVA using SPSS software to estimate the mean values of treatments after checking the normality and homogeneity of variance (Levene's test). Post hoc analysis along with Duncan's multiple range test (DMRT) was set to observe the significant differences among the means ($p < 0.05$).

3 Results

3.1 Proximate composition, anti-nutritional factors and amino acid composition

The crude protein content of *V. mungo, I. aquatica*, *H. spinosa* and mixed leaf meal (LM) were 238.5, 231.2, 221.2 and 235.4 g kg^{-1}, respectively which was around 7.5% higher than that of DORB. However, the crude fibre content was found 160.2, 162.1, 166.0 and 167.7 g kg^{-1} which were about 3% higher than DORB (Table 2).

There is a difference in quantity of the anti-nutrients such as total tannins, alkaloids, total oxalate and phytic acid in different leaves. The mixing of these three leaves at 1:1:1 ratio diluted the total tannins, alkaloids, total oxalate and phytic acid contents by 44.68, 62.64, 53.01 and 40.96%, respectively. Moreover, in vitro protein digestibility of LM for *L. rohita* fingerlings was found to be 28.42% (Table 2). The essential (EAAs) and non-essential amino acids (NEEAs) contents of LM were presented in Table 3.

3.2 Nutrient utilization, growth, survival, body indices and apparent digestibility coefficients (ADCs) of dietary nutrients

Fish fed with LM20 diet exhibited higher ($p < 0.05$) growth rates (specific growth rate, SGR, weight gain percentage, WG%, and protein efficiency ratio, PER) than control and LM40 fed groups. Dietary supplementation of exogenous xylanase and cellulase and limiting amino acids significantly ($p < 0.05$) improved the nutrient utilization and growth performance in the experimental fish. Maximum ($p < 0.05$) growth rates and lowest ($p < 0.05$) feed conversion ratio (FCR) was observed in LM20E fed group. However, LM20 and CE groups exhibited with similar ($p > 0.05$) FCR value. There was no death of experimental fish during the trial period (Table 4).

Table 3. Amino acid composition of mixed leaf meal (g kg^{-1} protein) and different experimental diets (g/kg feed) fed to *Labeo rohita* fingerlings for the period of 60 days.

Amino acids	Mixed leaf meal (g kg^{-1} protein)	DORB (g kg^{-1} protein) (Ranjan et al., 2018b)	C	LM20	LM40	CE	LM20E	LM40E	*R (g/kg diet)
Essential amino acids									
Arginine	133.6	128.2	2.31	2.31	2.29	2.3	2.3	2.31	2.3
Histidine	28.9	26.5	0.9	0.91	0.89	0.9	0.91	0.9	0.9
Isoleucine	48.2	44.5	1.22	1.2	1.21	1.21	1.2	1.2	1.2
Lysine	35.3	28.1	0.87	1.29	0.77	2.27	2.28	2.27	2.27
Leucine	81.8	87	1.5	1.51	1.51	1.49	1.5	1.5	1.5
Methionine	46	21.7	1.28	1.42	1.42	1.42	1.42	1.42	1.42
Phenylalanine	53	56.7	1.47	1.48	1.48	149	1.48	1.48	1.48
Threonine	61.7	69.2	1.71	1.7	1.72	1.71	1.71	1.7	1.71
Valine	59.1	57.5	3.76	3.75	3.75	3.75	3.74	3.75	3.75
Non-essential amino acids									
Alanine	53	91.2	–	–	–	–	–	–	–
Glycine	151.3	90.5	–	–	–	–	–	–	–
Aspartic acid	25.4	146.3	–	–	–	–	–	–	–
Glutamic acid	26.4	82.7	–	–	–	–	–	–	–
Serine	28	40.7	–	–	–	–	–	–	–
Tyrosine	47.1	28.5	–	–	–	–	–	–	–

[1] C, Control with 0 g kg^{-1} mixed leave meal (LM) and 400 g kg^{-1} de-oiled rice bran (DORB); LM20, 200 g kg^{-1} LM in replacement of 50% DORB; LM40, 400 g kg^{-1} LM in replacement of 100% DORB; CE, Control diet supplemented with 1 g kg^{-1} xylanase and cellulase enzyme mixture; LM20E, LM20 diet supplemented with 1 g kg^{-1} xylanase and cellulase enzyme mixture; LM40E, LM40 diet supplemented with 1 g kg^{-1} xylanase and cellulase enzyme mixture.
* R-Amino acid requirement of Rohu (FAO, 2017).

Table 4. Growth, nutrient utilization, survival, body indices and apparent digestibility coefficients of *Labeo rohita* fingerlings fed with different experimental diets for the period of 60 days.

Experimental group	C	LM20	LM40	CE	LM20E	LM40E	*p*- value
Fn. wt.[1](g)	$5.57^{a} \pm 0.04$	$5.93^{bc} \pm 0.05$	$5.59^{a} \pm 0.07$	$5.77^{b} \pm 0.05$	$6.40^{d} \pm 0.08$	$6.05^{c} \pm 0.04$	<0.001
WG[2] %	$68.05^{a} \pm 1.16$	$74.22^{b} \pm 1.08$	$67.27^{a} \pm 1.00$	$70.27^{a} \pm 0.80$	$88.65^{c} \pm 1.97$	$76.17^{b} \pm 0.30$	<0.001
SGR[3]	$0.82^{a} \pm 0.01$	$0.93^{c} \pm 0.01$	$0.82^{a} \pm 0.02$	$0.88^{b} \pm 0.01$	$1.06^{d} \pm 0.02$	$0.96^{c} \pm 0.02$	<0.001
FCR[4]	$2.30^{e} \pm 0.03$	$2.10^{c} \pm 0.01$	$2.20^{d} \pm 0.01$	$2.14^{c} \pm 0.02$	$1.90^{a} \pm 0.02$	$2.01^{b} \pm 0.02$	<0.001
PER[5]	$1.39^{a} \pm 0.02$	$1.54^{b} \pm 0.01$	$1.40^{a} \pm 0.01$	$1.43^{a} \pm 0.01$	$1.60^{c} \pm 0.02$	$1.54^{b} \pm 0.01$	<0.001
HSI[6]	1.36 ± 0.21	1.11 ± 0.11	1.16 ± 0.14	1.22 ± 0.08	0.93 ± 0.09	1.04 ± 0.12	0.305
ISI[7]	$4.17^{b} \pm 0.13$	$4.32^{b} \pm 0.24$	$4.34^{b} \pm 0.16$	$3.50^{a} \pm 0.20$	$3.74^{ab} \pm 0.24$	$3.30^{a} \pm 0.20$	0.001
Survival (%)	100.00	100.00	100.00	100.00	100.00	100.00	–
Apparent digestibility coefficients							
ADCDM[8]	64.39 ± 0.32	62.80 ± 0.57	64.24 ± 0.83	65.10 ± 1.03	68.10 ± 0.49	65.55 ± 0.36	0.243
ADCCP[9]	77.62 ± 1.37	80.25 ± 0.61	79.23 ± 1.52	78.99 ± 0.93	82.56 ± 0.35	80.55 ± 0.84	0.167
ADCEE[10]	94.25 ± 0.15	94.34 ± 0.30	94.59 ± 0.35	93.45 ± 0.23	94.15 ± 0.35	94.34 ± 0.40	0.359

Data are expressed as mean ± SE ($n = 3$); Mean values in the same row with different superscripts differ significantly ($p < 0.05$).
[1] Fn. wt., final body weight;
[2]WG, weight gain;
[3]SGR, specific growth rate;
[4]FCR, feed conversion ratio;
[5]PER, protein efficiency ratio;
[6]HSI, hepato-somatic index;
[7]ISI, intestine somatic index;
[8]ADCDM, apparent digestibility coefficient of dry matter;
[9]ADCCP, apparent digestibility coefficient of crude protein;
[10]ADCEE, apparent digestibility coefficient of ether extract.

Table 5. Digestive enzyme activities in the intestine of *Labeo rohita* fingerlings fed with different experimental diets for the period of 60 days.

Experimental group	Protease[1]	Amylase[2]	Lipase[3]
C	17.81 ± 0.63	4.98^{a} ± 0.16	0.21 ± 0.02
LM20	18.09 ± 0.90	6.20^{b} ± 0.38	0.22 ± 0.01
LM40	17.64 ± 0.47	4.98^{a} ± 0.32	0.18 ± 0.01
CE	18.56 ± 0.81	4.96^{a} ± 0.11	0.18 ± 0.01
LM20E	19.28 ± 1.33	8.88^{c} ± 0.25	0.23 ± 0.01
LM40E	17.25 ± 0.93	5.56^{ab} ± 0.15	0.21 ± 0.02
p-value	0.234	<0.001	0.113

[1] Data are expressed as mean ± SE (n = 3). Mean values in the same column with different superscripts differ significantly ($p < 0.05$). Protease activity is expressed as millimole of casein released min^{-1} mg protein^{-1} at 37 ° C
[2] Amylase activity is expressed as micromole maltose released min^{-1} mg protein^{-1} at 37 ° C
[3] Lipase activity is expressed as unit h^{-1} mg protein^{-1} at 37 ° C.

Table 6. Protein and carbohydrate metabolic enzyme activities of *Labeo rohita* fingerlings fed with different experimental diets for the period of 60 days.

Experimental group	AST[1]		ALT[2]		LDH[3]	
	Liver	Muscle	Liver	Muscle	Liver	Muscle
C	12.26^{a} ± 0.51	14.17^{a} ± 0.32	12.25^{a} ± 0.51	15.42^{a} ± 0.29	6.43^{c} ± 0.97	8.79^{c} ± 0.05
LM20	13.34^{b} ± 0.26	15.87^{b} ± 0.57	14.21^{b} ± 0.32	18.06^{b} ± 0.46	6.09^{c} ± 0.03	9.61^{c} ± 0.25
LM40	15.85^{c} ± 0.10	18.61^{c} ± 0.64	16.05^{c} ± 0.34	20.38^{c} ± 0.50	6.71^{c} ± 0.09	9.05^{c} ± 0.31
CE	14.07^{b} ± 0.17	14.25^{a} ± 0.34	14.96^{bc} ± 0.34	21.25^{c} ± 0.51	2.41^{a} ± 0.21	5.38^{a} ± 0.22
LM20E	17.64^{d} ± 0.27	24.56^{e} ± 0.65	21.29^{e} ± 0.25	25.82^{d} ± 0.57	3.20^{ab} ± 0.10	6.04^{ab} ± 0.03
LM40E	13.21^{ab} ± 0.38	22.63^{d} ± 0.50	18.49^{d} ± 0.31	24.88^{d} ± 0.43	3.70^{b} ± 0.18	5.71^{ab} ± 0.34
p-value	0.039	0.011	0.015	0.019	<0.001	<0.001

[1] Data are expressed as mean ± SE (n = 3). Mean values in the same column with different superscripts differ significantly ($p < 0.05$). AST, aspartate aminotransferase, the activity is expressed as nanomoles of oxaloacetate released min^{-1} mg protein^{-1} at 37 ° C.
[2] ALT, alanine aminotransferase, the activity is expressed as nanomoles of sodium pyruvate released min^{-1} mg protein^{-1} at 37 ° C.
[3] LDH, lactate dehydrogenase, the activity is expressed as unit min^{-1} mg protein^{-1} at 37 ° C.

There was no significant ($p > 0.05$) variation found in the hepato-somatic index (HSI) among the treatments (Table 4). Though intestine somatic index (ISI) values exhibited similar ($p > 0.05$) among the non-supplemented groups (C, LM20 and LM40), but supplemented groups like CE and LM40E exhibited significantly lower ($p < 0.05$) values of ISI (Table 4). Supplementation of exogenous enzymes and limiting amino acids could not produce any significant variation ($p > 0.05$) in the apparent digestibility coefficient of dry matter and nutrients of the experimental diets.

3.3 Digestive enzyme activities

The activity of amylase was significantly affected ($p < 0.05$) but lipase and protease activity of intestine was not affected ($p > 0.05$) due to feeding of different experimental diets (Table 5). *L. rohita* fingerlings of LM fed groups (LM20 and LM40) exhibited significantly ($p>0.05$) higher protease activity compared to DORB fed control group. No significant ($p > 0.05$) variation was observed in amylase activity between DORB fed control and LM40 groups, but higher ($p < 0.05$) activity was recorded in fish fed with LM20 diet. Though, dietary supplementation of exogenous enzymes and limiting amino acids enhanced the amylase activity in LM20E group in comparison to their non-supplemented counterparts. However, the activity was found similar ($p >0.05$) in fish of CE and LM40E diet fed groups.

3.4 Protein and carbohydrate metabolic enzyme activities

Protein metabolic enzymes like aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and carbohydrate metabolic enzyme like lactate dehydrogenase (LDH) activities in the liver and muscle of experimental fish were significantly ($p < 0.05$) affected due to feeding of various experimental diets (Table 6). Higher ($p < 0.05$) AST and ALT activities were observed in fish of LM fed groups (LM20 and LM40) than the DORB fed group. Exogenous enzymes and limiting amino acids supplementation exhibited higher ($p < 0.05$) hepatic AST activity in CE and LM20E groups, muscle AST activity in LM20E and LM40E groups and ALT activities in muscle and liver of CE, LM20E and LM40E groups as compared to the counter groups fed without supplements. However, hepatic AST

Table 7. Antioxidant or oxidative stress enzymes activities of *Labeo rohita* fingerlings fed with different experimental diets for the period of 60 days.

Experimental group	SOD[1]		CAT[2]	
Liver	Gill	Liver	Gill	
C	$20.49^{d} \pm 0.36$	$25.81^{bc} \pm 0.88$	2.60 ± 0.58	0.34 ± 0.01
LM20	$19.41^{cd} \pm 0.20$	$20.62^{a} \pm 0.68$	2.44 ± 0.56	1.39 ± 0.50
LM40	$18.34^{bc} \pm 0.25$	$20.94^{a} \pm 0.74$	1.74 ± 0.56	2.83 ± 0.12
CE	$17.06^{b} \pm 0.50$	$27.48^{c} \pm 0.17$	2.85 ± 0.27	1.94 ± 0.68
LM20E	$15.10^{a} \pm 0.08$	$21.80^{a} \pm 0.95$	2.78 ± 0.34	1.66 ± 0.55
LM40E	$19.01^{c} \pm 0.74$	$25.10^{b} \pm 0.26$	2.68 ± 0.57	1.59 ± 0.72
p-value	<0.001	<0.001	0.651	0.087

[1] Data are expressed as mean ± SE ($n = 3$). Mean values in the same column with different superscripts differ significantly ($p < 0.05$). SOD, superoxide dismutase, the activity is expressed as 50% inhibition of epinephrine auto-oxidation min^{-1} mg $protein^{-1}$ at 37 ° C.
[2] CAT, catalase, the activity is expressed as nanomoles H_2O_2 decomposed min^{-1} mg $protein^{-1}$ at 37 ° C.

Table 8. Haematological, haemato-biochemical and immunological parameters of *Labeo rohita* fingerlings fed with different experimental diets for the period of 60 days.

Experimental group	Hb[1] (g dL^{-1})	TEC[2] ($\times 10^6$ mm^{-3})	TLC[3] ($\times 10^3$ mm^{-3})	STP[4] (g dL^{-1})	SAlb[5] (g dL^{-1})	SGlob[6] (g dL^{-1})	SA:G[7]	NBT[8] ($OD_{620\ nm}$)
C	6.47 ± 0.09	1.70 ± 0.01	$225^{b} \pm 4.05$	3.89 ± 0.09	1.35 ± 0.01	2.53 ± 0.10	0.54 ± 0.03	$0.24^{a} \pm 0.00$
LM20	6.13 ± 0.15	1.60 ± 0.03	$212^{b} \pm 5.02$	3.87 ± 0.09	1.39 ± 0.03	2.49 ± 0.10	0.56 ± 0.03	$0.27^{a} \pm 0.02$
LM40	7.30 ± 0.44	1.86 ± 0.08	$246^{c} \pm 10.01$	3.81 ± 0.04	1.40 ± 0.03	2.42 ± 0.03	0.58 ± 0.01	$0.36^{b} \pm 0.01$
CE	5.77 ± 0.24	1.49 ± 0.04	$174^{a} \pm 2.84$	3.85 ± 0.06	1.38 ± 0.03	2.47 ± 0.05	0.56 ± 0.01	$0.37^{b} \pm 0.03$
LM20E	6.60 ± 0.85	1.74 ± 0.20	$264^{c} \pm 12.60$	3.99 ± 0.02	1.44 ± 0.04	2.55 ± 0.05	0.56 ± 0.03	$0.43^{c} \pm 0.01$
LM40E	6.57 ± 0.12	1.75 ± 0.01	$220^{b} \pm 4.40$	4.01 ± 0.02	1.37 ± 0.05	2.64 ± 0.05	0.52 ± 0.03	$0.39^{bc} \pm 0.00$
p-value	0.246	0.157	<0.001	0.200	0.580	0.370	0.620	<0.001

[1] Data are expressed as mean ± SE (n=3); Mean values in the same column with different superscripts differ significantly ($p < 0.05$).
Hb, haemoglobin
[2] TEC, total erythrocyte count
[3] TLC, total leucocyte count
[4] STP, serum total protein
[5] SAlb, serum albumin
[6] SGlob, serum globulin
[7] SA: G, serum albumin to globulin ratio
[8] NBT, nitrobluetetrazolium

activity of fish fed with LM40E diet was lower ($p < 0.05$) as compared to non-supplemented counterpart (LM40). While, muscle AST activity in fish of DORB based CE group was similar ($p > 0.05$) to non-supplemented counterpart (C, control).

Hepatic and muscle LDH activity exhibited a non-significant ($p > 0.05$) relationship among the non-supplemented groups (Control, LM20 and LM40). However, exogenous enzymes and limiting amino acids supplemented groups (CE, LM20E and LM40E) exhibited significantly ($p < 0.05$) lower hepatic and muscle LDH activities as compared to the counter groups fed without supplements (Table 6).

3.5 Activities of antioxidant or oxidative stress enzymes

Fish fed with LM based LM20 and LM40 diets lowered ($p < 0.05$) the hepatic and gill SOD activities than the DORB fed C group (Table 7). Likewise, dietary supplementation of exogenous enzymes and limiting amino acids lowered ($p < 0.05$) the hepatic SOD activity in CE and LM20E groups. Both hepatic and gill CAT activities exhibited with a non-significant ($p > 0.05$) relationship among the treatment groups.

Table 9. Economic evaluation of all the experimental diets.

Ingredients	Diet[1] (experimental group)					
	C	LM20	LM40	CE	LM20E	LM40E
De-fatted soybean meal	6.44	5.2	3.96	6.6	5.31	4.13
Groundnut oil cake	9	9	9	9	9	9
Wheat flour	0.5	1.29	2.07	0.32	1.17	1.92
De-oiled rice bran	5	2.5	0	5	2.5	0
Mixed leaf meal	0	0.6	1.2	0	0.6	1.2
Sunflower oil	1.6	1.6	1.6	1.6	1.6	1.6
Fish oil	2.9	2.9	2.9	2.9	2.9	2.9
Vitamin-mineral mix	1.26	1.26	1.26	1.26	1.26	1.26
Betaine	0.75	0.75	0.75	0.75	0.75	0.75
Choline chloride	0.06	0.06	0.06	0.06	0.06	0.06
Carboxymethyl cellulose	2.1	2.1	2.1	2.1	2.1	2.1
Butylated hydroxytoluene	0.04	0.04	0.04	0.04	0.04	0.04
Exogenous enzyme	0	0	0	0.75	0.75	0.75
DL-Methionine	0	0	0	0.36	0	0
L-Lysine	0	0	0	0.28	0.2	0.3
Processing charges	1.5	1.5	1.5	1.5	1.5	1.5
Cost (INR/Rs. kg^{-1} diet)	31.14	28.79	26.44	32.51	29.74	27.50

[1] C, Control with 0 g kg^{-1} mixed leave meal (LM) and 400 g kg^{-1} de-oiled rice bran (DORB); LM20, 200 g kg^{-1} LM in replacement of 50% DORB; LM40, 400 g kg^{-1} LM in replacement of 100% DORB; CE, Control diet supplemented with 1 g kg^{-1} xylanase and cellulase enzyme mixture; LM20E, LM20 diet supplemented with 1 g kg^{-1} xylanase and cellulase enzyme mixture; LM40E, LM40 diet supplemented with 1 g kg^{-1} xylanase and cellulase enzyme mixture.

3.6 Haematological, haemato-biochemical and immunological parameters

Haematological indices such as total erythrocyte counts (TEC) and haemoglobin (Hb) contents were independent of dietary treatment groups ($p > 0.05$). Fish fed with LM40E diet exhibited higher ($p < 0.05$) total leucocyte count (TLC) and nitrobluetetrazolium (NBT) value than control and LM20 groups, however it was found similar ($p > 0.05$) between these two groups (Table 8). Maximum ($p < 0.05$) and minimum TLC was observed in supplemented fed groups LM20E and CE, respectively. NBT values were significantly ($p < 0.05$) higher in supplement fed groups (CE, LM20E and LM40E) than non-supplement fed groups (C, LM20 and LM40). However, serum proteins concentrations and A:G ratio remains similar ($p > 0.05$) in fish of various treatments.

3.7 Economic evaluation of the experimental diets

Estimated costs (INR kg^{-1}) of the experimental diets were presented in Table 9. Inclusion of 400 g kg^{-1} LM (LM40, INR 26.44 kg^{-1}) resulted in 15.10% cost reduction (INR 4.7 kg^{-1} diet) in comparison to DORB based C diet (INR 31.14 kg^{-1}).

4 Discussion

In the present study, among the three leaf meals, higher levels of total tannin and total oxalate were found in *I. aquatica* leaf meal, while *H. spinosa* leaf meal contained higher levels of alkaloid and phytic acid. Therefore, mixing of leaf meals at 1:1:1 ratios diluted the ANFs and were found within the tolerable limit (<2 g 100 g^{-1}, <0.5 g 100 g^{-1} and <500 mg 100 g^{-1}, respectively) of fish (Francis et al., 2001; Rahman et al., 2013). Higher levels of ANFs are toxic to fish and known to hinder nutrient bioavailability (Maiti et al., 2019). Replacement of any aquafeed ingredient by targeted ingredient especially plant based one should be based on the essential amino acid profile, which influences the quality of dietary protein (Kaushik et al., 1995; Sahoo et al., 2020). In our study, it was observed that the essential amino acid profile was comparable, although levels of NEAAs like aspartic acid and glutamic acids in LM were much lower than DORB (Ranjan et al., 2018a). Moreover, most of the plant-based aquafeed are deficient in lysine and methionine (Sardar et al., 2009). In this study, control diet (C) was deficit in lysine and methionine but LM20 and LM40 diet exhibited the deficiency of lysine. Therefore, L-lysine and DL-methionine were supplemented in CE, and L-lysine was supplemented in both LM20E and LM40E diet to fulfill the amino acid requirement of *L. rohita* (Sardar et al., 2009, Ranjan et al., 2018b). Simultaneously, the diets were supplemented with 1 g kg^{-1} exogenous enzyme blend (xylanase and cellulase).

The in vitro protein digestibility (IVPD) of LM was found to be 28.42%, which was slightly higher than IVPD (26.72%) of single leaf (*H. spinosa*) meal (Maiti et al., 2019), but comparable with the IVPD (29.95%) of DORB (Udo and Umoren, 2011). The evaluation of feed digestibility of ingredients is crucial for aquafeed development (Glencross et al., 2007). In our present study, *L. rohita* fed with LM containing diets did not altered apparent digestibility coefficient (ADC) of dry matter (ADCDM) than DORB-based control group, suggesting optimum utilization of DORB and LM mediated nutrients and energy in the carp diet (Maiti et al., 2019). Further low ANFs (total tannin, total oxalate and phytic acid) levels in LM based diets that were further

enhanced with exogenous carbohydrases (xylanase plus cellulase) and limiting amino acids supplementation probably through enhancing the crude fibre digestibility. Sardar et al. (2009) also reported that nutrient digestibility of plant based carp diet could be enhanced through supplementation of exogenous enzymes.

Under the ample presence of non-protein energy sources in feed like lipids and carbohydrates, amino acids from protein supports growth of fish by production and retention of muscle protein (Maiti et al., 2019). In our study, 400 g kg^{-1} inclusion of LM (LM40 group) did not affected the nutrient utilization (PER) and growth performance (FBW, WG%, and SGR) in fish fed with DORB-based C diet. FCR value of 400 g kg^{-1} LM fed group was also lower than the DORB-based C group. This observation indicates that LM could be incorporated at 400 g kg^{-1} inclusion level in the diet without compromising the growth performance and nutrient utilization of fish. So far, no reports are available to evaluate the dietary effect of mixed leaf meal in fish on growth and nutrient utilization. However, several studies reported that leaf meal prepared from sweet potato leaf, *H. spinosa* and arhar, *Cajanus cajan* respectively could be incorporated in the carp diet up to 300 g kg^{-1} without any detrimental result on the nutrient utilization, feed conversion and growth of fish (Meshram et al., 2018; Maiti et al., 2019; Rani et al., 2021). Similarly, leaf meals from other terrestrial and aquatic plants like *Moringa oleifera* (Yuangsoi and Masumoto, 2012; Arsalan et al., 2016; Shahzad et al., 2016; Hussain et al., 2018), *Medicago sativa* (Vhanalakar and Muley, 2015), fermented *Eichhornia crassipes* (El-Sayed, 2003), and duckweeds (Mohapatra and Patra, 2013) etc. could be incorporated in aquafeed with replacement of conventional feed ingredients without compromising the performance of fish. Further, the supplementation of 1 g kg^{-1} exogenous carbohydrases (xylanase and cellulase mixture) and limiting amino acids to DORB and LM-based diets significantly improved the nutrient utilization, feed conversion and growth of fish. Highest growth performance were recorded in LM20E fed group which might be related to maximum utilization of dietary fibre for energy production and balancing of essential amino acids to synthesize more body protein leading to higher growth. In the similar line, Ranjan et al. (2018b) and Castillo and Gatlin (2015) reported that supplementation of exogenous carbohydrases could enhance the digestibility and utilization of dietary nutrients leading to increased growth of fish and that could be further assisted by dietary supplementation of limiting amino acids (Sardar et al., 2009). However, growth retardation was recorded in LM40 groups than LM20 groups attributed to little higher level of alkaloids in these diets. Alkaloids in small amount can act as growth promoter but little higher amount can alter brain functioning and growth retardation in fish (Maiti et al., 2019).

The body indices like HSI and ISI indicates fish metabolism along with efficiency of digestive enzymes secretion, digestion, absorption and assimilation of nutrients, health status of liver and intestine (Ighwela et al., 2014; Haque et al., 2021). In our study, though ISI exhibited with a non-significant relationship among the non-supplemented groups, supplementation of exogenous carbohydrases and limiting amino acids significantly decreased the ISI values in the fish of CE and LM40E groups as compared to the counter groups fed without supplements. However, ISI of LM20E supplemented group did not vary significantly with its non-supplemented groups. Similar to our observation, no significant variation of ISI value was found between DORB fed control and sweet potato leaf meal fed groups (Sereewatthanawut et al., 2008).

Regulation and functioning of digestive enzymes depends upon the nutrient profile of feed (Fernandez et al., 2001). The Digestive enzymes break the complex nutrients to simple one making them available either for maintenance and growth or for energy production in fish (Lemieux et al., 1999). Feeding plant-based diet to fishes influence the digestive enzymes activities and these are related to presence of ANFs and insoluble fibre (Olude et al., 2021). In our study, irrespective of exogenous carbohydrases and limiting amino acids supplementation, protease activity was not altered in LM fed groups. Whereas, amylase activities were significantly enhanced in fish due to feeding of 200 g kg^{-1} LM irrespective of exogenous carbohydrases and limiting amino acids supplementation. This might be due to presence of higher digestible carbohydrate in the gut of the fish. Similarly, feeding of *H. spinosa* leaf meal irrespective of exogenous carbohydrases supplementation did not cause any significant change in protease activity compared to DORB fed group (Maiti et al., 2019). But, author observed significantly higher amylase activity in rohu fingerlings at 200 g kg^{-1} inclusion of leaf meal supplemented with exogenous carbohydrases which is in agreement with our results. However, feeding of exogenous carbohydrases supplemented plant based diets to rohu fingerlings significantly enhanced the protease and amylase activities (Ranjan et al., 2018a). Moreover, Mondal et al. (2012) found that the α-amylase activity was significantly increased in *Labeo bata* due to feeding of 650 g kg^{-1} of mulberry leaf meal containing diet. However, lipase activity was found similar ($p > 0.05$) in fish of various treatments. Similarly, Nottanalan et al. (2021) did not found any significant variation of lipase activity among the *L. rohita* of DORB fed control and *Pisum sativum* leaf meal fed groups irrespective of exogenous carbohydrases supplementation. However, significant improvement was reported in lipase activity in *L. rohita* due to feeding of exogenous carbohydrases and limiting amino acids supplemented plant based diet (Ranjan et al., 2018a).

AST and ALT are crucial transaminases for the production of new tissue protein towards growth of the fishes (Shamna et al., 2015). In this current study, hepatic and muscle AST and ALT activities in fish fed with LM based diet was significantly higher as compared to DORB based control diet with highest activity in LM40 fed group. An increase in AST and ALT activities were related to synthesize the new amino acids to support the growth. While elevated aminotransferase activities in LM40 fed group was an indication of imbalance amino acids composition. Dietary supplementation of exogenous carbohydrases and limiting amino acids significantly enhanced the hepatic and muscle AST and ALT activities in *L. rohita* as compared to the counter groups fed without supplements. In agreement to our observation, Garg et al. (2019) reported significantly higher hepatic AST and ALT activity with higher growth trend in rohu fingerlings fed with *Houttuynia chordata* leaf meal containing diet. In contrast, non-significant change of hepatic and muscle AST and ALT activities in *L. rohita* was due to feeding of *H. spinosa* leaf meal containing diet (Maiti et al., 2019). Feeding *H. spinosa* leaf meal supplemented with exogenous carbohydrases enhanced the AST and ALT activities in muscle in

L. rohita and concluded that exogenous carbohydrases enhanced the utilization of non-soluble fibre and made them available to fulfill the energy satiation of fish, which improve the utilization of amino acids for protein accretion (Maiti et al., 2019). Similarly, Ranjan et al. (2018a) found that feeding of plant-based diet supplemented with exogenous carbohydrases and limiting amino acids could enhance the muscle AST and hepatic and muscle ALT activities in *L. rohita*.

Tissue lactate dehydrogenase (LDH) activity is considered as stress indicator in animals including fish (Jana et al., 2021b) Thus, higher LDH activity indicates the stress condition of fish, in which more amino acids degrades to satiate the high energy demand rather than new protein synthesis for growth (Maiti et al., 2019). Under oxidative stress mediated tissue hypoxic condition, pyruvate, the glycolysis end product instead of catabolizing via Krebs's cycle, is catabolized to lactate by LDH to supply energy in fish (Murray et al., 2000). In our study, dietary LM did not switch the LDH activities (liver and muscle) in C, LM20 and LM40 fed groups. It suggests that there was no shift of metabolism from aerobic to anaerobic condition. While, LDH activities decreased when fed with LM based diets supplemented with exogenous carbohydrases. It suggests that supplementation of exogenous carbohydrases and limiting amino acids enhance the nutritional profile of experimental diets, and are in corroboration with enhanced growth.

Oxidative stress of the organism arises when levels of reactive oxygen species (ROS) elevated, which may be mediated through either dietary (ANFs, high fibre content, feed deprivation or starvation etc.) or environmental (pollutants and bad water quality) or biological (high stocking density) factor (Schieber and Chandel, 2014; Naseemashahul et al., 2021). Living organisms can protect themselves from the ROS with the help of antioxidant enzymes such as SOD, CAT and glutathione peroxidase (GPx) (Kumar et al., 2018b). Thus, an increased activity of these enzymes is indicator of oxidative stress to living organism (Gupta et al., 2021). In our study, significantly, lower SOD activities were observed in liver and gill tissue of fish fed with 200 and 400 g kg^{-1} LM than DORB based C group. It might be due to the scavenging effects of free radicals by leaf meal mediated antioxidants like flavonoid and tannins (Popeskovic et al., 1980). Because low concentrations of these compounds act as antioxidants, but high concentrations interfere in dietary nutrient utilization and affects the immune responses of fish (Anand et al., 2020). It indicates that the incorporation of *H. spinosa*, *V. mungo* and *I. aquatica* leaf meal based LM in the carp diet up to 400 g kg^{-1} could not cause any stress mediated detrimental effect to the fish. In accordance with our finding, lower antioxidant enzymes activities also reported in *L. rohita* fed with leaf meal based diet (Garg et al., 2019).

Haematological and haemato-biochemical indices are meaningful indicators of health and non-specific immune responses of animals including fish (Houston, 1997; Abdullah et al., 2013; Karmakar et al., 2021). Here, haemato-biochemical parameters of fish did not altered among the treatments. Dietary LM at 400 g kg^{-1} inclusion level caused significant enhancement of TLC in rohu fingerlings. However, exogenous carbohydrases and limiting amino acids supplementation could not induce any further significant increment of this parameter. Enhanced TLC count in the blood up to certain limit is the indicator of enhanced innate or non-specific immunity of fish as they are the important component of fish immune system like lymphocytes to prevent the pathogens (Chen et al., 2013). In our study, increased TLC in the 400 g kg^{-1} LM group might be due to the action of dietary LM mediated low levels of alkaloids, flavonoid and tannins (Popeskovic et al., 1980). Similar reports concluded the significant enhancement of TLC in *L. rohita* fed with various leaf meals (Das et al., 2009; Garg et al., 2019; Nottanalan et al., 2021).

During respiratory burst of phagocytes, there is a generation of ROS or species with powerful microbicidal activity to kill the pathogens (Neumann et al., 2001; Babior and Bunn, 2005; Saha et al., 2021). Thus, phagocytes are considered as the cellular components of innate immunity and increased NBT value indicates the improved immune status of fish (Maiti et al., 2021). In the present study, dietary LM at 400 g kg^{-1} inclusion level in replacement of 100% DORB attributed significant enhancement of NBT value in fish, but there was no further significant change of this parameter due to the action of dietary supplemented exogenous carbohydrases and limiting amino acids. With the agreement of our finding, Kumala et al. (2018) reported significantly higher NBT of phagocytes in *O. niloticus* fed with algae and fungi. On the other hand, CE and LM20E fed groups exhibited significantly the higher NBT values compared to non-supplemented counterparts and concluded that exogenous carbohydrases supplemented plant based diet could improve NBT value in fish. In context of the present economics of the experimental diets, LM based diets decreased the cost by Rs. 4.7/kg (15.10 % cost reduction) in comparison to DORB based diets and thus attributed to higher production and profit to the farmers.

5 Conclusions and perspectives

Dietary mixed leaf meal (LM, *H. spinosa, V. mungo,* and *I. aquatica* in 1:1:1 ratio) affected the growth performance, feed conversion, digestive enzyme and metabolic enzyme activities in *L. rohita* fingerlings. Maximum growth performance indices were achieved in rohu fingerlings fed with 200 g kg^{-1} LM based diet, however, these were similar in DORB based C and LM40 fed groups. Dietary supplementation of exogenous carbohydrases and limiting amino acids enhanced the growth rates and feed conversion indices. Amylase and aminotransaminase activities were positively related with growth indices. Stress enzymes, superoxide dismutase activities were lower in LM fed groups while lactate dehydrogenase activity was independent of dietary treatments. Feeding of 200 g kg^{-1} LM with dietary supplementation of 1 g kg^{-1} exogenous xylanase and cellulase mixture along with 0.98 g kg^{-1} L-lysine exhibited best the growth performance, feed conversion, digestive and metabolic enzymes profile in fish. Based on the results, the complete substitution of DORB with mixed leaf meal (LM, *H. spinosa, V. mungo,* and *I. aquatica* in 1:1:1 ratio) in the diet of *L. rohita* fingerlings is recommended.

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Ethical approval

The animals used in present study were handled as per the standard procedure approved by Committee-Ethics and Animal Care of ICAR-Central Institute of Fisheries Education, Mumbai, India and The CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Environment and Forests (Animal Welfare Division), India.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data accessibility statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

Anakhy Mondal: Investigation, validation, software and original draft preparation. Parimal Sardar: Methodology, visualization, validation, review and editing of manuscript. Manish Jayant: Data curation, validation and editing of manuscript. Shamna N: Formal analysis, data visualization validation and manuscript editing. Gopika Radhakrishnan: Investigation, validation and software. Prasanta Jana: Formal analysis, data curation, review and editing of manuscript. Narottam Prasad Sahu: Conceptualization, supervision, reviewing and editing of manuscript.

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