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EVALUATION OF PARTIAL FISH MEAL SUBSTITUTION BY FERMENTED SOYBEAN MEAL ON GROWTH, ANTIOXIDANT AND IMMUNE STATUS OF *Labeo rohita*

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ABSTRACT

Due to the increase demand, market instability and the high price of fish meal (FM), the aqua-feed industry must find alternative protein sources to reduce its reliance on FM and to ensure cost-effective aqua-feed production. Due to its high protein content, soybean meal (SBM) can substitute costly FM in fish feed. This research evaluated the effects of substituting FM with SBM and fermented soybean meal (FSBM), supplemented with lysine and methionine, on growth performance, body composition, antioxidant enzyme activity and immune competency of *Labeo rohita.* Fingerlings (10.9 ±0.4g) were acclimatized for two weeks and divided into seven groups, each with three replicates. Seven experimental diets were formulated, replacing 0 (control diet), 25%, 50% %, and 75% FM with SBM and FSBM (SBM-25, SBM-50, SBM-75, FSBM-25, FSBM-50 and FSBM-75). In the present study, *Lactobacillus plantarum* was used to ferment SBM. Fish were fed two times a day for 4 months under laboratory conditions. Results showed that fermentation significantly improved the nutritional quality of SBM by reducing anti-nutritional factors (trypsin, glycinin, and β-conglycinin). The replacement of 25% FM with SBM and FSBM did not significantly affect growth performance compared to the control. However, a substantial decrease in weight gain (WG) and specific growth rate (SGR) was observed as FM replacement increased to 50% and 75%, with the lowest WG and SGR recorded in SBM-75 and FSBM-75 groups. Activities of antioxidant enzymes (SOD and CAT) and immune parameters (WBCs and IgM) decreased significantly in 75% SBM and FSBM groups compared to the control. The FSBM diets did not affect whole-body crude protein, fat and moisture content. However, SBM-75 diets significantly enhanced whole-body moisture content while reducing crude protein content. These results indicate that SBM and FSBM can replace 25 and 50% of FM with additional lysine and methionine supplementation without compromising growth and physiological health.
Keywords: Fermented soybean meal (FSBM), Anti-nutritional factors (ANF), *Labeo rohita,* immune competency, enzymatic indices.

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INTRODUCTION

Fish meal (FM) has long been utilized as the primary protein source in aqua-feed formulation (Wang *et al*., 2016). Feed accounts for 50-80% of total fish production costs. In recent years, the rapid expansion of aquaculture, increase in prices and diminishing fishery resources have led the nutritionist to explore sustainable protein sources to substitute FM (Rahimnejad *et al*., 2021). Plant proteins have gained more attention as potential candidates due to their low cost and abundant availability (Zhou *et al*., 2018). In particular, the substitution of FM with economically viable and eco-friendly soybean meal (SBM) (Rahimnejad *et al*., 2019) has drawn more interest because of its low cost, high protein content, consistent supply (Zhang *et al*., 2014) and widespread availability (Xu *et al*., 2022). However, SBM utilization in fish feed has been limited due to an unbalanced proportion of amino acids (such as lysine and methionine) and the presence of different anti-nutritional factors (ANF) like phytic acid, saponins and protease inhibitors (Liu *et al*., 2019) which negatively affect growth rate and feed efficiency of fish (Miao *et al*., 2018) by reducing nutrients digestion and absorption (Ding *et al*., 2015). Therefore, replacing FM with SBM above a certain level is difficult without affecting fish growth (El-Dakar *et al*., 2023). The high inclusion of SBM in feed can cause reduced fish growth due to inadequate lysine (Lys) and methionine (Met) levels. The supplementation of Met and Lys improves the growth of Grass carp (Jiang *et al.,* 2018) and rainbow trout (Hang *et al.,* 2022) that were fed plant-based feed. Various techniques have been employed to improve the bioavailability and absorption of nutrients in plant-based proteins; however, most of those techniques are costly, cause protein degradation, and affect environmental sustainability (Wang *et al*., 2016).

Fermentation has been suggested as a promising low-cost approach to improve the nutritional quality of plant proteins (Hassan *et al*., 2015) by degrading anti-nutritional factors. According to El-Dakar *et al*. (2023), FSBM can facilitate antioxidant activity, nutrient digestibility and immune function in fish by producing probiotics and prebiotics. A previous study by Adyemo *et al*. (2013) stated that fermentation by *Lactobacillus plantarum* caused a reduction in ANFs (trypsin, protease and phytate inhibitors) in soybean meal. Dietary supplementation of *L. plantarum* in aquaculture diets has been known to enhance immunity and disease resistance in various aquatic species (Wang *et al*., 2016). Choi *et al*. (2020) reported that approximately 40% FM can be substituted with SBM fermented by *Bacillus subtilis* in rainbow trout diet without adverse effects on feed utilization and fish growth. Similarly, Dai *et al*. (2017) observed that SBM fermented by Bacillus sp. has higher crude protein than that of SBM and the amount of trypsin inhibitor declined in FSBM by 51.1% compared to SBM.

Labeo rohita, a commercially important fish species, accounts for about 15% of gross global aquaculture production. This study aimed to assess the efficacy of SBM and *L. plantarum* fermented SBM as a partial substituent for FM along with additional supplementation of lysine and methionine in *L. rohita* feed by investigating the growth response and immune and antioxidant enzyme activity with the expectation that results attained could be beneficial for the development of sustainable and cost-effective dietary formulations for *L. rohita.*

MATERIALS AND METHODS

This study was carried out for 4 months under laboratory conditions. Fingerlings of *L. rohita* were taken from Fisheries Research Farm, University of Agriculture (Faisalabad- Pakistan). Before starting the experiment, fish were acclimatized for two weeks and fed on commercial feed during the acclimatization period. Fingerlings with an average initial body weight of 10.9 ±0.4g were divided into seven groups, each with three replicates with 10 fish per tank density, and fed twice daily. Experimental tanks were aerated continuously, and one-third of the water in every tank was renewed after siphoning. Fish in each aquarium were weighted fortnightly. The dissolved oxygen (>5mg/L), TAN (<0.05mg/L), pH (7.4-8.0) and temperature (27-32°C) of water were monitored throughout the feeding trial. For experimental feed formulation, wheat flour and fish oil were used as the primary carbohydrates and lipid sources. Soybean, fermented soybean meal, and fish meal were the primary protein sources. Seven experimental feeds were prepared by replacing 0 (control diet), 25, 50 and 75% fish meal with SBM and FSBM (25% SBM, 50% SBM, 75% SBM, FSBM-25, FSBM-50, and FSBM-75), respectively. Lysine and methionine were incorporated in FM-substituted diets to align the amino acid profile with the control diet. Dietary ingredients, including fishmeal, soybean meal, fish oil, wheat flour, vitamins, and rice bran, were brought from a local store. The collected ingredients were weighed carefully, then ground and mixed to formulate experimental diets according to the feed composition mentioned in the **table. 1**.

Table 1. Feed ingredients and feed composition (g/100g diet)

Ingredients	CON	SBM-25	SBM-50	SBM-75	FSBM-25	FSBM-50	FSBM-75
Fish meal (g)	48	36	24	12	36	24	12
Soybean meal (g)	--	12	24	36	--	--	--
Fermented Soybean meal	--	--	--	--	12	24	36
Wheat flour (g)	13	13	13	13	13	13	13
Wheat bran (g)	18	17.5	17.5	17.5	17.5	17.5	17.5
Rice bran (g)	15	14	14	14	14	14	14
Fish oil (ml/L)	4	4	4	4	4	4	4
Vit & Min premix (g)[a]	2	2	2	2	2	2	2
Lysine (g)	--	0.5	0.5	0.5	0.5	0.5	0.5
Methionine (g)	--	1	1	1	1	1	1
Total	100g	100g	100g	100g	100g	100g	100g
Proximate composition of experimental diets (%)							
Moisture	9.5	8.73	8.0	7.2	8.5	7.5	6.4
Crude protein	37.83	35.97	33.70	31.43	36.21	34.18	32.15
EE	11.9	11.0	9.9	8.7	11.1	10.1	9.08
Ash	8.8	8.0	7.2	6.5	7.7	6.7	6.0

CON (control group), **SBM**; soybean meal, **FSBM**; fermented soybean meal, [a] **Vitamin and mineral premix (g/kg):** vitamin B12 (0. 2 mg), thiamin (10mg), pyridoxine (10mg), riboflavin (8mg), pantothenic acid (20mg), folic acid (2mg), niacin acid (50mg), alphatocopherol (100mg), inositol (100mg), retinol acetate (400mg), Vitamin K (10mg), KI (0.8mg), CusO4 (10mg), zeolite (4.5mg), ZnSO4 (50mg), MnSO4 (25mg), FeSO4 (80mg), MgSO4 (200mg), CoCl2 (1mg), **EE**: Ether extract.

Fermentation of soybean meal: Soybean meal was fermented by following the method of Shiu *et al*. (2015). 500g defatted soybean meal was autoclaved for 20 min at 121℃ in beakers covered by aluminum foil and then cooled at room temperature. After that, 5 ml of *L. plantarum* (107cfu/mL) was inoculated into the SBM and evenly mixed. In an incubator, fermentation was carried out for 72h at 40°C. The fermented soybean was autoclaved at 121°C for 20 min to stop the fermentation process. After the fermentation, samples of SBM and FSBM were collected to determine the nutritional profile.

Growth performance: Feed utilization and growth indices, including body weight (grams), specific growth rate (SGR) and feed conversion ratio (FCR) were recorded fortnightly during the experimental period. Three fish from each tank were pooled to record the viscera and liver weight of fish for calculating hepatosomatic (HSI) and viscerosomatic index (VSI). The following formulas were used for the determination of these parameters:

Weight gain (g) = Final weight (g) - Initial weight (g)

$$\textbf{FCR} = \frac{Feed\ given\ (\text{g})}{\text{gain in weight}\ (\text{g})}$$

$$\textbf{SGR} = \frac{ln\text{final weight} - ln\text{initial weight}}{Number\ of\ day} \times 100$$

HSI (%) = Liver weight (g)/ fish weight (g) × 100

VSI (%) = Viscera weight (g)/ fish weight (g) × 100

Chemical analysis: The proximate composition of formulated feeds and fish samples was done according to AOAC (1995). Kjeldahl technique was performed to measure the crude protein content. The ether extraction method determined crude fat content in a Soxhlet apparatus. Moisture content was calculated by desiccating samples in the oven at 105°C. The main anti-nutritional factors (ANFs), i.e., trypsin inhibitor, phytic acid, glycinin, and β-glycinin, were also measured. Phytic acid content was calculated by following the protocol of Lee and Choi (2011). One gram of the sample was mixed with 20 ml of 2.4% HCl, incubated for 12h, then centrifuged at 3500 rpm for 30 min duration, and the supernatant was collected. 1ml of Wade's reagent was mixed with 3ml supernatant and centrifuged at 3500 rpm for 10 min. At 500nm, absorbance was measured by UV/VIS spectrophotometer. Trypsin activity was determined by the BAPA (benzoyl DL-arginine p-nitroanilide) method (Dai *et al*. 2017). The contents of glycinin and β-glycinin were measured by Competitive ELISA (Pederson *et al.* 2008).

Immune analysis: Three fish from each group were collected and anesthetized with clove oil to determine blood and serum biochemical analysis at the end of the experimental trial. Caudal vein blood samples were collected using a 3mL heparinized syringe and kept in EDTA tubes to prevent clotting. Serum was obtained by centrifuging the blood at 1500 rpm for 15 minutes and kept at 4℃. A white blood cell (WBC) count was done by using a hemocytometer. Turk solution was added to the blood sample to dilute the blood. WBC count was done in a hemocytometer and observed as 103/µL. Immunoglobulin (IgM) activity was determined using the ELISA technique. A microplate was pre-coated with an IgM antibody, and the wells were pipetted with the samples so that any IgM present in the sample adhered with the immobilized antibody. After washing away unbound components, a substrate was added, resulting in the development of color based on the amount of IgM present, and the intensity of color was measured (Khandige *et al*., 2022).

Antioxidant enzyme activity: Three fish from each group were dissected at the end of the experimental period to obtain liver tissue. The fish liver was homogenized in cold phosphate buffer. Homogenized organs underwent centrifugation for 15 min at 10,000rpm; the supernatant was taken and stored at -80°C. For determining Catalase activity (CAT), Chance and Maehly (1995) method was adopted. 100µL enzyme extract was taken, and then 2.5 mL phosphate buffer and 400 µL of 5.9 mM H2O2 were added. The spectrophotometer was set at 240nm, and a change in absorbance was noted after one minute. One unit of CAT activity was described as the enzymes' quantity, which catalyzes the breakdown of U/mg of H2O2 per minute.

Superoxide dismutase activity (SOD): This activity was assessed using the Maier *et al.* (2002) method. In a cuvette, 1 ml of blank buffer solution was taken, and the readings were noted by placing it into a spectrophotometer and adjusting it to zero. Then 0.05ml of enzyme extract, 1ml of buffer, and 0.016 ml of riboflavin were taken and incubated for 12 minutes. After that, the cuvette was placed into the spectrophotometer, where 0.067 ml of EDTA and the reaction mixture were added with 0.33 ml of NBT. Its absorbance was observed at A560 nm after 20 seconds of reaction. The activity of SOD was measured by using the following formula:

%age inhibition (Abs): Blank (Abs) – Sample × 100/ Blank (Abs)

Statistical analysis: All data of growth and biochemical parameters was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test, which was used to compare the difference among treatment groups (represented as Mean±S.E) with Statistix software version 8.1. Statistical differences between means were regarded as significant when $P \leq 0.05$. Principal

component analysis (PCA) was performed using R-version 4.1.2.

RESULTS

3Nutritional profile and anti-nutrients of SBM and FSBM: Fermentation with *L. plantarum* improved the nutritional value of SBM (**Table 2**). After fermentation, a significant increase in crude protein (4.3%) and crude lipid level (8.4%) was observed. Furthermore, *L. plantarum* fermentation decreased the ANF content in SBM, which includes glycinin (-74.6%), b-conglycinin (-54.4%), and trypsin inhibitors (-86.4%). At the same time, no significant difference was noticed in the phytic acid content of SBM and FSBM.

Growth performance: The growth performance of *Labeo rohita* fed with graded levels of FM replaced by SBM and FSBM is presented in Table 3. No fish mortality was observed during the feeding trial. Specific growth rate (SGR), feed conversion ratio (FCR) and weight gain (WG) of the fish in all treatments were affected significantly by different substitution levels of FM along with SBM and FSBM. Diets with 25% of FM substituted by SBM (SBM-25) and FSBM (FSBM-25) showed no significant differences in WG, SGR and FCR compared to the control diet. In contrast, 75% substitution of FM with SBM-75 and FSBM-75 exhibited significantly reduced WG and SGR compared to the control and 25% substitution feeds. The FCR of the experimental groups SBM-50, SBM-75, FSBM-50 and FSBM-75 was significantly higher than the control group ($p<0.05$). The hepatosomatic index (HSI) was significantly lower ($p<0.05$) in FSBM-75 and SBM-75 diets.

Table 2. Nutritional profile and anti-nutritional content of SBM and FSBM

Nutritional parameters	SBM	FSBM
Crude protein (g/100g)	46.1±0.36^{b}	48.1±0.39^{a}
Crude lipid (g/100g)	1.9±0.3^{b}	2.06±0.20^{a}
Trypsin inhibitors (mg/g)	2.8±0.26^{a}	0.40±0.20^{b}
Phytic acid (mg/g)	17.3±0.35^{a}	16.6±0.27^{a}
Glycinin (mg/g)	140.9±0.40^{a}	34.5±0.61^{b}
β-Conglycinin (mg/g)	121.7±0.76^{a}	55.3±0.60^{b}

Values are presented as mean ± standard error. SBM; soybean meal FSBM; fermented soybean meal. Values carrying different superscripts in a row are significantly different ($P\leq0.05$)

Table 3. Growth performance of *L. rohita* fed graded levels of SBM and FSBM

Parameter	Treatments						
	Control	SBM-25	SBM-50	SBM-75	FSBM-25	FSBM-50	FSBM-75
IBW[1] (g)	11.1±0.14	10.9±0.2	10.9±0.21	10.8±0.17	11.2±0.23	10.9±0.26	10.7±0.23
FBW[2] (g)	90.2±0.21^{a}	89.3±0.2^{a}	84.8±0.26^{c}	80.2±0.21^{d}	89.9±0.23^{a}	86.5±0.26^{b}	83.8±0.17^{c}
WG[3] (g)	79.1±0.23^{a}	78.4±0.24^{a}	73.9±0.21^{c}	69.4±0.2^{d}	78.7±0.21^{a}	75.6±0.28^{b}	73.1±0.19^{c}
SGR[4] (%per day)	2.93±0.23^{a}	2.86±0.20^{a}	2.6±0.20^{c}	2.2±0.20^{d}	2.9±0.23^{a}	2.7±0.20^{b}	2.53±0.20^{c}
FCR[5]	1.4±0.20^{d}	1.43±0.19^{d}	1.66±0.23^{b}	1.9±0.20^{a}	1.4±0.25^{d}	1.56±0.23^{c}	1.7±0.20^{b}
HSI[6]	1.73±0.24^{a}	1.70±0.26^{a}	1.63±0.20^{a}	1.36±0.21^{b}	1.73±0.20^{a}	1.63±0.20^{a}	1.33±0.18^{b}
VSI[7]	12.06±0.78^{a}	11.86±0.62^{a}	11.8±0.62^{a}	11.56±0.67^{a}	11.86±0.57^{a}	11.8±0.58^{a}	11.46±0.57^{a}

Values are the mean of three replicates (mean ± SE**)**; Means in the same row with different superscripts are significantly different ($p < 0.05$). Control means basal diet.[1]Initial body weight; [2]Final body weight, [3]Weight gain, [4]Specific growth rate, [5]Feed conversion ratio, [6]Hepatosomatic index; [7] Viscerosomatic index. Values carrying different superscripts in a row are significantly different ($P\leq0.05$)

Proximate body composition: The results of the proximate body composition of *L. rohita* are shown in Table 4. Analysis of the proximate body composition of fish showed that with the increase in dietary FSBM levels, crude protein, fat, ash and moisture levels presented no significant differences to the control. By comparison, with the rise in substitution level of FM protein, fish whole body composition was affected significantly in SBM groups. Compared to the other groups, significantly higher ash and moisture content were observed in the SBM-75 group ($p\leq0.05$). However, an opposite pattern was recorded in crude protein and fat content, which tend to decrease with the increase in fish meal substitution level.

Antioxidant and Immune Analysis: The antioxidant enzyme activities in fish liver reduced significantly with increasing replacement levels of FM protein by fermented and non-fermented soybean meal (**Fig.1**). Experimental fish in the control, SBM-25, SBM-50, FSBM-25, and FSBM-50 groups exhibited higher CAT activities than those in SBM-75 and FSBM-75 groups ($p<0.05$). SOD activity remained relatively stable across most dietary treatments, except in SBM-75 and FSBM-75% groups, where a significant reduction in SOD activity was observed ($p\leq 0.05$). Immune response parameters, including WBC count and immunoglobulin (IgM) levels, varied across treatments (**Fig. 1**). WBC count was highest in the control, SBM-25 and FSBM-25

groups, with no significant difference ($p>0.05$) among them. At the 50% replacement level, the SBM-50 and FSBM-50 groups maintained comparable WBC and IgM levels, with slight reductions compared to the control group. However, a significant decline in IgM level was recorded in the SBM-75 and FSBM-75 groups ($p\leq0.05$).

Principal component analysis: Principal component analysis (PCA) was employed as a multivariate analysis method, which enables the simultaneous examination of multiple variables and their relationship among them (**Fig.2**). Growth and biochemical parameters were used as variables. The first two main component axes explained 91.7% of the total variability. The first PCA accounted for 76.7% of the variance and clearly showed a highly positive correlation among WG, SGR, SOD, CAT, WBCs, IgM and CP. The second PCA accounted for 15% of total variability, showing a high negative correlation with the variable in PCA 1; specifically, FCR and ash content were highly negatively correlated to the variables in PCA 1.

Table 4. Whole body proximate composition of *L. rohita* fed graded levels of SBM and FSBM

Parameter	Treatments						
	Control	SBM-25	SBM-50	SBM-75	FSBM-25	FSBM-50	FSBM-75
Crude protein	17.83±0.19^{a}	17.25±0.21^{ab}	16.9±0.23^{ab}	16.4±0.2^{b}	17.52±0.21^{a}	17.3±0.23^{ab}	16.98±0.17^{ab}
Crude fat	4.13±0.2^{a}	4.06±0.23^{a}	3.63±0.21^{ab}	2.96±0.17^{c}	4.3±0.20^{a}	3.97±0.2^{ab}	3.1±0.11^{bc}
Moisture	77.1±0.2^{b}	77.3±0.17^{b}	78.03±0.34^{ab}	78.7±0.23^{a}	77.1±0.19^{b}	77.4±0.26^{b}	77.7±0.21^{ab}
Ash	3.3±0.15	3.4±0.2	4.1±0.17	4.16±0.19	3.36±0.16	4.01±0.17	4.13±0.14

Values are the mean of three replicates (mean ± SE); Means in the same row with different superscripts are significantly different ($p \leq 0.05$).

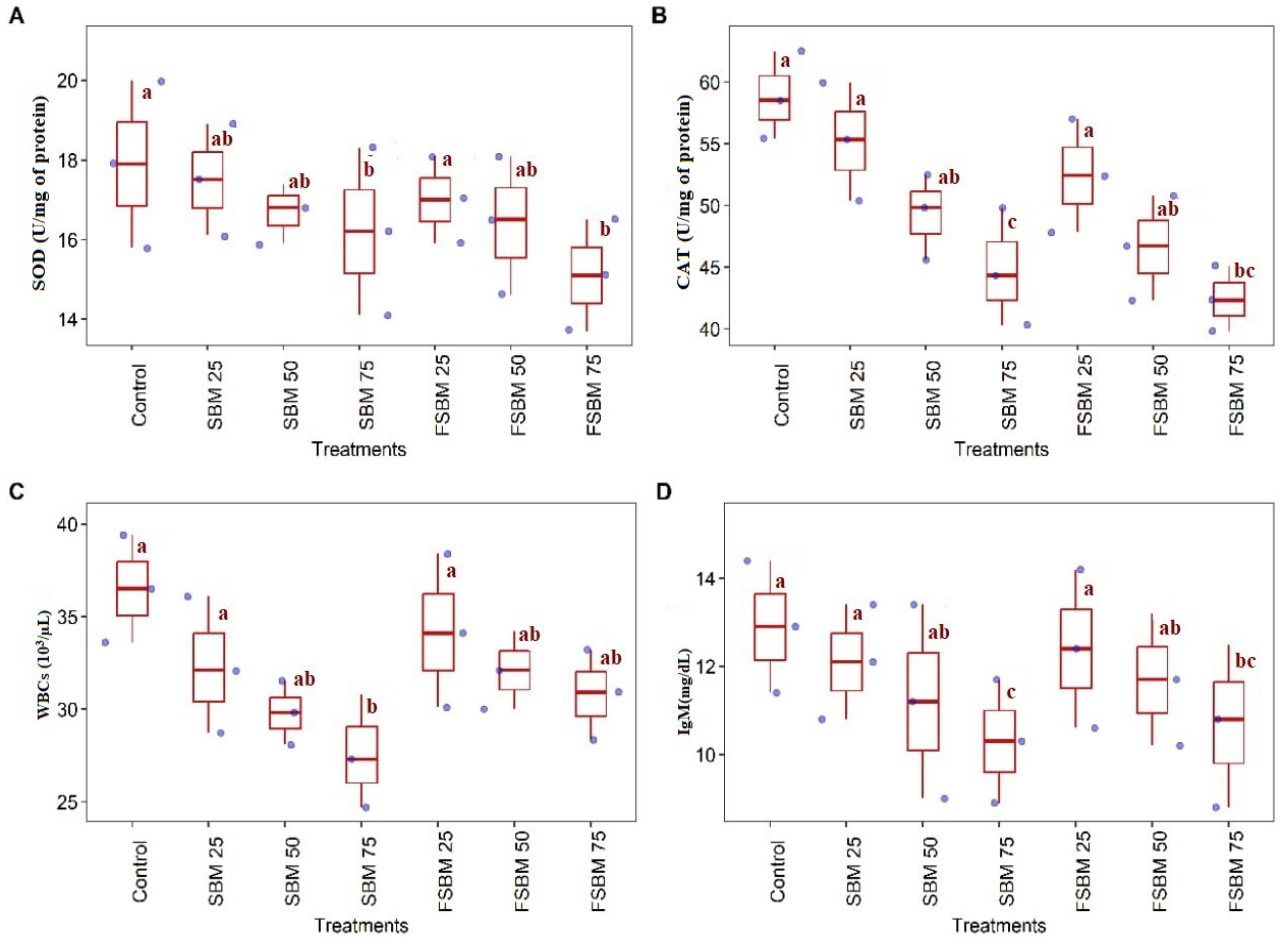

Fig. 1 Antioxidant enzymes and immune function of fish (a) Superoxide dismutase (b) Catalase (c) White blood cells (d) Immunoglobulin

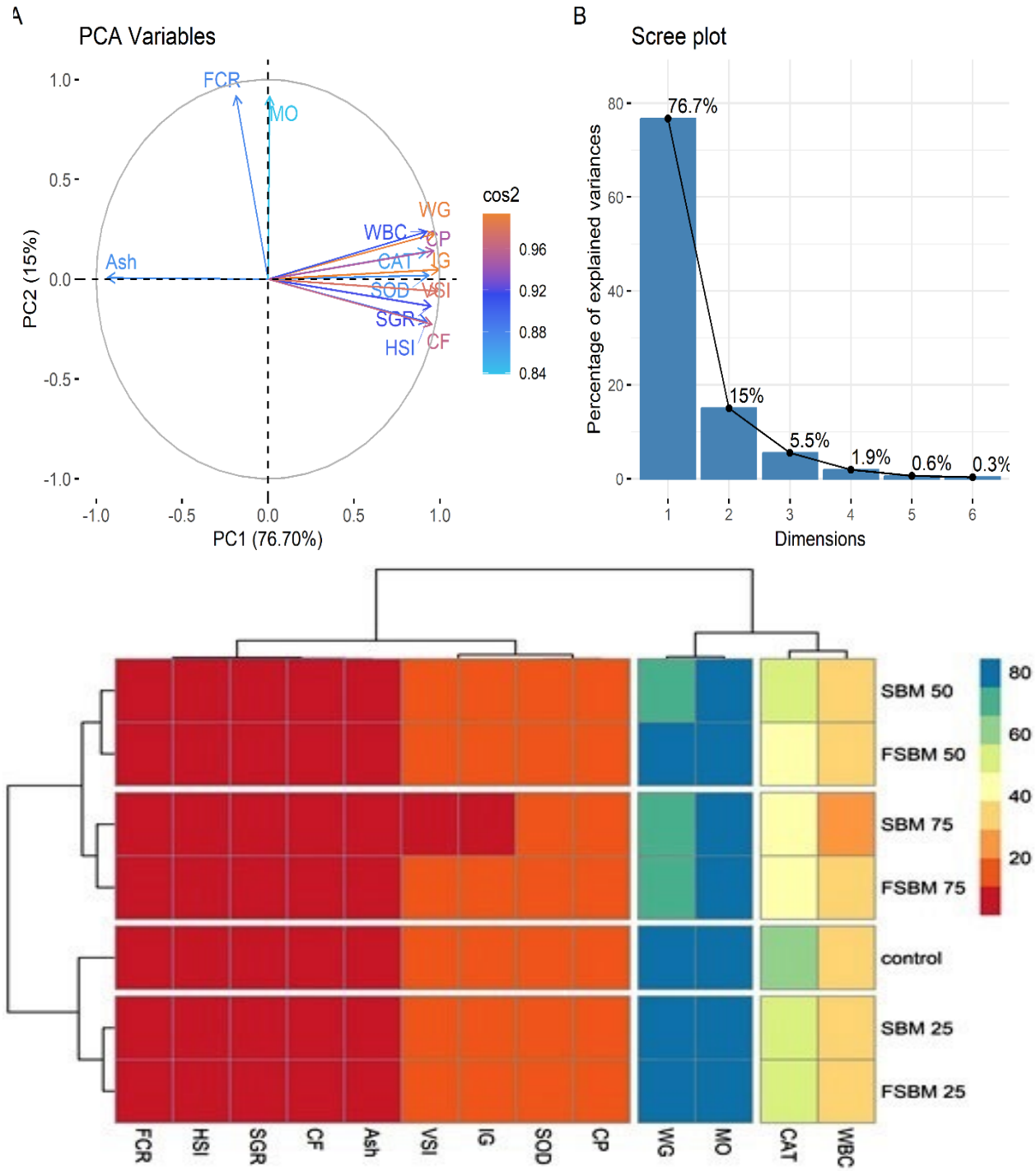

Fig. 2 Principal component analysis (PCA) and heat map: relationship between dietary fish meal replacement with SBM, FSBM and fish growth and biochemical parameters. According to Spearman heat map correlation, a close relationship was observed among growth, immunity, antioxidant and proximate body composition and color variation indicates the level of correlation between them. The color intensity is represented in the right column. WG=weight gain; FCR= feed conversion ratio; SGR= specific growth rate; HSI= Hepatosomatic index; VSI= Viscerosomatic index; SOD= Superoxide dismutase; CAT= catalase; WBCs= white blood cells; IgM= immunoglobulin; CP=crude protein; CF= crude fat; MO=moisture content.

DISCUSSION

The selection of appropriate feed components is a critical factor in formulating high-quality feed for aquatic species. One of the primary objectives of sustainable aquaculture is to achieve maximum growth by utilising the lowest inputs at the lowest cost (Zhang *et al*., 2023). Given the increasing demand for FM and concerns about its environmental impacts and availability, assessing the viability of SBM as a substitute for FM has gained significant attention. Generally, high levels of fishmeal replacement with plant protein sources have led to poor growth in fish (Abdel-Warith *et al*., 2013). The deficiency of lysine (Lys) and methionine (Met) in SBM is another critical factor limiting its replacement level in fish feeds (Wu *et al.,* 2015). Recently, fermented SBM has drawn considerable attention as a promising cost-efficient means to enhance plant protein quality (He *et al.,* 2020) by degrading ANFs and producing beneficial components such as probiotics and prebiotics, which serve as prophylactic agents (Rahimnejad *et al.,* 2021). This study aims to evaluate the impact of graded levels of FM substitution with Lactobacillus plantarum fermented and non-fermented SBM on the general health and nutritional aspects, such as body composition, growth, immune response, and antioxidant capacity of L. rohita.

In this study, substituting SBM and FSBM for a portion of FM led to a notable enhancement in growth performance, particularly at lower replacement levels. Significantly higher body weight gain (WG) and specific growth rate (SGR) were observed in the control, SBM-25, FSBM-25, SBM-50, and FSBM-50 groups compared to the experimental group fed 75% SBM and FSBM. Notably, fish fed fermented SBM diets showed better growth than those fed unfermented SBM, as evidenced by higher WG and SGR. This indicates that FSBM can effectively substitute FM in the feed without compromising growth performance, and the impact of FSBM was better than that of unfermented SBM. The improved growth performance in FSBM-fed fish is likely due to the reduced ANFs, the enhanced nutrient profile of FSBM, and improved digestibility, which facilitates better nutrient absorption and utilization. In agreement with our findings, Hassan *et al.* (2015) noted that SBM fermentation improved its nutritional value and that fermented SBM could substitute FM in the Nile Tilapia diet without adverse effects on growth and physiological conditions. Wang *et al.* (2016) demonstrated that 30% of FM can be substituted with SBM in the diet of Scophthalmus maximums, while L. plantarum FSBM could replace up to 45% of FM. These observations suggest that FSBM can be more efficiently utilized and absorbed by fish than non-fermented soybean meal.

When FM is replaced by plant protein sources, the unbalanced amino acid content is the typical shortage. Thus, the addition of amino acids to the diet can be an effective method to reduce the shortage of amino acids in feed (Wu *et al*., 2015). In the present study, the supplementation of Lys and Met likely lessened the adverse effects of high SBM inclusion, as evidenced by the improved growth performance at 25% and 50% replacement levels. This is consistent with findings in other species, such as Black Sea Bream (Jiang et al., 2018), Grass carp (Xie *et al*., 2022), Yellow Catfish (Jiang *et al*., 2018) and Rainbow Trout (Hang *et al*., 2022), where dietary Lys and Met supplementation reversed the adverse effects of high SBM inclusion and improved growth performance.

However, a significant reduction in growth response was recorded when the SBM substitution level increased to 75%. This is consistent with the findings of previous studies on FM replacement with SBM in fish feeds, where substitution levels surpassing 50% led to reduced growth and feed utilization efficiency. As reported by Pervin *et al*. (2020) and El-Dakar *et al*. (2023), substituting 50% of FM with SBM did not affect growth and immune parameters in the case of Nile Tilapia. Similar results were observed in the Silver Barb (Jahan *et al*., 2020) and Redlip mullet (Liu *et al*., 2021), where 75% dietary substitution of FM by SBM significantly reduced growth and feed efficiency. This reduction in the growth rate of fish at higher inclusion levels may be attributed to various factors, including poor palatability, reduced uptake of essential amino acids (Parveen *et al*., 2021) and the occurrence of ANFs such as phytate, trypsin inhibitors, saponins, and tannins (Li *et al*., 2022). These factors can inhibit protein digestion, nutrient absorption and amino acid availability, leading to increased FCR and, consequently, reduced growth performance (Zhang *et al.,* 2023).

To assess the efficacy of fermentation on SBM's nutritional quality, the content of crude protein, lipids, and ANFs in SBM and FSBM were mainly determined. The present study showed that fermentation with *L. plantarum* significantly decreased the ANFs (trypsin, glycinin and β-conglycinin) content; nonetheless, phytic acid level was not affected after fermentation. During fermentation, the production of several extracellular enzymes accounts for ANF degradation. Trypsin inhibitor (TI) is a major ANF in plant-based protein and could impair fish feed utilization and growth rate (Adeyemo and Onilude, 2013). In this study, trypsin inhibitor content decreased by -86.4%, glycinin level by -74.6% and b-conglycinin level by -54.4% in *L. plantarum* fermented SBM compared to non-fermented SBM. This ability of *L. plantarum* to degrade ANFs was similar to that of *L. acidophilus*, which lowered the trypsin inhibitor, glycinin and b-conglycinin content of SBM through fermentation (Li *et al*., 2022).

Furthermore, fermentation also improved the nutritive value of SBM and a moderate increase in the

crude protein level of FSBM (4.3%) was observed, which was similar to the results of Li *et al*. (2022), who stated significant increase in the crude protein level of SBM after fermentation with *L. acidophilus*. Recent works have confirmed that fermentation could enhance the level of macromolecular protein, which could improve the nutritive value of SBM and essential amino acids (El-Dakar *et al*., 2023). Increasing protein content increases protein deposition in animal muscles (Xu *et al*., 2020).

Whole body proximate composition such as protein and lipid, moisture and ash content deeply determined the nutritional value of fish and the inclusion of high plant protein levels in fish feed has been reported to cause a significant increase in moisture and decreased lipid and protein content of Japanese Sea bass (Liang *et al*., 2017), Nile Tilapia (Pervin *et al*., 2020) and Turbot (Wang *et al*., 2016). The protein content of an animal's body indicates the degree of feed absorption and utilization in a specific context. In our study, proximate body analysis revealed that increasing the replacement levels of FM with FSBM didn't result in a significant difference in crude protein, fat, moisture, and ash content in *L. plantarum-treated* FSBM groups compared to the control group. However, a substantial reduction in crude protein and fat content was recorded in 75% of SBM groups. These findings are in agreement with Wang *et al*. (2016), who reported no significant differences in proximate composition among groups fed with FSBM-based diets. Similarly, Pervin *et al.* (2020) reported a decrease in whole-body lipid and protein content in Nile Tilapia with increasing levels of FM replacement with SBM. The observed reduction in crude protein contents in the groups fed with SBM-based feeds could be attributed to the presence of ANFs in untreated SBM, which can interfere with protein digestion. In contrast, SBM fermentation with *L. plantarum* enhances nutrient availability by breaking down ANFs, thereby improving protein digestibility (Hussain *et al*., 2024).

Fish health and immune status are highly dependent on the antioxidant system. In addition to demonstrating the antioxidant capacity of aquatic organisms, assessment of SOD and CAT activity can also be considered as the biomarker for oxidative stress (Dawood *et al.*, 2016). In this study, the replacement of FM with SBM significantly reduced the activities of key antioxidant enzymes SOD and CAT, particularly at higher inclusion levels. This reduction in antioxidant activity could result from oxidative stress induced by reactive oxygen species (ROS), which can interfere with the functionality of these enzymes, ultimately compromising the fish defense mechanism (Wang *et al*., 2017). Additionally, the deficiency of essential amino acids, particularly Met and Lys, in SBM-based diets can aggravate oxidative stress by restraining the synthesis of antioxidant enzymes and other proteins crucial for immune function and cellular repair (Jiang *et al*., 2018). In contrast, fish-fed FSBM diets exhibited a significant increase in CAT and SOD activities, suggesting that fermentation of SBM enhances the antioxidant defense and immune response of *L. rohita* by stimulating the production of bioactive peptides that regulate oxidative stress through the modulation of enzymatic antioxidant pathways (Zhang *et al*., 2023). Similar results were reported by Lee *et al*. (2016), who observed increased SOD and CAT activities in *Sebastes schlegelii* fed diets containing *Bacillus subtilis* fermented SBM.

The immune response of fish to dietary ingredients has gained significant consideration in recent years, with blood biochemical and immunological parameters increasingly being used as valuable diagnostic tools in fish nutrition studies (Rahimnejad *et al*., 2021). Fish rely on white blood cells (WBCs), which act as the first line of defense against pathogens, while IgM serves as the primary antibody responsible for neutralizing infections (Kiron, 2012). In this study, replacing FM with SBM and FSBM revealed significant effects on WBCs and IgM levels. Although fermentation can decrease the ANFs in SBM, higher inclusion levels of FSBM have been shown to impair the immune response of aquatic animals, likely due to the residual ANFs and imbalance in essential nutrients, which may affect immune function at increased inclusion levels (Zhang *et al.,* 2021). However, in this study, fish-fed diets with 25% and 50% SBM and FSBM diets exhibited a significant increase in WBC levels, indicating an enhanced immune response. These findings align with those of Kader *et al*. (2012), who reported no adverse effects on WBC levels with up to 48% FM replacement by SBM. Similarly, Howlader *et al*. (2023) observed no significant difference in WBCs and IgM levels up to 50% replacement of FM protein by SBM in fish feeds. However, a significant decrease in these parameters was recorded at the 75% replacement level, indicating that the fish's immune system was compromised due to potential physiological stress or nutrient imbalance at higher inclusion levels (Wang *et al*., 2016).

Conclusion: In conclusion, the results of this study indicated that SBM and FSBM protein can substitute 25% and 50% of FM in the diet of *Labeo rohita* without significant adverse effects on growth, immune response and antioxidant activities when supplemented with lysine and methionine. The supplementation of these essential amino acids is crucial to compensate for the reduced protein and amino acid content in plant-based diets.

Authors' contribution*:* AM and NA designed the study**,** NA performed the research experiment, AM devised ideas and supervised the whole experiment, SP helped in data analysis and helped formulate plant-based feed. SP and AM provided the critical revision and final approval of the article.

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