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Supplementation of acidifiers & exogenous enzymes improves the growth performance of *Labeo rohita* fed with leaf meal-based diet

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Abstract
The present study aimed to investigate the effect of combinatorial supplementation of bile acid, citric acid and xylanase enzymes on the growth performance of *Labeo rohita* fed with fermented sesbania leaf meal-based diet. Eighteen hapas were fixed in an earthen pond (2m×1m×1m dimension**)** and each hapa was stocked with 10 *Labeo rohita* juveniles (18.67±0.26 g.). *Labeo rohita* juveniles were fed with a basal diet containing fermented sesbania leaf-meal (30%) as a control. Four experimental diets T1, T2, T3 & T4 were supplemented with bile acid & citric acid, bile acid & xylanase, citric acid & xylanase and bile acid, citric acid & xylanase, respectively in the basal diet. The positive control group was fed with commercial carp feed. The result showed that weight gain percentage, specific growth rate (SGR) and FCR were significantly improved compare to the control group among all the treatment groups. The weight gain percentage was significantly higher in the Positive Control group (PC) (111.11%) and T4 (104.96%) with no significant difference among them. The least weight gain % recorded in control group (64.13%). In comparison to the positive control, T4 group did not illustrate any significant difference (P>0.05) in any growth parameters like weight gain percentage, specific growth rate (SGR) and FCR. Treatments supplemented with a combination of bile acid, xylanase and citric acid showed enhanced lipase & amylase activity along with increased bioavailability of minerals. The present study has demonstrated that dietary bile acid, citric acid and xylanase enzyme supplementation in fermented sesbania leaf meal-based diet led to enhanced utilization of nutrients from plant ingredient-based diet and improved the growth performance of *Labeo rohita*.

Keywords: Bile acid (BA), Citric acid (CA), Sesbania leaf meal (SLM), Fermented sesbania leaf meal (FSLM)

1. Introduction
Aquaculture is being forecasted as the central core for economic growth in the agriculture sector in India. As per one of the fascinating predictions of the Food and Agriculture Organization, worldwide capture fishery production has been drenched. FAO (2018) [8] has projected that the global yield of capture fishery will be 91 million tons in 2030, only 1% higher than that in 2016. But aquaculture sector will remain to play its major role, with an estimated annual production of 109 million tons by 2030, almost 37% higher compare to 2016. With the increase in diversification and intensification of fish farming, the aquaculture sector has maintained its high level of production, but it's also resulting in increased demand for fish feed ingredients and another aspect of environmental stress, which are the major limiting factor for the aquaculture sector (Nawaz *et al*., 2018) [19]. Hence, there is an urgent need to develop cost-effective and environmentally friendly fish feed, for enhancing growth rate and nutrient utilization efficiency without putting fish under stress. With the growth of the aquaculture sector, production of aqua-feeds at the world level is also growing and these days, the fish feed includes a wide range of feed ingredients that were not previously utilized for feeding fish such plant feed ingredient.
With the increased use of plant feed ingredients in fish feed production, supplementation of bile acids (BAs) is attracting the attentions of aquaculture industry because some of the key compounds of the BAs like taurine and cholesterol, are usually limited in plant leaf meal. Some recent studies had showed that, BAs as a feed additive have confirmed their positive effects on enhancing fish performance (Jiang *et al*., 2018) [12]. Citric acid (CA) is another exogenous enzyme which is extensively used in animal feed industry as an acidifier in diet. (Baruah *et al*. (2007) [7] reported that supplementation of citric acid (3%) acidifier in fish feed

enhances the bioavailability of several minerals, this decreases the requirement for supplementation minerals which decreases the price of feed. As per the existing studies on CA in fish feed, CA has potential to improve growth and nutrient utilization by fish. Its acidification in fish feed can be used to formulate cost effective and environment friendly feed. Xylanase is an exogenous enzyme which disrupts integrity of plant cell wall and in that way, it decreases the size of non-starch polysaccharides (NSPs). Subsequently, it enhances digestion by reducing viscosity in fish gut (Adeola & Cowieson, 2011) [1]. The present study was designed to evaluate the effect of feeding fermented Sesbania leaf meal supplemented with acidifiers and exogenous enzymes (citric acid, Bile Acid and xylanase enzyme) on growth of *Labeo rohita* in five different combinatorial form.

2. Material and Method

2.1 Collection and processing of sesbania leaf (dhaincha) leave: Sesbania plants were collected from the agriculture field of Gorakhpur, a northeast district of Uttar Pradesh state of India. The leaves were hand-picked and leaflets were separated from the longleaf stalk. The picked leaves were dried at 40 °C for 48hrs in a hot air oven, powdered, sieved, labelled and packed in an airtight container.

2.2 Solid state fermentation: Two kilograms of the leaf powder were weighed and put into two conical flasks and autoclaved. This autoclaved powder was subjected to solid-state fermentation (50% w/v) in the conical flasks using *Bacillus subtilis* bacteria by properly mixing it using an autoclaved glass rod. The mouth of the conical flasks was then tightly plugged using autoclaved cotton. The fermentation was carried out for 6 days with regular mixing with a glass rod once a day.

2.3 Proximate analysis: The water content of sesbania leaf sample was determined by AOAC, (1990) method by weighing and drying in a hot air oven at 105 °C till a constant weight was achieved. Total nitrogen was estimated by using an automated nitrogen analyser (KEL Plus-Classic DX VA, Pelican Equipment, India) and Crude protein was calculated by multiplying the total nitrogen with a factor of 6.25. The ether extract was estimated by an automatic fat extraction system (SOCS PLUS-SCS 08 AS, Pelican Equipment, India) using petroleum ether (Boiling point 60-80 ºC) as the solvent. Total ash content was estimated in a muffle furnace (AI-7981, Expo Hi-Tech, Mumbai) at 550 °C for 6 hours. The crude fibre was estimated following AOAC (1990). The total carbohydrate of the experimental diets was calculated by subtracting the percentage of other nutrients from 100.

Determination of Anti-nutritional Compounds

The method of AOAC (1990) was used in the determination of cyanogenic glycosides using alkaline titration. Phytic acid was carried out following method by spectrophotometer (Shimadzu, UV1800 and Kyoto, Japan). Tannin content was determined using the standard method described by AOAC (1990) using Oxalic (0.1N). Saponin was estimated following the standard method of AOAC (1990) using gravimetric method employing the use of a soxhlet extractor and two different organic solvents. The mimosine content of the leaf powder was determined by the rapid colorimetric method of (Megarrity, 1978) [22] by measuring the absorbance at 535 nm in Shimadzu, UV1800 and Kyoto, Japan visible spectrophotometer. The mimosine concentration was then calculated from a standard curve using pure mimosine (Sigma Chem., Ltd. London). For Oxalate determination, the titration method was used as per Day and Underwood (1986)).

2.5 Diet preparation

Six isonitrogenous (30% crude protein) practical diets for the experiment were prepared (Table 1) to replace DORB with fermented sesbania leaf meal (FSLM) viz. C (Control 30% inclusion of FSLM), Positive control (PC): Commercial feed (purchased), T(1): 30% inclusion of FSLM supplemented with bile Acid and citric acid, T(2): 30% inclusion of FSLM supplemented with bile acid and xylanase enzyme, T(3): 30% inclusion of FSLM supplemented with citric acid and xylanase enzyme, T(4): 30% inclusion of FSLM supplemented with bile acid (0.015%), citric acid (0.3%), xylanase enzyme (0.01%).

2.6 Experimental design

The experiment was conducted in hapa of size: 2mx1mx1m in an earthen pond (dimension: 115m x 35m) at the Livestock research station, Assam Agricultural University, Hekra, Assam. Experimental fish were divided into three groups as Control, Positive Control and Experimental hapas. Fish in the control hapa were fed only with a fermented Sesbania leaf meal-based diet (30% inclusion), fish in the positive control hapa was fed with commercial feed, the other experimental hapa were fed with experimental diet supplement with exogenous enzymes in four different combinations. Each treatment had triplicates. The feed was given twice a day of 6 am and 6 pm. The experiment was conducted for 60 days. Sampling was done at intervals of 15 days to assess the bodyweight of the fish. Fishes were starved overnight before taking the weight in an electric balance.

2.7 Growth performance and nutrient utilization

Parameters pertaining to growth and nutrient utilization were calculated using standard formula. Weight gain (%) = [(final weight-initial weight)/initial weight] ×100; specific growth rate (%) = [ln (Final weight)-ln (Initial weight)/[no. of days] ×100; feed conversion ratio = {feed consumption (g on dry weight basis)/body weight gain (g on wet weight basis)}; protein efficiency ratio = {net weight gain (g on wet weight basis)/protein fed (g on dry matter basis).

2.8 Sample collection

While sampling, all the ethical guidelines of the animal cares for ICAR-Central Institute of Fisheries Education, Mumbai India was strictly followed. Randomly selected fish from each treatment's group, that is, two fish from each replicate (n = 6), were anaesthetized by using clove oil (50 µl/L).

2.9 Statistical analysis

Statistical analysis of the data was carried out with statistical software Package 231 SPPS (ver. 22), in which all data were subjected to one-way ANOVA and Duncan's multiple range tests for determination the significant differences between the means.

3. Result & discussion

Use of plant-derived materials in fish feed ingredients is limited due to the presence of a wide variety of ANFs and NSPs (Halver, 2002). Hence, solid state fermentation of sesbania leaf meal was done for 6 days with *Bacillus subtilis*

(1x10^8 CFU) to improve its nutritional profile. The proximate analysis of fermented sesbania leaf meal showed that compare to raw sesbania leaf meal, there was change in chemical composition of fermented sesbania leaf meal. The crude protein was of 32.20%, ether extract (EE)-2.67%, crude fibre (CF)-10.41%, ash-9.10%, Nitrogen free extract (NFE)-46.02%. There was also reduction in level of anti-nutritional factors in fermented sesbania leaf meal *viz.* tannin, oxalate, saponin, cyanid, phytate, alkaloids, TIA and Mimosin (table 1) but was not entirely eliminated. Since, no study has been reported to investigate the beneficial effect of dietary supplementation of acidifier and exogenous enzymes in fermented sesbania leaf meal, hence, this study was conducted to evaluate the combinatorial impact of acidifiers and exogenous enzymes on fish growth.

Under the present study, along with the commercial feed (positive control) four combinatorial supplementations of exogenous enzymes of citric acid, bile acid and xylanase enzyme were taken with a basal fermented leaf meal-based diet (table 2) to study nutrient utilization and growth performance by *Labeo rohita*.

All experimental feeds were isonitrogenous (30.41-31.46%). After analysis, it was found that the ether extract in the diets varied from 6.12 to 6.59%. The ash content in the diets varied from 7.10 to 9.06%. The crude fibre of the experimental feed was estimated in the range of 7.86 to 8.90%. The calculated nitrogen-free extract ranged from 48.95 to 49.02%. The result of the proximate composition of the diets is shown in Table 3.

A 60-day of feeding trial was conducted with design feed in order to study the growth and the physiological performance of *Labeo rohita*. The weight gain % was significantly higher in the positive control (PC) group (111.11%), fed with commercial feed followed by the T4 group (104.96%) with no significant difference (P>0.05) among them. The least weight gain percentage was recorded in control (64.13%) group (Table 5). Similar trend was also recorded for SGR and PER. The mean of FCR was higher for the Control Group (2.27) followed by T3(1.97) and the least value recorded in Positive control (1.60). The highest growth in the Positive control might be due to certain undisclosed ingredients of the commercial feed which might be enhancing the better digestibility and mineral absorption from the feed. Whereas under formulated experimental feed (C, T1, T2, T3 and T4), the result of this experiment shown that combinatorial supplementation of bile acid, xylanase, and citric acid (T4 group) in fermented sesbania leaf meal-based diet shown better result compare to other treatments, and were parallel to Positive Control (PC) group. There was no significant difference in growth and feed efficiency parameters.

Whereas growth in T1 (Bile Acid+ Citric Acid) was not significantly different from T2 (Bile Acid+ Xylanase Enzyme) and T3 (Citric Acid +Xylanase) but T1, T2 and T3 shown better growth compared to the control group. This increase in growth rate in treatments compare to the control group might be due to the effects of the bile acid, citric acid, and xylanase incorporated in the experimental feed, as xylanase supplementation in fish feed seem to be increased protein utilization and growth (Jiang *et al*., 2014) [13], whereas, Bile acid facilitates the digestion of lipid by enhancing the intestinal absorption of fat or fat-soluble vitamins (Maldonado-Valderrama, Wilde, Macierzanka & Mackie, 2011) [18] and citric acid improves the bioavailability of minerals (Baruah *et al*., 2007) [7].

In the present study, the addition of xylanase improved the weight gain % and FCR in T2, T3 and T4 groups compared to the Control group. Similarly, Jiang *et al*., (2014) [13] reported that the growth performance of juvenile Jian carp was improved with xylanase supplementation. Improvement of final body weight with dietary xylanase supplementation was also found in Japanese sea bass (*L. japonica*) (Ai *et al*., 2007) [4] and African catfish (*C. gariepinus*) (Babalola *et al*., 2006) [6]. Overdose of xylanase diet in Juvenile Jian carp showed poor weight gain, which can be correlated with the overproduction of xylose and xylooligosaccharides. In addition, excess xylose may result in osmotic diarrhoea or poor performance in chickens (Schutte 1990). Similar results reported that xylanase supplementation enhanced the growth performance of fish (Ai *et al*., 2007; Babalola, 2006) [4, 6]. The improvement in growth and nutrient utilization of a fish feed diet supplemented with xylanase or complex with other enzymes could be attributed to the degradation of NSPs to the level that the viscosity property of these fractions is largely reduced (Jiang *et al*., 2014) [13].

A range of studies has demonstrated that bile acid supplementation promoted fish growth even though they were fed with diets containing fishmeal or other animal origin ingredients the effective dose of supplementation of BAs had been reported in different studies. Zheng *et al*. (2016) [31] estimated that the optimum dietary BA supplementation was 0.22-0.27 g/kg based on WG and FCR of prenant's schizothoracin (*Schizothora xprenanti*) fed test diets containing 42% fishmeal. The optimum dietary bile acid (mixtures of hyodeoxycholic acid and lithocholic acid) supplementation was 0.17-0.19 g/kg for grass carp fed a diet containing 100 g/kg fishmeal and 40 g/kg fish oil based on the growth performance (Zeng *et al*., 2017) [30]. In another study, Sun *et al*., (2014) [27] suggested higher level of BA up to 1.5 g/kg for juvenile turbot even though when test diets contained 400 g/kg of fishmeal and 50-130 g/kg of fish oil. Hence, these studies shows that the required optimal level of BAs may vary depending on species of fish, type of feed formulation, and other specific culture conditions.

As per the result, incorporation of the citric acid (CA) in the experimental diet of T1, T3 and T4 improved the growth performance of the *Labeo rohita* compare to the Control (C) group. As per the studies conducted, CA supplemented diet have shown inspiring results (Sarker *et al*., 2005; Pandey and Satoh, 2008) [24, 21]. There was an increase the weight gain and specific growth rate along with decreased the feed conversion rate in Rohu (Baruah *et al*., 2007) [7], when 3% citric acid was incorporated in diet. Similarly, feed performance was improved in red sea bream (Sarker *et al*., 2005) [24] and protein efficiency ratio in Beluga (Khajepour and Hosseini, 2012) [14], due to incorporation of 3% CA in fish feed. In other studies, it was reported that 1% CA enhanced weight and feed conversion ratio in red sea bream (Hossain *et al*., 2007) while a similar increase in weight was also observed in Yellowtail (Sarker *et al*., 2012) [23]. However, in another study, Sarker *et al*., (2012) [23] observed that there was no effect of CA (0.5%) on specific growth rate and feed conversion ratio parameters in Yellowtail. Studies with aquatic animals showed that adding 2.0 g kg^{-1} of CA in the diet increased the weight gain of tilapia, *Oreochromis niloticus x O. aureus* (Pan *et al*., 2004) by 15.3% and adding 3.0 g kg^{-1} of CA in the diet of allogynogenetic crucian carp, *Carassius auratus gibelio* (Leng *et al*. 2006) [17] by increased weight gain by 10.3%, while decreased FCR by 15.2% and 7.2% respectively. Dietary 30.0 g kg^{-1} CA improved the WG of red sea bream,

Pagrus major by 19.1% (Sarker *et al*. 2005) [24]. The addition of 30 g kg^{-1} CA (Khajepour and Hosseini 2012) [14] in the diet with fish meal partly replaced by soybean meal, the weight gains of beluga sturgeon, *Huso huso*, was improved by 11.1% and FCR decreased by 7.6%. Su *et al*., (2014) [26] reported that there was increased WG and decreased FCR of the shrimp ($P<0.05$), when shrimp fed with 2.0 g kg^{-1} CA in diet. Studies above indicated that a proper level of dietary CA can improve the growth performance of aquatic animals. It is noteworthy that the additional level of CA in the diet of aquatic animals mentioned above is remarkably different, for example, the suitable level of CA was 2.0 g kg^{-1} for *Carassius auratus gibelio* (Leng *et al*. 2006) [17], 3.0 g kg^{-1} for *Oreochromis niloticus* x *O. aureus* (Pan *et al*. 2004), 30.0 g kg^{-1} for Pagrus major (Sarker *et al*. 2005) [24] and *Huso huso* (Khajepour and Hosseini 2012) [14]. Baruah *et al*. (2007) [7] reported significant improvements to the growth performance, feed conversion ratio (FCR) and P availability in rohu fed with 30 g kg^{1} citric acid supplemented soya bean meal-based diet. However, it did not affect the protein efficiency ratio (PER) of fish. Baruah *et al*. (2007) [7] reported that this growth improvement might be due to beneficial effect of citric acid, which might have released minerals from the phytic acid complex of the soya bean meal-based diet.

Further, there was an increase in the ash content of the carcass of *Labeo rohita* among the citric acid supplemented diets *viz.* T1, T3 and T4 compare two T2 and control (C) groups (table 4). It might be due to the positive effect of citric acid in mineral absorption. The ash content is a very well-documented parameter to estimate the mineralization of bones and muscles in fish. The administration of 3% CA improves the ash contents of muscle (Baruah *et al*., 2005) in rohu due to increase in bioavailability of minerals from the fish diet. In Beluga also, increase in the muscle ash content was reported by Khajepour and Hosseini (2012) [14]. Similarly, the addition of lower levels of citric acid (1-3 g/kg) had also resulted in increased weight gain in tilapia (Ng *et al*., 2009; Koh *et al*., 2014) [20, 16]. Results of the present study demonstrated a positive effect of citric acid for improving the growth performance of *Labeo rohita* fingerlings. The present positive effect of citric acid supplements to improve growth performance is in accordance to Baruah *et al*. (2005; 2007) [7] for same fish species.

Endogenous enzymes play a major role in digesting nutrients for fish, directly enhance the digestive ability of animals (Adeoye *et al*., 2016; Wen, Zhou, Feng, Jiang, & Liu, 2009) [2, 28]. In the present study, lipase activity was higher in T1, T2 and T4 compared to Control (C) and T3 groups. This might be due to the presence of bile Acid in the experimental diet of T1, T2 and T4, which improves lipid digestion and metabolism. One of the major roles of bile acids is known to be involved in lipid emulsification that assists in breaking down large lipid molecules into small globules, which provides lipase with increased surface area for lipid digestion (NRC, 2011). Increased lipase activity was observed in tilapia when supplemented with Dietary BAs (Ogata *et al*., 2003). Similar results were observed in juvenile of rainbow trout (Adhami *et al*., 2017) [3], grass carp (Zeng *et al*., 2017) [30] and Japanese founder, *Paralichthys olivaceus* (Alam *et al*., 2001) [5]. This increase in lipase activity can be correlated with the decrease in the lipid level in the carcass of the *Labeo rohita* fingerlings fed with an experimental diet supplemented with Bile Acid viz. T1, T2 and T4. Results of this study demonstrates that bile acid supplementation led to low lipid accumulation in body tissues of *Labeo rohita*. Similar results have been observed in grass carp (Zeng *et al*., 2017) [30], prenant's schizothoracin (Zheng *et al*., 2016) [31] and turbot (Sun *et al*., 2014) [27]. This decrease in lipid accumulation in the carcass may be attributed to increased lipid metabolism for energy by enhancing lipolysis, a process for breaking down triglyceride.

The present study showed that the 30% inclusion level of fermented sesbania leaf meal (Control group) recorded the lowest activity of digestive enzymes (table 6). This decrease in digestive enzyme activity of Control group can be attributed to the presence of antinutritional factors, fibre and NSP in fermented sesbania leaf meal which reduced the digestion (Hassaan *et al*., 2017) [10]. Amylase activity was higher in treatment groups fed xylanase supplemented diets (table 6). The activities of amylase in the intestine improved with the increasing xylanase levels up to a certain level (Jiang *et al*., 2014) [13]. The positive effect of dietary xylanase on the activities of digestive enzymes can be related to the degradation products of arabinoxylans. The addition of xylanase increased the activities of amylase in the intestine of *Labeo rohita.* It is also reported that exogenous enzymes improve the activity of endogenous enzymes (Hlophe-Ginindza *et al*., 2016) [11]. This improved activity of endogenous enzymes might be due to the action of xylanase in the degradation products of arabinoxylans, hydrolyse cell wall components in the plant material; thereby, it reduced the molecular size characteristics of NSPs content of the plant materials. Therefore, this finding can be related to the role of the exogenous xylanase in releasing the bound nutrients and promoting rapid digestion in *Labeo rohita* by reducing the digesta viscosity in the gut of *Labeo rohita.*

In the present study, positive control (commercial feed) as the higher activities of AST and ALT in the muscle and liver shows that there was a synthesis of non-essential amino acids finally it resulted in good growth or better weight gain in the positive control group and T4 group compare to control and other treatments (table 6). So, these metabolic enzyme activities positively correlated with growth parameters. The least activity of AST and ALT found in the control group, shows that in the absence of exogenous enzymes, there was less utilization/absorption of the nutrients from the experimental diet by *Labeo rohita*.

Antioxidant enzymes are the primary defence to counter reactive oxygen species, hence their activity levels are considered as the indicator of oxidative stress. This study showed that SOD and catalase activities were not affected significantly among different experimental groups (table 7). Hence, it can be concluded that fish fed with 30% Fermented sesbania leaf meal inclusion level and along with supplementation feed additives *viz*. citric acid, bile acid and xylanase enzyme does not cause any stress on the *Labeo rohita.* However, there are no parallel study available about the effect of fermented sesbania leaf meal supplemented with feed additives on antioxidant enzymes in fish.

Haematological parameters including red blood cells (RBCs) count, haemoglobin (Hb) and white blood cells (WBCs) count are considered valuable indices to assess fish health (Roberts and Rodger, 1978) [22]. In the present study, no change in haematological indices was recorded, which reflects that supplementation of exogenous enzymes did not cause any stress (table 8). In Beluga, exogenous enzymes showed no effect on RBCs and WBCs count which reflects that acidifier does not cause any stress. Likewise, mean cell volume

(MCV), mean cell haemoglobin (MCH), serum glucose and total proteins were not affected by the inclusion of CA. This indicates that acidification does not cause any metabolic stress. Protein is the most important compound in the serum with albumin and globulin being the major serum protein, which plays a very important role in the immune response. In the present study total protein level in the serum of fish were significantly different, whereas albumin, globulin and A/G ratio found to be similar among the treatments (table 9), which are in agreement with the findings of Zhou *et al*., (2014), who also observed significant changes in the total protein level but did not find any significant change in the globulin level and A/G ratio of *L. rohita*, suggesting that supplementation diet did not have an immunosuppressive effect.

Tables 1: Effect of solid-state fermentation on proximate composition and antinutritional factors of Sesbania leaf meal

Raw & Fermenation Value→	RSLM	FSLM	%(-inc./+dec.)
CP (%)	25.37	32.20	26.92↑
EE (%)	4.70	2.67	43.22↓
CF (%)	11.60	10.41	10.25↓
Ash (%)	8.57	9.10	6.14↑
NFE (%)	49.70	46.02	7.40↓
Tannin (mg/100g)	0.20	0.08	60.14↓
Oxalate (%)	0.60	0.38	35.89↓
Saponin (%)	3.01	1.16	61.53↓
Cyanid (mg/100g)	21.23	11.90	43.94↓
Phytate (mg/100g)	15.26	6.57	56.90↓
Alkaloids (%)	24.06	10.93	54.58↓
Tia (mg/100g)	0.44	0.22	49.18↓
Mimosin (%)	2.25	1.54	31.75↓

Abbreviations: ASH, total ash content; CF, crude fibre; CL, crude lipid; CP, crude protein; RSLM, raw sesbania leaf meal; FSLM, fermented sesbania leaf meal; NFE, nitrogen-free extract; RSLM, raw sesbania leaf meal.

Table 2: Composition of different experimental diet experimental diets (% dry matter)

Ingredients %	Control (C)	Positive control (PC)	T1	T2	T3	T4
DSBM	20.2	*	20	20.2	20	20
GNOC	18	*	18.4	18	18.2	18.5
Wheat Flour	11.17	*	10.66	11.15	10.86	10.55
DORB	12	*	12	12	12	12
Fermented sesbania	30	*	30	30	30	30
Vit-Mineral mix	2	*	2	2	2	2
VIT C	0.1	*	0.1	0.1	0.1	0.1
CMC	0.5	*	0.5	0.5	0.5	0.5
BHT	0.03	*	0.03	0.03	0.03	0.03
OIL	6	*	6	6	6	6
Bile Salt	0	*	0.015	0.015	0	0.015
Acidifier: Citric acid	0	*	0.3	0	0.3	0.3
Enzyme: Xylanase	0	*	0	0.01	0.01	0.01
Total	100	*	100	100	100	100

GNOC=Ground Nut Oil Cake, DORB=De-oiled Rice Bran, CMC=Carboxy Methyl Cellulose, BHT-Butylated Hydroxy Toluene, DM=dry matter.

Composition of Vitamin-mineral mix (PREMIX PLUS) (quantity.kg^{-1}), 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 B1 (100 mg), Vitamin B2 (200 mg), Vitamin B6(1,000 mg); Vitamin B12 (6 mcg); 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) (10g); Selenium (125mg).

*feed ingredients composition was not disclosed by the firm.

Table 3: Proximate composition of experimental diets (% dry matter basis)

Treatment	C	PC	T1	T2	T3	T4
Moisture	5.98±0.31	5.42±0.80	5.63±0.16	6.00±0.01	5.64±0.01	5.38±1.07
Crude protein	30.44±0.59	30.41±0.80	31.46±0.01	30.47±0.03	31.38±0.03	31.21±0.62
Ether extract	6.59±0.24	6.12±0.25	6.33±0.21	6.17±0.02	6.12±0.02	6.32±0.41
Nitrogen free extract	48.95±0.30	48.99±0.86	49.02±0.06	48.97±0.03	48.96±0.03	48.97±0.64
Ash	7.10±0.05	9.06±0.64	8.39±0.05	7.56±0.02	8.04±0.24	8.11±0.46
Crude fiber	7.86±0.44	8.37±0.01	8.90±0.03	7.87±0.21	8.14±0.03	8.22±0.50

All values are Mean ± SE, obtained from three replicates. Values in the same row with different superscript letters are significantly different (P<0.05).

Control (C)-Fermented sesbania leaf meal-based diet (30% inclusion); Positive Control (PC)- commercially available carp feed.; T1-Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid and citric acid; T2: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid and Xylanase Enzyme,T3: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Citric Acid and Xylanase Enzyme, T4: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid, Citric Acid and Xylanase Enzyme.

Table 4: Carcass composition of whole body of *Labeo rohita* fingerlings fed with each experimental diets (% wet weight basis ± SE) at the end of 60 day of experimental feeding

Treatment	Moisture	CP	Lipid	ASH	NFE
Control(C)	75.50±0.19	15.35±0.07	3.37±0.07	3.39^{a}±0.15	1.24±0.08
Positive Control (PC)	75.42±0.11	15.5067±0.09	3.58±0.16	4.08^{b}±0.12	1.4±0.18
T1	76.01±0.39	15.58±0.36	3.69±0.09	4.14^{b}±0.18	1.43±0.04
T2	75.42±0.33	15.56±0.17	3.33±0.13	3.49^{a}±0.06	2.18±0.49
T3	75.33±0.23	15.03±0.21	3.43±0.12	4.33^{b}±0.08	1.86±0.18
T4	75.46±0.23	15.37±0.11	3.56±0.17	4.35^{b}±0.13	1.56±0.12
p-value	0.55	0.43	0.41	<0.01	0.13

All values are Mean ± SE, obtained from three replicates. Values in the same row with different superscript letters are significantly different (P<0.05).

Control (C)-Fermented sesbania leaf meal-based diet (30% inclusion); Positive Control (PC)-commercially available carp feed.; T1-Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid and citric acid; T2: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid and Xylanase Enzyme,T3: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Citric Acid and Xylanase Enzyme, T4: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid, Citric Acid and Xylanase Enzyme.

Table 5: Growth performance of *L. rohita* fingerlings fed with different experimental diets

Treatment	Initial weight (g)	Final weight (g)	WG%	SGR	FCR	PER
C	18.66±0.24	30.62^{a}±0.47	64.13^{a}±1.64	0.82^{a}±0.01	2.27^{e}±0.04	1.47^{a}±0.03
PC	19.10±0.38	40.30^{e}±0.3	111.11^{d}±4.28	1.24^{d}±0.03	1.48^{a}±0.04	2.25^{d}±0.06
T1	18.48±0.21	33.25^{b}±0.19	80.03^{bc}±3.05	0.98^{bc}±0.03	1.90^{cd}±0.05	1.75^{bc}±0.05
T2	18.43±0.18	35.30^{c}±0.47	91.57^{c}±4.39	1.08^{c}±0.03	1.72^{bc}±0.06	1.94^{c}±0.07
T3	18.74±0.29	33.08^{b}±0.52	76.62^{b}±4.46	0.94^{b}±0.04	1.97^{d}±0.09	1.69^{b}±0.08
T4	18.60±0.25	38.10^{d}±0.36	104.96^{d}±4.64	1.19^{d}±0.03	1.54^{ab}±0.05	2.16^{d}±0.07
p-value	0.138	<0.01	<0.01	<0.01	<0.01	<0.01

All values are Mean ± SE, obtained from three replicates. Values in the same row with different superscript letters are significantly different (P<0.05).

Control (C)-Fermented sesbania leaf meal-based diet (30% inclusion); Positive Control (PC)-commercially available carp feed.; T1-Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid and citric acid; T2: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid and Xylanase Enzyme, T3: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Citric Acid and Xylanase Enzyme, T4: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid, Citric Acid and Xylanase Enzyme.

WG % = Weight Gain %, SGR = Specific Growth Rate, FCR = Feed Conversion Ratio, PER= Protein Efficiency Ratio.

Table 6: Digestive enzymes activity and Tissue ALT (Alanine aminotransferase) and AST (Aspartate aminotransferase) activities of *L. rohita* fed with different experimental diet

Treatment	Lipase	Protease	Amylase	GOT (AST) Liver	GOT (AST) Muscle	GPT (ALT) Liver	GPT (ALT) Muscle
C	0.10^{a} ±0.01	1.05^{a}±.041	0.97^{a}±0.07	29.69^{a}±1.16	24.13^{a}±0.13	23.83^{a}±0.28	27.23^{a}±0.10
PC	0.15^{ab} ±0.01	1.54^{b}±.036	1.58^{c}±0.03	37.84^{d}±0.31	33.37^{d}±0.29	33.06^{e}±0.19	36.98^{d}±0.24
T1	0.18^{b} ±0.03	1.14^{a}±.017	0.97^{a}±0.07	32.17^{ab}±0.66	26.30^{b}±0.16	25.68^{b}±0.31	29.11^{b}±0.43
T2	0.18^{b}±0.01	1.08^{a}±.058	1.19^{b}±0.06	34.91^{c}±0.57	26.95^{b}±0.34	27.26^{cd}±0.49	34.22^{c}±0.28
T3	0.12^{a}±0.02	1.13^{a}±.028	1.05^{ab}±0.06	33.69^{bc}±0.44	26.76^{b}±0.32	26.92^{c}±0.45	29.50^{b}±0.22
T4	0.18^{b}±0.01	1.11^{a}±.018	1.04^{ab}±0.02	35.55^{cd}±1.24	30.54^{c}±0.35	28.18^{d}±0.43	34.51^{c}±0.32
p-value	0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01

All values are Mean ± SE, obtained from three replicates. Values in the same row with different superscript letters are significantly different (P<0.05).

Control (C)-Fermented sesbania leaf meal-based diet (30% inclusion); Positive Control (PC)-commercially available carp feed. T1-Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid and citric acid; T2: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid and Xylanase Enzyme, T3: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Citric Acid and Xylanase Enzyme, T4: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid, Citric Acid and Xylanase Enzyme.

Protease activity expressed as micromol of tyrosine released min-1mg $protein^{-1}$ Amylase activity expressed as micromol of maltose released min^{-1} mg $protein^{-1}$ Lipase activity expressed as unit's mg $protein^{-1}$

ALT: Specific activities expressed as nano moles of sodium pyruvate formed/mg protein/minute at 37 ^{o}C. AST: Specific activities expressed as nano moles of oxaloacetate released/min/mg protein at 37 ^{o}C.

Table 7: Catalase and Superoxide dismutase (SOD) activities of *L. rohita* fed with different experimental diet

Treatment	SOD Liver	SOD Gill	CAT Liver	CAT Muscle
C	1.8417±0.0	1.5647±0.03	22.2933±0.61	16.34±0.19
PC	1.7742±0.02	1.5022±0.0	22.1767±0.31	16.3033±0.39
T1	1.8049±0.03	1.5504±0.01	23.1433±0.41	15.7967±0.43
T2	1.8604±0.02	1.5342±0.0	22.7±0.50	15.6±0.23
T3	1.7915±0.03	1.5652±0.01	22.2267±0.32	15.7867±0.50
T4	1.7938±0.03	1.5686±0.01	22.35±0.58	15.6833±0.25
p-value	0.222	0.212	0.677	0.549

All values are Mean ± SE, obtained from three replicates. Values in the same row with different superscript letters are significantly different ($P<0.05$).

Control (C)-Fermented sesbania leaf meal-based diet (30% inclusion); Positive Control (PC)-commercially available carp feed. T1-Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid and citric acid; T2: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid and Xylanase Enzyme, T3: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Citric Acid and Xylanase Enzyme, T4: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid, Citric Acid and Xylanase Enzyme.

Data expressed as mean ± SE, (n=3) SOD activity was expressed as 50% inhibition of epinephrine auto oxidation/mg protein/min. Catalase activity was expressed as nanomoles H_2O_2 decomposed/min/mg protein.

Table 8: Blood parameters of *L. rohita* fed with different experimental diet

Treatment	Hb (g dL−1)	RBC (106 cells mm^{-3})	WBC (103 cells mm^{-3})	MCV (fL)	MCH (pg)	MCHC (g dL−1)
C	7.63 ± 0.09	2.24± 0.05a	36.37 ± 2.46	161.90 ± 17.40	34.07 ± 0.92	21.57 ± 2.35
PC	7.93 ± 0.70	2.27 ± 0.04ab	36.67 ± 1.27	154.67 ± 15.78	34.93 ± 2.38	22.73 ±0.83
T1	5.97 ± 0.03	2.26 ± 0.02ab	34.67 ± 1.89	139.80 ± 10.44	31.17 ± 0.19	22.53 ± 1.69
T2	6.67 ± 1.09	2.25 ± 0.02a	32.93 ± 2.72	132.53 ± 7.26	31.83 ± 5.46	24.37 ± 5.04
T3	6.57 ± 0.09	2.26 ± 0.04ab	34.53 ± 1.62	133.13 ± 4.28	30.73 ± 0.66	23.13 ± 0.28
T4	6.46 ± 1.03	2.27 ± 0.06ab	33.22 ± 1.04	132.5 ± 3.73	31.24 ± 0.71	23.58 ± 0.82
p-value	0.181	<0.001	0.708	0.37	0.76	0.959

All values are Mean ± SE, obtained from three replicates. Values in the same row with different superscript letters are significantly different ($P<0.05$).

Control (C)-Fermented sesbania leaf meal-based diet (30% inclusion); Positive Control (PC)-commercially available carp feed.; T1-Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid and citric acid; T2: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid and Xylanase Enzyme,T3: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Citric Acid and Xylanase Enzyme, T4: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid, Citric Acid and Xylanase Enzyme.

Hb haemoglobin, RBC red blood cell, WBC white blood cell, MCV mean cell volume, MCH mean cell haemoglobin, MCHC mean cell haemoglobin concentration Mean values in the same column with different superscript differ significantly. Data Expressed as mean ± SE, (n=3).

Table 9: Serum protein, albumin, Globulin and A: G ratio of *L. rohita* fed with different experimental diet

Treatment	Serum protein (g/dl)	Albumin (g dL^{-1})	Globulin (g dL^{-1})	A/G ratio	Glucose (g/dl)
c	4.43 ± 0.02^{a}	1.07 ± 0.09	1.35 ± 0.07	0.80 ± 0.11	20.122 ± 0.28
PC	4.98 ± 0.05^{c}	1.00 ± 0.05	1.29 ± 0.20	0.83 ± 0.18	20.72 ± 0.19
T1	4.30 ± 0.17^{ab}	0.94 ± 0.02	1.04 ± 0.04	0.91 ± 0.04	20.28 ± 0.30
T2	4.16 ± 0.03^{abc}	0.98 ± 0.05	1.18 ± 0.02	0.83 ± 0.06	20.72 ± 0.24
T3	4.11 ± 0.06^{bc}	1.03 ± 0.04	1.08 ± 0.09	0.97 ± 0.12	20.39 ± 0.67
T4	4.15 ± 0.02^{bc}	0.92 ± 0.03	1.04 ± 0.01	0.92 ± 0.04	20.12 ± 0.82
p-value	0.033	0.519	0.219	0.802	0.222

All values are Mean ± SE, obtained from three replicates. Values in the same row with different superscript letters are significantly different ($P<0.05$)

Control (C)-Fermented sesbania leaf meal-based diet (30% inclusion); Positive Control (PC)- commercially available carp feed.; T1-Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid and citric acid; T2: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid and Xylanase Enzyme, T3: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Citric Acid and Xylanase Enzyme, T4: Fermented sesbania leaf meal-based diet (30% inclusion) supplemented with Bile acid, Citric Acid and Xylanase Enzyme.

Data Expressed as mean ± SE, (n=3). Total protein expressed as (g %), albumin expressed as (g %) and globulin expressed as (g %).

4. Conclusion

In conclusion, the present study has demonstrated that dietary bile acid, citric acid and xylanase enzyme supplementation in fermented sesbania leaf meal-based diet can enhance the utilization of nutrients from dietary plant ingredients and improved the growth performance of *Labeo rohita.* More research will be required to address their optimal levels due to the nature of feed formulation and the difference in feeding nature of targeted fish species.

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6. Conflict of interest

The authors declare no conflict of interest.

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