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Evaluation of Fermented Rain Tree Seed Meal as an Alternative Protein Source: Effects on Growth, Nutrient Utilization, Digestive Enzyme Activity and Digestibility in Tilapia, *Orechromis niloticus*

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Abstract

Microbial fermentation improves the nutritional value of plant ingredients by decreasing the natural toxicants and reducing the complexity of the nutrients. The current study was conducted to replace soybean meal (SM) with yeast (*Saccharomyces cerevisiae)* fermented rain tree seed meal (FRTSM) and to evaluate its effect on the growth, nutrient utilisation, carcass composition, digestive enzymes activity, and degree of digestion of tilapia fry *(Oreochromis niloticus)*. Five iso-nitrogenous and iso-lipidic diets were formulated with replacing SM (0, 25%, 50%, 75% and 100%) with FRTSM and was denoted as FRTSM0 (Control, without FRTSM), FRTSM25, FRTSM50, FRTSM75 and FRTSM100. The feeding experiment was conducted for 60 days in FRP tanks of 150L capacity taking twenty tilapia fry (6.27 ± 0.09 g) per tank in triplicates. The tilapia fed with FRTSM0 diet showed significantly higher ($P < 0.001$) final weight, weight gain and weight gain percentage compared to other treatments. The quadratic regression analysis showed that replacing up to 37 and 38.64% of SM with FRTSM led to optimal specific growth rate (SGR) and weight gain. The FRTSM0 diet-fed group also showed lower body moisture content and increased body protein content. The quadratic regression analysis revealed that digestive enzymes, nutrient digestibility, and serum biochemical parameters were optimized with less than 20% replacement of SM. Despite notable enhancements in nutritional status and a decrease in antinutritional factors, replacing SM with FRTSM did not emerge as a superior ingredient choice. However, it is important to highlight that a cautious replacement of 38% SM with FRTSM can be achievable in the Nile tilapia diet.

Keywords: Fermented rain tree seed meal, tilapia, antinutritional factors, digestive enzymes, digestibility

Introduction

Aquaculture heavily relies on protein-rich aqua feed to achieve increased production, with fishmeal (FM) being the traditional primary protein source. However, it has become increasingly impractical for inclusion of fishmeal in aqua feed formulations due to production shortages and rising costs (Song et al., 2014). As an alternative, soybean meal is being utilized in aqua feeds due to its significant amount of protein, reasonable pricing, and consistent supply (Han et al., 2019). It is also crucial to recognize that soybean production, an integral part of industrial agriculture, exerts a significant environmental impact. This impact stems from the excessive use of nutrients and chemical inputs, runoff leading to eutrophication; deforestation of sensitive lands, high energy consumption, and the emission of greenhouse gases (Dreoni, Matthews, & Schaafsma, 2022). Additionally, the widespread occurrence of antinutritional components like trypsin inhibitors, phytates and plant lectins in soybean meal (SM)

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imposes constraints on its suitability for use in feed formulations (Francis, Makkar, & Becker, 2001). Furthermore, the shortage of the essential amino acid methionine, along with increased inclusion of SM is also linked to growth and intestinal issues in numerous fish species (Wang, Wang, Zhang, & Song, 2017). The aquaculture sector is also exerting growing pressure on crop sources, as it competes with terrestrial animal production systems like pigs and poultry. Hence, there exists a potential necessity to substitute soybean meal with alternative suitable non crop ingredients in aqua feed.

Rain tree seed (*Samanea saman)* is one of the best-known tropical trees in the world. The high protein content, well-balanced amino acid and affordablility of rain tree seeds make them useful as a good source of protein in aqua feed (Staples & Elevitch, 2006). The application of entire pod as both feed and nutrient supplements for ruminants and livestock has been reported earlier (Barcelo & Barcelo, 2012). The application of rain tree seed pod in fish diets is constrained by the presence of antinutritional components such as phytates, trypsin inhibitors, anti-vitamin factors, and toxic amino acids (Hagan, 2013). Various processing methods are available for the removal of these antinutritional factors from ingredients, such as heat treatment (Peres, Lim, & Klesius, 2003), phytase supplementation (Dalsgaard, Ekmann, Pedersen, & Verlhac, 2009) and microbial fermentation (Dileep et al., 2021). Among these techniques, microbial fermentation stands out as a promising approach for the removal of antinutritional factors from feed stuffs. It also offers other benefits that include significant changes in the characteristic structure, texture, and organoleptic profile of the raw ingredients. During fermentation microorganisms digest the carbohydrates in raw ingredients and use them for their own growth and development. Reduction in dry matter and the increased weight of microorganisms results in the increased ratio of protein content (Song et al., 2014). Fermented ingredients in aqua feed have been shown to enhance nutrient efficiency and enhance the overall nutritional quality of the feed (Dawood & Koshio, 2020). Fermented feeds provide several other benefits, such as enhancing metabolic processes, including the hematological and biochemical profiles of blood (Czech, Grela, Kiesz, & Kłys, 2020), as well as improving the organ antioxidant capacity (Verni, Rizzello, & Coda, 2019) in animals.

Tilapia is the world's second most important cultivated fish, surpassed only by carp. In 2018, global tilapia production reached 6.03 million metric tonnes, up from 3.49 million metric tonnes in 2010, whereas the share of tilapia in the global fish supply increased from 8.29 % in 2010 to 10.20% in 2018 (FAO, 2020). Nile tilapia, in particular, benefits from a low trophic feeding level and an herbivorous feeding strategy, which makes them less expensive to feed compared to other carnivorous finfish species. The effect of fermented rain tree seed meal (FRTSM) in the feed for tilapia production has not been investigated. Therefore, the present study was designed to evaluate the effect of substitution of SM with FRTSM on the growth performance, nutrient utilization, digestive enzyme and digestibility of Nile tilapia *Oreochromis niloticus*.

Materials and Methods

Fresh rain tree pods were harvested from the Kerala University Fisheries and Ocean Studies (KUFOS) campus in Panangad, Kerala. The pods underwent manual extraction, with the seeds removed and sun-dried. The seeds were then ground to a homogeneous size. The ground seeds were subjected to fermentation using *S. cerevisiae* at a cell density of 3×10^6 cells/g. In a 5L glass jar, 50% distilled water was added per kilogram of rain tree seed meal, and the combined mix was homogenized for 15 minutes, following the method outlined by Hassaan, Soltan, and Abdel-Moez (2015).

The diets were formulated by substituting SM with FRTSM at levels of 25%, 50%, 75% and 100% of SM, designated as FRTSM25, FRTSM50, FRTSM75, and FRTSM100, respectively. The control diet, denoted as FRTSM0, was formulated without FRTSM. Table 1 provides a breakdown of the components of the experimental diet. Components 1-7 listed in Table 1 were obtained from the market, while others were procured from Associated Scientific Company, Kochi, India. Coarse plant ingredients were ground at the feed mill at KUFOS, and all ingredients were sieved through a 400 μm mesh. The ingredients were thoroughly mixed, and an appropriate amount of water was added before being compressed using a semi-industrial, 2 hp motor-driven pelletizer with a 2 mm die to form pellets. The pellets were subsequently dried using a dryer (Servo Enterprises, Chennai, India) and stored in an air-tight container for future use.

Nile tilapia fry were obtained from the Marine Products Exports Development Authority (MPEDA), Kochi, Kerala, India. The fish were acclimatized to laboratory conditions and were fed a control diet for one week. For the initial proximate composition analysis, 15 fish were sacrificed. The feeding experiment utilized fibre reinforced plastic (FRP) tank with a capacity of 150 liters. A total of 15 tanks were employed, with three tanks assigned to each dietary treatment. Each FRP tanks was stocked with 20 fish fries, each having an average initial weight of 6.27±0.05 g. The fish were fed twice a day at 09:30 and 16:00 hours until visual satiation was achieved. The daily feed allocation was meticulously recorded. Faeces were collected twice daily using a siphoning pipe, ensuring minimal disturbance to the fish throughout the entire experimental period and stored at -20°C for subsequent nutrient digestibility analysis.

Water quality parameters were monitored regularly. Temperature was measured using a mercury thermometer (Duvcon Instruments, India), and dissolved oxygen levels were measured with an oxygen probe (HACH, HQ40D, USA). Water alkalinity was determined through titration (Boyd, 1990). Weekly recordings of ammonia-N (<0.02 mg/L) were conducted according to APHA (1995). pH levels were assessed using pH meter (pH510, Eutech Instruments, Singapore). The feeding experiment spanned for a duration of 60 days. Upon conclusion of the feeding experiment, whole fish, intestine and blood samples were collected for the determination of proximate composition, digestive enzymes activity and serum parameters in tilapia.

Upon completion of the experiment, five fish from each tank were pooled together according to their respective treatments and processed for proximate composition analysis following the AOAC (1998) methods. Whole tissue samples were oven dried to a constant weight at 105°C for 24hr to remove moisture content. Crude lipid, crude protein and ash were determined by SoxPlus System (Pelican Equipments, SCS 06 R (E-TS), Chennai, India), Kjelplus, Classic-DX VATS (B) (Pelican Instruments, Chennai, India) and muffle furnace (Nabertherm, LE 2/11/R6, Lilienthal, Germany), respectively. The nitrogen-free extract (NFE) percentage was calculated by using the formula, NFE = 100- (crude protein% + crude lipid% + ash %).

The total amount of amino acid (AA) content was determined by hydrolyzing the dried fish samples for 24 hours at 110°C using hydrochloric acid (HCl, 19.6N) (Ren, Li, Yin, & Blachier, 2013). Quantification of AAs in the whole body of fish was performed at the SAIF, ICAR-IIHR, Bengaluru, India.

Phytate content was determined by spectrophotometric method as described by Wheeler and Ferrel (1971). The phytate content was computed as g $100g^{-1}$ of the sample. The Total phenolic content (TP was quantified using a gallic acid standard curve and expressed as mgGAE per gram of the dried sample (Swain & Hillis, 1959). Trypsin inhibitor (TI) activity was determined by BAPNA (Benzoyl-DL-arginine-Para nitroanilide) substrate method (Kakade, Rackis, McGhee, & Puski, 1974) and it was expressed as the amount of TI present in mg g^{-1} of the sample. For detailed analytical procedures, refer to Dileep et al. (2021).

Moisture, protein, and lipid of fecal matter were analyzed following AOAC (1998) methods. Chromium oxide (Cr_2O_3) in the fecal samples was also determined (Furukawa & Tsukahara, 1966). Briefly, 100 mg of sample (feed and faecal matter) was digested with 5ml of nitric acid (HNO_3) in a digestion flask under a low flame for 30 minutes, until ejection of yellowish fumes. The digested sample turned greenish on completion of the reaction. In the cooled sample 3 ml of perchloric acid ($HClO_4$) was added and boiled until the solution turned lemon yellow. A reddish ring was observed around the edge and after that the sample was cooled. Sample was then transferred into a 25 ml volumetric flask, and the concentration was measured at 350 nm using a spectrophotometer (Thermo Scientific Evolution 201, Thermo Fisher Scientific, Waltham, MA). Concentration of chromium oxide as a marker was calculated using the following equation: Y= 0.2089 X + 0.0032 (where, Y= absorbance and X = Cr_2O_3 mg $100ml^{-1}$).

After determining the nutrient composition and marker percentage in the faecal matter, nutrient digestibility was calculated using the following formula.

Apparent nutrient digestibility coefficient=

$$100 - \left[\frac{100\ \%\ \text{Marker in feed} \times \%\ \text{Nutrient in faeces}}{\%\ \text{Marker in faeces} \times \%\ \text{Nutrient in feed}}\right]$$

Protease activity in the gut was evaluated using Kunitz (1947) casein digestion method. Protease enzyme activity was measured as μg of hydrolyzed proteins produced per minute per millilitre of the sample. Amylase activity was determined by the method of Bergmeyer and Grasl (1983) using a glucose standard curve and expressed as μg of reducing sugars released per min per mililitre. Lipase assay was carried out as per the method by Winkler and Stuckmann (1979) using PNPP (Para Nitro phenyl Palmatate) as substrate. It can be defined as 1 μmol of p-nitrophenol enzymatically released from substrate per mililitre per minute. For a thorough description of the analytical procedures, consult the work of Dileep et al. (2021).

Protein, albumin, globulin, Serum Glutamic Oxalo-acetic acid (SGOT), Serum Glutamic Pyruvic Transaminase (SGPT), alkaline phosphatase (ALP) and glucose in serum samples were analysed using the Aura Diagnostic kit (Aura Diagnostic, Kerala, India). Globulin was calculated by subtracting the albumin value from the total protein.

The data is displayed as Mean ± standard error. One-way analysis of variance (ANOVA) was utilized for

Table 1. Feed formulation, biochemical composition, ant nutritional factors and amino acid profile in experimental diets with graded level of replacement of soyabean meal by fermented rain tree seed meal (FRTSM)

Ingredients (g kg^{-1})	FRTSM0	FRTSM25	FRTSM50	FRTSM75	FRTSM100
Soyabean meal	420	315	210	105	0
FRTSM[a]	0	125	250	375	500
Fish Meal	50	50	50	50	50
Maize	145	125	105	85	65
GNOC[b]	170	170	170	170	170
Rice Polish	145	145	145	145	145
Vegetable oil	20	20	20	20	20
Vit[c] + Min[d] mix	10	10	10	10	10
Tricalcium phosphate	10	10	10	10	10
L- Methionine	5	5	5	5	5
L - Lysine	5	5	5	5	5
Choline chloride	10	10	10	10	10
CMC[e]	5	5	5	5	5
Chromium (lll) oxide	5	5	5	5	5
Biochemical Composition (g kg^{-1})					
Dry matter	930	920	910	930	940
Crude Protein	298	292	291	302	305
Crude Lipid	59	58	60	58	59
Fibre	70	107	110	115	119
Ash	81	76	79	80	82
NFE[f]	408	433	440	455	465
Antinutritional Factors					
Total phenolic compound (mg g^{-1})	1.85	2.6	8.1	10.75	15.1
Trypsin inhibitors (mg g^{-1})	4.8	4.9	5.2	5.6	5.9
Phytate-P (g $100g^{-1}$)	3.6	4.1	5.3	6.6	6.9

Abbreviations: [a]FRTSM: Fermented rain tree seed meal; [b]GNOC: ground nut oil cake; [c]Vitamin (IU or g kg^{-1}premix): retinol palmitate: 50000 I.U; thiamine: 5; riboflavin: 5; niacin: 25; folic acid: 1; pyridoxine: 5; cyanocobalomin: 5; ascorbic acid: 10; cholecalciferol: 50000 IU; α-tocopherol: 2.5; menadione: 2; inositol: 25; pantothenic acid: 10; choline chloride: 100; biotine: 0.25; [d]Minerals (g kg^{-1}): $CaCO_3$: 336; KH_2PO4: 502; $MgSO_4.7H_2O$: 162; NaCl: 49.8; Fe(II) gluconate: 10.9; $MnSO_4.H_2O$: 3.12; $ZnSO_4.7H_2O$: 4.67; $CuSO_4.5H_2O$: 0.62; KI: 0.16; $CoCl_2.6H_2O$: 0.08; ammonium molybdate: 0.06; $NaSeO_3$: 0; [e]CMC: carboxy methyl cellulose; [f]Nitrogen free extract (NFE)= 100-(crude protein%+ crude lipid%+ ash%) SEM (Standard error of the mean) the statistical analysis, and a significance threshold of 5% ($P \leq 0.05$) was established. The software package IBM SPSS Version 20.0 was employed for the statistical analyses. Additionally, multi-objective regression equations, both linear and quadratic, were analyzed to identify the optimal replacement of soybean meal.

Results and Discussion

In the present experiment, yeast *S. cerevisiae* fermented RTSM showed a 27% increase in protein content and a 50% reduction in fibre content (Table 3). The yeast, *S. cerevisiae* as a fermentation agent increased the protein content and reduced the antinutritional factors in guar meal, copra meal and rapeseed meal (Dileep et al., 2021; Vlassa et al., 2022) in previous studies. The increase in protein in the fermented ingredient observed in our study aligns with the findings of our previous study (Dileep et al., 2021) where the fermentation of copra meal with *S. cerevisiae* demonstrated an approximate 26% increase in protein content. Similarly, in our preceding study (Dileep et al., 2021) we also observed a decrease in fiber and phytic acid by 17% and 60%, respectively, in copra meal due to fermentation. The present study also demonstrated a 50% reduction in fibre and an 18% reduction in phytic acid. This suggests that yeast fermentation selectively reduces fibre and antinutritional factors

Table 2. Amino acid composition of the experimental diets

EAA[a] (mg g^{-1})	FRTSM0	FRTSM25	FRTSM50	FRTSM75	FRTSM100	Requirements *
Arginine	8.13	6.40	6.38	6.17	6.55	1.18
Histidine	3.60	3.29	3.35	3.28	3.61	0.48
Leucine	4.68	7.20	8.35	8.41	8.20	0.95
Lysine	6.38	5.09	4.35	5.53	5.56	1.43
Methionine	4.41	6.74	6.52	6.44	5.55	0.75
Phenylalanine	2.50	2.21	3.51	3.58	3.58	1.05
Threonine	3.53	2.33	3.28	3.16	3.54	1.05
Tryptophan	0.63	0.49	0.85	1.46	1.56	0.28
Valine	1.53	1.75	1.50	1.37	2.28	0.78
ΣEAA	35.39	33.50	38.09	39.40	43.78	
NEAA[b] (mg g^{-1})						
Alanine	7.36	6.27	8.15	8.10	8.56	
Aspartic acid	6.66	6.58	6.42	7.26	7.40	
Cysteine	1.36	0.27	0.6	0.93	1.21	
Glutamic acid	10.38	10.45	10.35	10.33	10.38	
Serine	9.54	9.39	9.35	9.57	9.74	
Proline	9.60	7.62	8.10	8.55	9.51	
Glycine	1.37	1.06	1.51	1.84	1.65	
Tyrosine	1.21	1.51	2.31	2.77	2.65	
Asparagine	1.53	1.56	2.30	1.39	1.46	
Ethionine	0.41	0.03	0.67	0.26	0.71	
Citrulline	4.64	4.52	3.31	1.36	2.44	
Beta3-4 dihydroxy phenylalanine	0.08	0.13	0.11	0.15	0.13	
ΣNEAA	43.01	49.39	53.18	52.51	47.81	
ΣAA[c] (mg g^{-1})	78.4	82.89	91.27	91.91	91.59	

Abbreviations: [a]EAA: essential amino acid; [b]NEAA: non-essential aminoacids; ΣAA: sum of total amino acid

* Santiago and Lovell (1988)

Table 3. Proximate composition, amino acid profile and anti-nutritional factors of raw rain tree seed meal (RTSM), yeast fermented rain tree seed meal (FRTSM) and soybean meal.

Attributes	RTSM	FRTSM	% of increase/ decrease	SBM (FAO/WHO Reference protein)
Proximate composition (g $100g^{-1}$)				
Crude protein	30.85±0.02	39.23±0.34	27.16↑	46.43
Crude lipid	11.36±0.01	12.37±0.12	08.89↑	1.36
Crude fibre	9.01±0.08	4.45±0.78	50.61↓	4.60
Ash	7.39±0.05	7.51±0.65	01.62↑	5.41
Nitrogen free extract	41.39±0.25	36.44±0.45	11.95↓	42.20
EAA (mg g^{-1})				
Arginine	2.64±0.20	2.99±0.12	13.25↑	7.2
Histidine	1.56±0.31	1.62±1.15	05.12↑	2.5
Leucine	3.00±0.02	3.20±0.05	06.66↑	7.8
Lysine	2.82±0.01	4.39±0.57	55.67↑	6.4
Methionine	0.42±0.35	2.15±1.22	41.19↑	1.3
Phenylalanine	2.11±0.14	3.70±0.26	75.35↑	4.9
Threonine	1.05±0.21	2.62±0.09	149.5↑	3.9
Tryptophan	0.01±0.31	0.34±0.01	33.00↑	1.3
Valine	6.03±0.21	7.90±0.11	31.01↑	4.8
ΣEAA	17.53	28.91	64.91↑	
NEAA (mg g^{-1})				
Alanine	1.51±0.02	1.66±1.92	09.93↑	4.3
Asparagine	0.17±0.01	4.20±2.38	23.70↑	11.7
Cystine	0.41±0.23	0.39±0.00	04.87↓	1.3
Glutamic acid	4.59±0.12	7.51±1.12	63.61↑	18.7
Glycine	0.24±0.25	2.41±0.20	90.41↑	4.2
Proline	1.49±0.21	4.98±1.35	234.2↑	5.5
Serine	3.84±0.01	4.66±1.99	21.35↑	5.1
Tyrosine	2.31±0.05	3.01±0.16	30.30↑	3.9
Citrulline	0.92±0.09	3.92±0.03	32.60↑	
ΣNEAA	13.80	32.74	137.4↑	
Antinutritional factors				
Total phenolic compound (mg $100g^{-1}$)	4.67±0.24	2.45±0.48	47.53↓	2.01
Phytate-P (g $100g^{-1}$)	1.76±0.21	1.43±0.12	18.75↓	1.72
Trypsin inhibitor (mg g^{-1})	5.35±0.09	3.01±0.03	43.73↓	7.61

↑Percentage increase

↓Percentage decrease

EAA: Essential amino acid

NEAA: Non-essential amino acid

based on the substrate involved.

The FRTSM0 diet fed group showed significantly ($P<0.05$) higher final weight, weight gain, weight gain % and SGR in tilapia compared to other treatments (Table 4). All these parameters decreased both linearly as well as quadratically with the substitution of SM with FRTSM in the feeds. The incorporation of FRTSM against SM did not promote weight gain or feed consumption. The drastic reduction in weight gain, growth and nutrient consumption could be attributed to the presence of ANFs in the experimental feeds due to the replacement of SM with FRTSM. We observed that incorporating FRTSM as a substitute for SM in the feed led to a gradual increase in antinutritional factors such as phenolic compounds, trypsin inhibitors, and phytate. Earlier reports suggest that the inclusion of phenolic compounds from plant extracts can negatively affect growth performance and

Table 4. Growth performance and nutrient utilization in tilapia fed fermented rain tree seed meal (FRTSM) replacing soybean meal in experimental diets for 60 days

Attributes	FRTSM0	FRTSM25	FRTSM50	FRTSM75	FRTSM100	SEM	Regression	
							L	Q
IW	6.32±0.03	6.27±0.01	6.26±0.01	6.20±0.10	6.31±0.05	0.062	NS	NS
FW^{a}	14.62±0.46^{a}	9.89±0.14^{b}	7.71±0.11^{c}	7.36±0.21^{cd}	6.63±0.06^{d}	0.201	***	***
WG^{b}	8.30±0.49^{a}	3.62±0.15^{b}	1.44±0.10^{c}	1.15±0.10^{c}	0.32±0.02^{d}	0.200	***	***
WG %^{c}	131.26±8.50^{a}	57.65±2.48^{b}	23.03±1.69^{c}	18.53±1.51^{c}	5.09±0.41^{d}	3.165	***	***
SGR^{d}	2.64±0.02^{a}	2.25±0.01^{b}	2.00±0.01^{c}	1.96±0.02^{c}	1.86±0.01^{d}	0.019	***	***
FCR^{e}	1.50±0.01^{e}	1.74±0.03^{d}	2.76±0.08^{c}	3.23±0.20^{b}	3.94±0.10^{a}	0.063	***	***
PER^{f}	0.27±0.17^{b}	0.33±0.00^{a}	0.04±0.00^{c}	0.03±0.00^{cd}	0.01±0.02^{d}	0.006	***	***
PRE^{g}	51.92±0.60	51.11±0.52	51.51±0.44	50.64±0.55	50.72±1.50	0.087	NS	NS
LRE^{h}	43.15±2.77	42.55±0.13	42.77±1.97	43.53±1.23	38.37±0.60	0.206	NS	NS
SURV%^{i}	100±0.00	99.54±0.66	99.78±0.00	100±0.00	98.74±.66	0.282	NS	NS

Abbreviations: ^{a}FW: Final weight; ^{b}WG: Weight gain (g) = Final weight “ Initial weight; ^{c}WG%: Weight gain percentage = (Weight gain/Initial weight) × 100; ^{d}SGR: Specific growth rate = 100 × [(ln final weight “ ln initial weight)]/days of experiment; ^{e}FCR: Feed conversion ratio = Feed intake/wet weight gain; ^{f}PER: Protein efficiency ratio= Weight gain/crude protein fed; ^{g}PRE: Protein retention efficiency = 100 × (Protein gain/Total crude protein fed); ^{h}LRE: Lipid retention efficiency = 100 × (lipid gain/Total crude lipid fed); ^{i}SURV%: Survival % = (Number of fish on final harvest/Number of fish at initial stocking) × 100.

SEM: Standard error of the mean; NS: No significance; * - $P<0.05$; ** - $P<0.01$; *** - $P<0.001$.

L: Linear; Q: Quadratic

Table 5. Body composition in tilapia fed fermented rain tree seed meal (FRTSM) replacing soybean meal in experimental diets for 60 days

Attributes	FRTSM0	FRTSM25	FRTSM50	FRTSM75	FRTSM100	SEM	Regression	
(g $100g^{-1}$)							L	Q
Moisture	78.18^{b}	79.46^{b}	80.86^{ab}	82.22^{a}	81.95^{a}	0.136	NS	NS
Protein	15.78^{a}	13.76^{b}	13.51^{b}	13.02^{b}	12.90^{b}	0.091	NS	NS
Lipid	4.26^{a}	2.37^{b}	1.38^{c}	1.34^{c}	1.21^{d}	0.079	***	***
Ash	4.39^{a}	3.20^{b}	1.50^{c}	1.45^{c}	1.38^{c}	0.083	***	***

SEM: Standard error of the mean NS, No significance; * - $P<0.05$; ** - $P<0.01$; *** - $P<0.001$.
L: Linear; Q: Quadratic

feed efficiency in fish (Buyukcapar, Atalay, & Kamalak, 2011). Tannins, a well-known group of polyphenol compounds represent most of the secondary metabolites found in terrestrial plants, can be divided into hydrolysable and condensed tannins (Das, Islam, Faruk, Ashaduzzaman, & Dungani, 2020). Hydrolysable tannins have demonstrated both positive and negative effect to fish (Omnes et al., 2017). However, the biological activity of tannins depends on their dietary concentration and animal species (Qiu et al., 2023). Buyukcapar et al. (2011) showed that Nile tilapia (*O. niloticus*) could tolerate 0.5% dietary hydrolysable tannin. However, dietary tannins had negative effects on digestive enzymes, such as intestinal trypsin and lipase, in Chinese seabass (*Lateolabrax maculatus*) when fed at levels of 2 g/kg or more (Qiu et al., 2023). The gradual increase in the concentration of phenolic compounds with the incorporation of FRTSM could be the reason for the growth retardation observed in the experimental fish.

Table 6. Serum biochemical parameters in tilapia fed with fermented rain tree seed meal (FRTSM) replacing soybean meal in experimental diets for 60 days

Attributes	FRTSM0	FRTSM25	FRTSM50	FRTSM75	FRTSM100	SEM	Regression	
							L	Q
Glucose (mg dl^{-1})	69.20^{b}	91.26^{a}	62.69^{c}	69.90^{b}	46.02^{d}	1.004	NS	NS
Total Protein (g dl^{-1})	1.12^{b}	1.29^{a}	1.03^{c}	0.69^{d}	0.61^{e}	0.024	***	***
Albumin (g dl^{-1})	0.64^{c}	1.16^{a}	0.81^{b}	0.59^{d}	0.55^{e}	0.015	NS	NS
Globulin (g dl^{-1})	0.47^{a}	0.21^{b}	0.13^{c}	0.13^{c}	0.02^{d}	0.010	***	***
SGOT^{a} (U l^{-1})	9.35^{e}	11.19^{d}	13.44^{c}	16.43^{b}	19.53^{a}	0.252	NS	NS
SGPT^{b} (U l^{-1})	1.38^{c}	1.51^{c}	5.29^{a}	2.38^{b}	5.50^{a}	0.126	NS	NS
ALP^{c} (U l^{-1})	2.66^{c}	2.73^{c}	1.75^{d}	4.24^{b}	6.28^{a}	0.110	NS	NS

[a]SGOT: Serum glutamic oxaloacetic acid; [b]SGPT: Serum glutamic pyruvic transaminase; [c]ALP: alkaline phosphatase
Values are means ± SEM of three replicates
In the rows, different letters indicate statistical difference at P<0.05.
SEM: Standard error of the mean; NS- No significance; * - P<0.05; ** - P<0.01; *** - P<0.001.
L: Linear; Q: Quadratic

Table 7. Digestive enzyme activity in tilapia fed fermented rain tree seed meal (FRTSM) replacing soybean meal in experimental diets for 60 days

Attributes (g 100mg^{-1})	FRTSM0	FRTSM25	FRTSM50	FRTSM75	FRTSM100	SEM	REGRESSION	
							L	Q
Protease^{a}	7.34^{a}	5.49^{b}	2.44^{c}	2.40^{c}	2.55^{c}	0.140	***	***
Amylase^{b}	1.40^{a}	1.29^{ab}	1.19^{ab}	1.12^{bc}	0.96^{c}	0.012	***	**
Lipase^{c}	3.54^{a}	3.26^{b}	2.51^{c}	2.35^{c}	1.47^{d}	0.051	***	***

[a]Activity expressed as micromoles of tyrosine released min^{-1} mg^{-1} protein; [b]Activity expressed as micromoles of maltose released min^{-1} mg^{-1} protein; [c]U/mg protein

SEM: Standard error of the mean; NS: No significance; * - P<0.05; ** - P<0.01; *** - P<0.001.

L: Linear; Q: Quadratic

The water temperature during the experimental periods ranged between 27.5 and 29.5°C. The levels of dissolved oxygen, alkalinity, ammonia-N and pH were 4.8 to 5.6 mg/L, 134.7 to 135.1 mg/L, <0.02 mg/L and 6.9 to 7.2, respectively. Water quality was properly maintained throughout the experimental period.

The effect of substitution of SM with FRTSM on digestive enzymes such as amylase, protease and lipase, as well as nutrient digestibility in tilapia, was analysed. The multi objective regression analysis for protease (y = 0.5692x3 - 4.8163x2 + 10.444x, R^2 = 0.784, optimal SM replacement dose = 10.56%) and amylase (y = -0.0083x3 + 0.07x2 - 0.2717x + 1.612, R^2 = 0.9975, optimal SM replacement dose = 17.08%) is presented in Table 9. Lipase activity in tilapia ranged between 1.47 to 3.54 U/mg protein, with significantly higher (P<0.001) lipase activity noted in the FRTSM0 treatment (Table 7). In the present study, there was a consistent decline in digestive enzymes, including protease, amylase, and lipase, as well as nutrient digestibility parameters such as dry matter, protein, and lipid digestibility. Significantly higher (P<0.001) apparent crude protein digestibility (ACPD) was noted in FRTSM0 (Table 8). As per regression

Table 8. Nutrient digestibility in tilapia fed fermented rain tree seed meal (FRTSM) replacing soybean meal in experimental diets for 60 days

Attributes ($g\ 100g^{-1}$)	FRTSM0	FRTSM25	FRTSM50	FRTSM75	FRTSM100	SEM	Regression L	Regression Q
ADMD[a]	85.98[a]	86.03[a]	82.65[b]	79.64[c]	78.85[c]	0.217	***	***
ACPD[b]	84.92[a]	83.90[a]	79.00[b]	76.14[c]	76.43[c]	0.258	***	***
ACLD[c]	82.37[a]	78.69[b]	76.43[c]	74.43[d]	65.24[e]	0.396	***	***

[a]ADMD: apparent dry matter digestibility; [b]ACPD: apparent crude protein digestibility; [c]ACLD: apparent crude lipid digestibility
SEM: Standard error of the mean; NS: No significance; * - P<0.05; ** - P<0.01; *** - P<0.001.
L: Linear; Q: Quadratic

Table 9. Multi objective regression equations of growth performance, digestive enzymes, and digestibility and blood parameters with soybean meal replacement by fermented rain tree seed meal (FRTSM)

Parameters	Linear	Quadratic	Optimal value of x as per quadratic curve
WG[a]	$y = -0.074x + 12.944$ $R^2 = 0.8147$	$y = 0.0011x^2 - 0.1864x + 14.348$ $R^2 = 0.9788$	38.62
SGR	$y = -0.0074x+2.512$ $R^2 = 0.872$	$y = 9E\text{-}05x^2 - 0.0164x + 2.6249$ $R^2 = 0.9856$	37.00
Protease enzyme	$y= -1.267x+7.845$ $R^2=0.7869$	$Y=0.5007x^2-4.2713x+11.35$ $R^2=0.9561$	10.56
Amylase enzyme	$y = -0.105x + 1.507$ $R^2 = 0.9854$	$y = -0.005x^2 - 0.075x + 1.472$ $R^2 = 0.9886$	17.08
Protein digestibility	$y = -2.474x + 87.5$ $R^2 = 0.8997$	$y = 0.3329x^2 - 4.4711x + 89.83$ $R^2 = 0.9225$	17.03
Lipid digestibility	$y = -3.902x + 87.078$ $R^2 = 0.9288$	$y = -0.5986x^2 - 0.3106x + 82.888$ $R^2 = 0.9594$	16.16
Serum albumin	$y = -0.0028x + 0.892$ $R^2 = 0.2021$	$y = -1E\text{-}04x^2 + 0.0071x + 0.7677$ $R^2 = 0.4189$	20.30

[a]WG: Weight gain

analysis, ACPD was ($y = 0.5858x^3 - 4.939x^2+9.3545x+79.988$, $R^2 = 0.9951$) recorded at SM replacement dose of 17.03% (Table 9). The apparent crude lipid digestibility (ACLD) was significantly lower ($P<0.001$) in the FRTSM100 group and higher in FRTSM 0. As per regression analysis ACLD was ($y =-0.6467x^3+5.2214x^2-15.572x+93.752$, $R^2 = 0.9962$) recorded at SM replacement dose of 16.16 %. This decline in nutrient digestibility with increasing incorporation of FRTSM followed a linear trend with increased substitution of SM with FRTSM. The higher incorporation of FRTSM increased the antinutritional factors such as total trypsin inhibitor, phenolic compound and physic acid. These antinutritional factors can reduce the digestibility of dietary proteins, potentially causing harm to the pyloric cecal region of the intestine and reducing nutrient absorption in fish. Liu, Liang, Li, Yuan, and Fang (2018) reported that a 4.7g/kg phytic acid concentration decreased the apparent digestibility and utilization of amino acids and minerals in grass carp (*Ctenopharyngodon idellus*). Similarly, Nile tilapia is sensitive to trypsin inhibitors, and optimal growth occurs when trypsin inhibitor levels are below 0.09% (Wee & Shu, 1989). Noteworthy, in the present experiment all the three antinutritional factors exceeded the tolerable levels for tilapia despite using FRTSM, thereby influencing fish performance negatively. Nevertheless, regression analysis of our experiment showed that the higher activity of protease and amylase was at 10.56 and 17.08% of SM replacement, respectively. The fermentation process reduced the fibre content in the rain tree seed by about 50%. However, the fibre content in FRTSM 25 to FRTSM 100 showed a range of 107 to 119 g kg^{-1} diets which was notably higher than the control diet (70 g kg^{-1}). The increased fibre content of diets above 100 gkg^{-1} with plant ingredients, has been shown to negatively affect weight gain, growth response, and protein utilization in Nile tilapia in previous studies (Fagbenr, Akande, Fapohunda, & Samsons, 2004). Similar result was reported in the water hyacinth (WH) based diet of Nile tilapia,where poor performance of Nile tilapia was noted compared to the control diet. This could be due to the relatively high fibre (cellulose) content of water hyacinth, since these fish lack the ability to secrete cellulase; the main cellulose-digesting enzyme (Buddington 1980).

In the current study the greater substitution level of FRTSM were also reflected in body protein, lipid, and ash levels (Table 5). The body protein content did not show any linear or quadratic trend across the experimental groups, indicating that substituting SM with FRTSM did not alter the body protein content. The whole-body lipid content in the tilapia fed with FRTSM0 showed significantly greater ($P<0.001$) value and decreased both linearly and quadratically by increasing the soybean meal replacement levels with FRTSM. The significantly higher ($P < 0.001$) whole body ash content was found in the FRTSM0 group. The body ash content reduced up to 50% replacement level and plateaued thereafter, showing both linear and quadratic trends. This result aligns with previous study where the body fat of juvenile red sea bream significantly decreased with increasing substitution of fish meal with a fermented mixture of SM and scallop by-products (Kader et al., 2011). The body protein content was significantly lower in treatment groups in comparison to control, which may be related to limited utilization of dietary essential amino acids due to the presence of ANFs, which have a negative effect on protein utilization (Lim et al., 2004). Previous studies on Nile tilapia fed with alternate fermented plant protein, such as guar and copra meal sources, against fish meal, have shown a decline in whole body protein content (Dileep et al., 2021). The reduced body lipid may also be attributed to the reduced lipid digestibility and negative correlation with body moisture content. Since body fat content is directly associated with weight increase in fish, this was also anticipated, as the fish gained less weight in comparison to replacement levels (Kim, Lim, Kang, Kim, & Son, 2012). Furthermore, the ash content decreased in the current study with higher level of FRTSM, suggesting limited mineral utilization.

The blood parameters of tilapia were analysed to assess the effect of substitution of SM with FRTSM on the health condition (Table 6). The quadratic regression analysis showed 20.30% replacement of SM with FRTSM resulted in better albumin levels. This suggests that tilapia may not tolerate higher inclusion level of FRTSM in their diet, leading to compromised liver function. Consequently, not only did the serum protein parameters decreased, but the SGOT and SGPT parameters also increased, indicating potential liver damage (Javed, Ahmad, Usmani, & Ahmad, 2017). Previous study on tilapia with fermented guar and copra mixture (Dileep et al., 2021) described that the SGOT and SGPT value for control fish was in the series of 10.47 and 1.74 U l^{-1}, respectively. After 50% inclusion of FGCM these values significantly increased compared to the control group, which could likely be due to the responsiveness of SGOT and SGPT towards minor cellular and tissue injury (Palanivelu, Vijayavel, Balasubramanian, & Balasubramanian, 2005). Similar results were observed in Nile tilapia (*O.niloticus*) fed with fermented SM and red seabream provided with fermented rapeseed meal, where higher inclusion levels significantly increased SGOT and SGPT levels (Dossou et al., 2018). In this current study, change in serum ALP (2.66 – 6.28 U l^{-1}) was noticed in tilapia fed with the SM replaced by FRTSM diets, indicating potential damage to normal liver function. The existence of greater level of ANFs in the fish diet due to the FRTSM substitution could be blamed for the damages to the internal mechanism of the fish.

Yeast fermentation improved the nutritional quality of rain tree seed meal. Nevertheless, incorporating FRTSM as a replacement for SM in the tilapia diet did not result in favourable outcomes for growth, nutrient utilization, digestive enzymes, nutrient digestibility, and blood parameters, even at lower (25%) SM replacement levels compared to control. Regression analysis indicated that for optimal SGR and weight gain, 37% and 38% of SM could be replaced, respectively. The analysis revealed that <20% SM could be replaced with FRTSM for better digestive enzymes, nutrient digestibility, and blood parameters. In conclusion, the study suggests that FRTSM may not be a suitable ingredient in the Nile tilapia diet as a substitute for SM. Nonetheless, a maximum replacement of 38% SM with FRTSM is feasible. Overall, fermentation of rain tree seed meal failed to meet the necessary criteria to serve as an alternative protein to soybean meal (SM) in tilapia diet. Consequently, exploring other processing techniques like enzyme hydrolysis, protein concentrate, or isolate from rain tree seed meal may be considered for improving the utilization of this ingredient resource.

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