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Nutritional evaluation of protein isolate from rubber seed in the diet of *Labeo rohita*: Effects on growth performance, nutrient utilization, whole body composition and metabolic enzymes activity

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ABSTRACT

The nutritional potential of protein isolate from rubber seed (RPI) in the diets of *Labeo rohita* (initial average weight 4.45 ± 0.01 g) was assessed in a 60 days feeding trial. Five isonitrogenous (32.62 ± 0.13 CP Kg^{-1}) and isocaloric (18.47 ± 0.08 MJ kg^{-1}) experimental diets were formulated with graded level of RPI like 0%, 25%, 50%, 75%, or 100% in replacement for soybean protein isolate (SPI), and designated as Control, RPI25, RPI50, RPI75, RPI100, respectively. The RPI contributed 0%, 13%, 26%, 39% or 52% of the total dietary protein in the diets. Each diets were randomly assigned to 15 experimental tanks containing 12 fish in triplicates and fed to satiation twice daily at 10:00 h and 18:00 h. At the end of the feeding trial, the growth performance and nutrient utilization indices such as percent weight gain (WG%), specific growth rate (SGR), daily growth coefficient (DGC), feed intake (FI), protein efficiency ratio (PER), feed conversion ratio (FCR) and protein retention (PR) values were not significantly ($p > 0.05$) affected by the dietary treatments irrespective of inclusion levels of RPI. A significantly higher ($p < 0.05$) hepatosomatic index (HSI) was recorded in the control and RPI 50 group compared to other treatment groups ($p < 0.05$). The intestinal somatic index (ISI) and Survival rate were similar ($p > 0.05$) in all the groups. The apparent digestibility coefficients (ADCs) of dry matter and protein for fish fed the control and RPI 100 diets were found to be similar, while RPI 50 and RPI 75 groups exhibited a significantly lower value corresponding to the protease enzyme activity. The whole body compositions and digestive/metabolic enzymes activities among the various groups did not differ significantly ($p > 0.05$). The serum cholesterol and triglyceride levels were found to be significantly higher ($p < 0.05$) in the control compared to the RPI fed groups. Significantly higher serum glucose level was recorded in RPI 50, while a reverse was seen in the liver glycogen contents. Overall, this study clearly showed that RPI from rubber seed can serve as alternative protein source in the diets of *L. rohita* fingerlings without any adverse effects on growth, nutrient utilization and physio-metabolic responses.

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1. Introduction

The current world population of about 7.3 billion is expected to reach 9.7 billion by 2050 (UN-DESA, 2015), and the global demand for animal protein and hence protein rich feed ingredients for production of animal protein are expected to increase accordingly. Although, aquaculture production continues to grow at a relatively high rate compared to other animal production sector (SOFIA, 2007), but not immune from ingredients shortage occasioned by the high demand and constraint in fish meal production (Tacon and Metian, 2008; Shamna et al., 2015). Aside from the limited availability, the price of the commonly used ingredients is on the rise due to increased demand in the feed of poultry and livestock as well (Coffey et al., 2016). Presently, soybean meal (SBM) is one of the most available plant protein source commonly used in aquafeed production for many fish species including *Labeo rohita* (El-Sayed, 1999; Storebakken et al., 2000). This is due to the high protein and energy contents, high digestibility and relatively well-balanced amino acid profile of soybean meal (Hertrampf and Piedad-Pascual, 2000; Yue and Zhou, 2008). However, the price of SBM is becoming high and competitive, thereby making it less cost efficient for aquafeed production. Hence, there is the need for its replacement with a potential non-conventional plant protein sources for cost efficient and sustainable aquafeed development. Therefore, in the present scenario, non-edible oil seeds cake or kernel would be one of the most preferred choice provided they are made free of deterrent factors, and nutritionally compatible to cultured species (Marrufo-Estrada et al., 2013; Suprayudi et al., 2015).

Rubber seed cake obtained from rubber tree seed (*Hevea brasiliensis*) is a potential non-conventional feedstuff, which have received scant research attention is fish nutrition (Alegbeleye et al., 2004; Sharma et al., 2014; Suprayudi et al., 2015; Deng et al., 2015). According to the Natural Rubber Statistic Report (2015), the world natural rubber plantation by acreage in 2014 stand at 12 million hectares. Rubber seeds has enormous potential for aquaculture feed industry if well harnessed. The protein content of rubber seed cake ranges between 17–25% (Sharma et al., 2014), and are very rich in n-3 and n-6 fatty acid (Eka et al., 2010). Despite the moderate protein content, the nutritive value of rubber seed cake is undermined by the presence of anti-nutritional factors (ANFs) especially cyanogenic glycosides, whose metabolic product is hydrogen cyanide that impacts negatively on the physio-metabolic responses of fish (Francis and Becker, 2001; Sharma et al., 2014; Deng et al., 2015). Other deterrent factors include phytate, tannin, high fiber and complex carbohydrate, which also have detrimental effects on feed palatability, phosphorus availability, digestibility and growth (Watson et al., 2012). However, improvement in plant protein processing technologies has proven to overcome many of these problems, not only by inactivating the anti-nutritional factors or reducing the level of the toxic component but also by improving the nutritional value of the by-products. Many studies have been conducted in this regard, and the concept of protein concentrates/isolates attained relevance in fish nutritional research.

Extraction of protein from defatted seed cake has been described as a way of reducing the contents of antinutrient and toxic components (Marrufo-Estrada et al., 2013) with high levels of protein, which often have digestibility similar or greater than that of fishmeal protein (Makkar et al., 2008). Since rubber kernel meal contain less protein, a large amount of indigestible material and anti-nutrients, there is need to convert them into a better useful products by isolating the protein for optimum utilization. Several studies were conducted recently to explore the inclusion level of various plant protein isolates in fish such as soybean protein isolate in *Acipenser schrenckii* (Xu et al., 2012), rapeseed (*Brassica napus*) protein isolate in juvenile *Psetta maxima* (Nagel et al., 2012), canola (*Brassica campestris*) protein isolate in *Oncorhynchus mykiss* (Slawski et al., 2013), jatropha protein isolate in *Cyprinus carpio* (Kumar et al., 2012; Latif et al., 2015), and pea protein isolate in juvenile *Oreochromis niloticus* (Schulz et al., 2007). The results from these findings were encouraging, and to the best of our knowledge, no work on protein isolates from rubber seeds has been reported in fish and livestock. With this backdrop, protein isolate was prepared from rubber kernel meal and fed to *Labeo rohita* (rohu) to assess the potential utilization for aquafeed production. Presently, rohu is the most popular and widely cultured freshwater fish in South-east Asia and command high demand among the consumer. Therefore, *L. rohita* was selected as the candidate species with the aims of investigating the nutritional potential of protein isolate prepared from rubber kernel meal and examine its impact on growth performance, nutrient utilization, whole body composition, digestibility, metabolic enzyme activity, and serum metabolites.

2. Materials and methods

2.1. Production of protein isolate from rubber seed (RPI) and amino acid analysis

Dried rubber seeds were collected from Tripura (22°56′N–24°32′N, 90°09′ E–92°20′E), India. The seed were dehulled and the kernel obtained were powdered and defatted with hexane (three sequential times) to obtain defatted rubber kernel meal used for the preparation of protein isolate. RPI was prepared by alkali extraction and acid precipitation at its iso-electric point following the method of Saetae and Suntornsuk (2011) with little modification. Ground defatted rubber kernel meal of 2000 g was dispersed in distilled water in the ratio of 1:15 (w/v). The pH of the dispersion was adjusted to 12 with 1 N NaOH and stirred for 2 h at room temperature. The slurry was centrifuged at 7000 rpm for 20 min, and supernatant was collected and adjusted to pH 4 with 1 N HCl to precipitate the protein. The precipitated protein was centrifuged and washed twice with distilled water to obtain the protein isolate. The wet protein isolate was lyophilized (Scanvac^{TM} cool safe 100-9 pro), and the final protein content was found to be 908 g Kg^{-1} (Table 1). Amino acid analysis was carried out following the method described by Devappa and Swamylingappa (2008), and quantitfied by High performance liquid chromatography (HPLC).

Table 1
Proximate composition (g kg^{-1} DM), antinutrient contents and amino acid composition of rubber protein isolate (RPI) and soybean protein isolate (SPI).

Variables	RPI	SPI	RKM[a]
Dry matter	942	968	940
Crude protein	908	845	221
Crude lipid	15	35	330
Ash	20	40	39
[c]Total carbohydrate	57	80	410
[d]Gross energy (MJ kg^{-1})	23.3	23	25.6
Indispensable Amino acids (g kg^{-1})		AA[b]	
Arginine	109.5	67	–
Histidine	26.4	23	–
Isoleucine	34.1	43	–
Leucine	62.9	72	–
Lysine	16.6	55	–
Phenylalanine	47.2	46	–
Methionine	17.0	12	–
Threonine	30.8	33	–
Tryptophan	ND	12	–
Valine	78.7	44	–
Dispensable amino acids (g kg^{-1})			
Alanine	33.1	38	–
Glycine	41.5	37	–
Aspartate	102.8	102	–
Glutamic acid	140.5	169	–
Proline	43.8	45	–
Serine	49.6	46	–
Cystine	19.4	11	–
Tyrosine	28.5	34	–
Antinutritional factors			
Cyanide (mg HCN Kg^{-1})	27	–	75.6
Phytic acid (g Kg^{-1})	15.4	14.3	31.5
Tannin (g Kg^{-1})	0.4	8.2	5.2

[a] Rubber kernel meal (g kg^{-1} DM).
[b] AA – based on company analysis (Soy Growth™, Medicamen Organics Limited, India) ND – not determine.
[c] Total carbohydrate = 1000 – (Crude protein + Crude lipid + ash).
[d] Calculated Gross energy (MJ kg-1) = [23.9 x CP (g kg^{-1}) +39.8 x CL (g kg^{-1}) + 17.6 x TC (g kg^{-1})]/1000.

2.2. *In vitro* protein digestibility

In vitro protein digestibility assay was carried out by pH-drop method following the method described by Ali et al. (2009). Fresh tissue of alimentary canal was homogenized under cold condition and diluted with distilled water (1:10 w/v) followed by centrifuging at 12000 rpm for 15 min at 4 °C. An equivalent amount of RPI that provided 160 mg of crude protein was weighed and dispersed in 20 mL of distilled water, and 2 mL of intestinal homogenenate (enzyme source) to obtain 8 mg crude protein per milliliter, and the pH was adjusted to 8:00 (Eutop pH tutor, Thermo Fisher Scientific, Singapore). The pH drop was recorded at every minutes interval for 10 min. Casein was used as the reference protein.

Relative Protein Digestibility was calculated using the following formula:

Relative Protein Digestibility (RPD%) = (ΔpH of rubber protein isolate/ΔpH of casein) $\times$ 100.

2.3. Diets preparation

Five experimental diets were prepared to be isonitrogenous (32.62 $\pm$ 0.13% CP) and isocaloric (18.47 $\pm$ 0.08 MJ kg^{-1}), with control diet containing soybean protein isolate (SPI) as the major protein source (Table 2). The SPI protein was replaced at 25%, 50%, 75%, or 100% with RPI (designated as RPI 25, RPI 50, RPI 75, RPI 100, respectively). The RPI thereby contributed 0%, 13%, 26%, 39% or 52% of the total dietary protein, respectively. The diets were supplemented with vitamins-mineral mix, and dicalcium phosphate to ensure that all diets satisfied the dietary requirement of *L. rohita* fingerlings (Debnath et al., 2007). Carboxymethyl cellulose (CMC) was added as a binder and butylated hydroxytoluene (BHT) was incorporated as antioxidant in the diets. All the ingredients were milled and mixed thoroughly to form homogenous blend, then oil and water were added to form dough. The prepared dough was passed through a hand pelletizer using 2 mm die and the pellets were air dried, and stored at –20 °C until use. A 5 g Kg^{-1} chromium oxide was added to each diet as an inert marker for the estimation of apparent digestibility coefficients (ADC) as described by Hardy and Barrows (2002).

Table 2
Feed and proximate composition of the experimental diets (DM basis) fed to *Labeo rohita* fingerlings during the 60 days experimental period.

Ingredients(g kg^{-1})	C	RPI25	RPI50	RPI75	RPI100
Soy protein isolate[a]	200	150	100	50	0
Rubber protein isolate	0	47	93	139	187
Ground nut cake[b]	126	126	126	126	126
Fish meal[b]	50	50	50	50	50
Rice bran[b]	240	240	240	240	240
Wheat flour	290	293	297	301	303
Sunflower oil:cod liver oil(1:1)[a]	60	60	60	60	60
Dicalcium phosphate[c]	10	10	10	10	10
Vitamin/mineral mix	10	10	10	10	10
Choline Chloride[c]	2	2	2	2	2
Butylated hydroxytoluene[c]	2	2	2	2	2
Carboxymethyl cellulose[c]	10	10	10	10	10
Total	1000	1000	1000	1000	1000
Cr_2O_3	5	5	5	5	5
Proximate composition (g kg^{-1} DM)					
Dry matter	92.1	92.1	91.8	91.7	92.1
Crude protein	329	329	323.8	323.8	325.5
Crude lipid	62.5	62.5	65	65	67.5
Ash	84.6	79.6	79.6	75	75
Crude fibre	65	70	70	70	70
NFE[d]	459	459	462	466	462
Gross energy (MJ kg^{-1})[e]	18.4	18.4	18.4	18.5	18.6
Antinutritional factors[f]					
Cyanide (mg HCN Kg^{-1})	0.00	1.27	2.51	3.75	5.05
Phytic acid (g kg^{-1})	2.86	2.87	2.86	2.86	2.88
Tannin (g kg^{-1})	1.64	1.25	0.86	0.47	0.07

Composition of vitamin-mineral mix (PRE-EMIX PLUS) (quantity/kg): Vitamin A, 55, 00 000 IU; Vitamin B, 2 000 mg; Vitamin D3, 11, 00 000 IU; Vitamin E, 750 mg; Vitamin K, 1000 mg; Vitamin B6, 1000 mg; Vitamin B1, 2,6 mcg; Calcium Pantothenate,2500 mg; Nicotinamide, 10 g; Mn, 27 000 mg; I, 1000 mg; Fe, 7500 mg; Zn, 5000 mg; Cu, 2000 mg; Co, 450; L-lysine, 10 g; DL-Methionine, 10 g; Selenium, 125 mg.

[a] Soy Growth™, Medicamen Organics Limited, India.

[b] Purchased from local animal feed ingredient dealer, Mumbai, India.

[c] Himedia Pvt, Mumbai, India.

[d] NFE (Nitrogen free extract) = 1000 − (Crude protein + Crude lipid + crude fibre + ash).

[e] Calculated gross energy (MJ kg^{-1}) = [23.9x CP (g kg^{-1}) +39.8x CL (g kg^{-1}) + 17.6x NFE (g kg^{-1})]/1000.

[f] Calculated based on cyanide, phytic acid and tannin concentration in RPI, and SPI.

2.4. Experimental set-up

Rohu (*Labeo rohita*) fingerlings were obtained from Srushti Aquaculture, Sudhagad, Raigad District, Maharashtra, India, and transported to the wet laboratory of the Division of Fish Nutrition, Biochemistry and Physiology, ICAR-Central Institute of Fisheries Education, Mumbai. Fish were carefully transferred to two circular fibre tank (1000 L) for acclimatization under aerated condition and fed with control diets. One hundred and eighty rohu fingerlings were randomly distributed in 15 plastic rectangular tubs (75 L capacity) each containing 12 fish (initial average weight 4.45 ± 0.01 g) per tank, in triplicates. Fish were hand fed with their respective diets to apparent satiation twice daily at 10:00 h and 18:00 h under normal light regime. The fish in each tub were weighed at the start and every two weeks over a period of 60 days. The fish were not fed on the day of weighing. Water quality parameters were monitored and maintained at optimal level (temperature 25–29 °C; pH 7.5–8.1; DO 6.3–7.5 mg/l; ammonia 0.05–0.09 ppm). Prior to the commencement of the feeding trial, a total of 15 fish were sacrificed and stored at − 20 °C for the analysis of initial whole body composition.

2.5. Fish sampling and chemical analysis

Fish were fasted for 24 h on the completion of 60 days feeding trial, weighed and the growth performance and nutrient utilization parameters like percent weight gain (WG%), specific growth rate (SGR), daily growth coefficient (DGC), feed intake (FI), protein efficiency ratio (PER), feed conversion ratio (FCR) and protein retention (PR) were calculated according to the formulae given beneath Table 3. Difference in number of fish stocked at the beginning and end of the experimental trial were determined for calculation of survival. The liver and intestine from six fish per treatment were pooled together and weighed for the calculation of hepatosomatic index (HSI) and intestinal somatic index (ISI). Three fish from each tank were sampled for the analysis of whole body composition. The moisture, crude protein, crude lipid and ash content in the diets and whole body were carried out as per the standard methods of the Association of Official Analytical Chemists (AOAC, 1995). Moisture was determined after drying in an oven at 105 °C until constant weight. Ash content was determined by muffle furnace at 550 °C for 6 h. Crude protein content (N × 6.25) was determined by the Kjeldahl method after acid digestion using an Auto Kjeldahl System (2200 Kjeltec auto distillation; Foss Tecator, Hoganas Sweden). Crude lipid was determined

Table 3
Growth performance, nutrient utilization, survival and apparent digestibility coefficient of *Labeo rohita* fingerlings fed different experimental diets.

Variables	Diets					SEM[a]	P-values		
	Control	RPI 25	RPI 50	RPI 75	RPI 100		Overall	Linear	Quadratic
Weight gain (%)[b]	131.66	121.04	114.28	117.08	121.39	4.485	0.147	0.115	0.041*
SGR[c]	1.40	1.32	1.27	1.29	1.32	0.034	0.151	0.115	0.043*
FI[d]	6.08	5.98	5.83	5.93	6.16	0.148	0.584	0.837	0.133
DGC[e]	3.24	3.00	2.83	2.90	3.01	0.114	0.185	0.148	0.050
PER[f]	1.98	1.90	1.87	1.87	1.85	0.070	0.688	0.202	0.600
FCR[g]	1.55	1.61	1.65	1.64	1.66	0.059	0.661	0.189	0.552
Protein retention[h]	33.77	32.03	31.49	31.54	31.49	1.974	0.905	0.436	0.603
HSI[i]	0.88	0.70	0.76	0.64	0.65	0.042	0.014*	0.003*	0.259
ISI[j]	2.99	2.47	2.10	2.22	2.01	0.304	0.233	0.045*	0.353
Survival[k]	100	97.22	100	100	97.22	1.756	0.580	0.628	0.682
ADC of DM[l]	67.04	63.03	62.01	63.22	67.98	0.648	0.004*	0.359	<0.001*
ADC of protein	85.27	86.24	80.49	80.97	84.51	0.236	<0.001*	<0.001*	<0.001*
ADC of lipid	89.44	88.17	72.37	71.56	85.75	6.343	0.247	0.285	0.111
ADC of P[m]	40.02	45.26	39.63	39.25	40.87	0.729	0.010*	0.121	0.495
Liver glycogen[n]	33.18	35.10	23.34	50.26	50.06	3.626	0.001*	0.002*	0.033*

* Estimated Marginal Means ($P < 0.05$).
[a] Standard error of the mean.
[b] Weight gain% = (final body weight − initial body weight)/initial body weight × 100.
[c] Specific growth rate (SGR, %/day) = 100 × (ln final body weight−ln initial body weight)/experimental duration in days.
[d] Feed intake (g kg^{-1}MBW/day) = total dry feed given per fish (g $fish^{-1}$)/MBW/experimental duration in days; mean metabolic body weight (MBW) = {(initial body weight in g/1000)$^{0.75}$ + (final body weight in g/1000)$^{0.75}$}/2 (Deng et al., 2015).
[e] Daily growth coefficient (DGC, % $days^{-1}$) = 100 × {(final body weight in g)$^{1/3}$ −(initial body weight in g)}$^{1/3}$/experimental duration in days.
[f] Protein efficiency ratio (PER) = Net weight gain (g)/protein fed (g).
[g] Feed conversion ratio (FCR) = total dry feed given (g)/wet weight gain (g).
[h] Protein retention (PR) = 100 x {(final body weight x final body protein content) − (initial body weight × initial body protein content)}/(protein content in diet × total feed intake).
[i] Hepatosomatic index (HSI) = 100 x wet weight of liver (g)/whole body weight of fish (g).
[j] Intestinal somatic index (ISI) = 100 x wet weight of intestine (g)/whole body weight of fish (g).
[k] Survival (%) = 100 x (total number of fish harvested)/(total number of fish stocked).
[l] ADC – Apparent digestibility coefficient; DM- Dry matter.
[m] P – Phosphorus.
[n] (mg/g wet tissue).

by the ether-extraction method in a soxhlet extraction apparatus (Socsplus, SCS-08-As, Pelican equipment, Chennai, India). The cyanide content of the RPI was analysed according to alkaline titration method of AOAC (2000), phytic acid and tannin contents were analysed as per the method of Gao et al. (2007) and Schanderi (1970), respectively.

2.6. In vivo digestibility

Faecal samples were collected daily by siphoning for 15 days (45 to 60th day). Three hours after feeding each day, the tanks were checked for uneaten feed, but there was no feed left out in the tubs. Faecal sample were collected 7 h after feeding, centrifuged at 5000 rpm for 15 min to separate the water, the wet faecal sample was stored at −20 °c and lyophilized. Analysis of crude protein and lipid contents were carried out according to AOAC (1995) and chromium content of feed and faecal matters was determined by the method of Furukawa and Tsukahara (1966). The apparent dry matter digestibility of diets and nutrients were calculated according to Law (1986): ADC of dry matter of diet (%) = 100 x {1- (% Cr_2O_3 in feed)/(% Cr_2O_3 in faeces)}; ADC of Nutrients/phosphorus in the diets (%) = 100 x [1 − {(% Cr_2O_3 in feed/% Cr_2O_3 in faeces) × (% Nutrients/phosphorus in faeces/(% Nutrients/phosphorus in feed)}].

2.7. Serum biochemistry and enzyme assay

After 24 h of fasting, blood samples were collected from randomly selected fish (four from each replicate). The fish were anaesthetized with clove oil (50 μL of clove oil per litre of water; Debnath et al., 2007) and blood was collected from the caudal vein without anticoagulant into a dried eppendorf tube and allowed to stand in a slanted position at room temperature. The blond were centrifuged at 5000*g* for 10 min in a cooling centrifuge (REMI CPR-24, India), and transferred into another eppendorf tube and kept in − 20 °C until use. Serum cholesterol, triglyceride and glucose were estimated using a commercial kit (Erba® Diagnostic Mannheim, Transasia Bio-medicals Ltd, Solan, HP, India). The liver, muscle and intestinal tissues were dissected out and homogenized in cold 0.25 M sucrose solution in 15 mL plastic tubes using a Teflon-coated mechanical homogenizer (MICCRA D-9, ART Prozess and Labortechnik, Germany). The tubes were continuously kept in ice to avoid heating. The homogenate was centrifuged (5000*g* for 10 min at 4 °C) and the supernatant stored at −20 °C until analysis. The protease enzyme activity was determined by the casein digestion method (Drapeau, 1974). Amylase activity was estimated as the reducing sugars produced due to the action of glucoamylase and α-amylase on carbohydrate using dinitro-

salicylic-acid method (Rick and Stegbauer, 1974). The aspartate amino transferase (AST) and alanine amino transferase (ALT) activities were measured by the method described by Wooten (1964). Lactate dehydrogenase (LDH) enzyme activity was measured following the method of Wroblewski and Ladue (1955). Protein contents of the tissues were determined according to Bradford (1976), adapted to microplate in other to express enzyme activities as a function of the protein content. Bovine serum albumin was used as the protein standard.

2.8. Hepatic glycogen content

Liver glycogen content was estimated colorimetrically by the method described by Hassid and Abraham (1957). The tissue was digested with 30% KOH and 95% ethanol was added. Glycogen was precipitated upon centrifugation at 5000*g* for 5 min, which was dissolved in distilled water. To a known quantity of aliquot, anthrone reagent was added and mixed by swirling the tube. The tubes were covered with glass marble and heated for 10 min in boiling water followed by cooling. The absorbance was recorded at 590 nm. The reading was compared with standard glycogen and expressed as mg/g wet tissue.

2.9. Statistical analysis

All data were subjected to a one way ANOVA using the General Linear Model Procedure of IBM SPSS package, version 20. The overall treatments effects were determined and polynomial contrasts were used to test linear and quadratic effects of dietary inclusion of RPI. Significance was defined at $P < 0.05$.

3. Results

3.1. Protein isolate production, antinutritional factors and in vitro protein digestibility

The protein, lipid, ash and carbohydrate contents of the RPI were found to be 908, 15, 20 and 57 g kg^{-1}, respectively (Table 1). The protein content of RPI increased more than threefold with a corresponding decrease in the lipid, fibre and total carbohydrate contents compared to the rubber kernel meal (RKM). The cyanide, phytic acid and tannin contents estimated in the RKM (75.6, 31.5, 5.2 g kg^{-1}, respectively) and RPI (27, 15.4, 0.4 g kg^{-1}, respectively) samples are given in Table 1. The amino acid composition of RPI is comparable to that of soybean protein isolate (SPI) except lysine, which is lower in RPI. The RPI protein digestibility *in vitro* was found to be 93.2%.

3.2. Growth performance, nutrient utilization and survival rate

After the 60 days feeding trial, the weight gain (%), SGR, DGC, PER, PR and FCR values did not differ significantly among the various groups ($p > 0.05$) and no linear trend was observed (Table 3). However, weight gain (%) and SGR values showed a quadratic trend ($P < 0.05$). The HSI value showed overall significant effects ($p < 0.05$) and followed linear trends. The ISI and fish survival rate were not significantly ($p > 0.05$) affected as a result of feeding RPI. Overall, the groups of fish fed RPI-based diets showed similar performance in terms of growth and nutrient utilization with the control group.

3.3. Apparent digestibility coefficients (ADC)

The apparent digestibility coefficient of dry matter showed similar value between the fish fed control and RPI 100 diets (Table 3), whereas RPI 50 and RPI 75 fed groups registered a significantly lower value ($p < 0.05$). The ADC of protein showed both linear and quadratic effects, with the highest value recorded in RPI 25. Similar results were recorded in ADC of phosphorus with a significantly higher value recorded in RPI 25, but showed no linear and quadratic trends. Nonetheless, phosphorus digestibility was observed to be lower in all the groups (39.25%–45.26%). No significant variation was observed in the ADC of lipid (71.56%–89.44%) among the various groups ($p > 0.05$).

3.4. Whole body composition

The dietary inclusion of RPI showed no significant difference ($p > 0.05$) in the whole body composition of the treatment groups compared with the control (Table 4). The initial fish body composition showed higher ash content compared to the experimental fish (final body ash), wherein the reverse was found in the case of protein and lipid contents.

3.5. Digestive and metabolic enzyme activitivies, serum metabolites and liver glycogen contents

There was no significant difference ($p > 0.05$) in the protease (0.37- 0.47 U mg^{-1} protein) and amylase (0.99–1.32) enzymes activities among the various groups (Table 5). The activities of the metabolic enzymes (AST, ALT and LDH) in the liver and muscle were not significantly ($p > 0.05$) affected as a result of feeding RPI, and no observable trends recorded (Table 6). The cholesterol and triglyceride levels in the serum were found to be significantly higher ($p < 0.05$) in the control compared to the RPI fed groups (Table 5). The serum cholesterol level tends to decrease linearly with the increasing dietary RPI levels,

Table 4
Whole body (wet weight basis) composition of *Labeo rohita* fingerlings fed different experimental diets.

Diets	Variables			
	Moisture	Crude protein	Crude lipid	Ash
Control	70.72	15.46	7.82	2.61
RPI 25	73.62	15.28	6.36	2.76
RPI 50	72.25	15.17	7.31	2.71
RPI 75	72.36	15.24	6.08	2.80
RPI 100	73.04	15.38	6.11	2.92
SEM[a]	0.666	0.413	0.585	0.148
Overall	0.096	0.988	0.208	0.686
Linear	0.140	0.885	0.075	0.198
Quadratic	0.264	0.607	0.724	0.900

Initial fish – 79.42 moisture; 13.35 crude protein; 2.28 crude lipid; 3.91 ash.
Estimated Marginal Means ($P < 0.05$).
[a] Standard error of the mean.

Table 5
Intestinal protease and Amylase, serum cholesterol (mg/dl), serum triglyceride (mg/dl), and serum glucose (mg/dl) levels in *Labeo rohita* fingerlings fed different experimental diets.

Diets	Variables				
	Protease[b]	Amylase[c]	Cholesterol	Triglyceride	Glucose
Control	0.47	1.32	92.70	132.95	105.47
RPI 25	0.47	0.99	84.13	124.90	116.41
RPI 50	0.41	1.00	77.46	110.73	124.84
RPI 75	0.37	1.00	79.37	111.11	81.21
RPI 100	0.45	1.12	73.33	122.99	111.02
SEM[a]	0.025	0.233	3.914	4.504	2.208
Overall	0.104	0.834	0.046*	0.025*	<0.001*
Linear	0.130	0.605	0.006*	0.039*	0.006*
Quadratic	0.119	0.344	0.373	0.009*	0.114

* Estimated Marginal Means ($P < 0.05$).
[a] Standard error of the mean.
[b] Protease: U mg protein^{-1}.
[c] Amylase specific activity expressed as micromole of maltose released min^{-1} g protein^{-1}.

Table 6
Metabolic enzymes activity in the liver and muscle of *Labeo rohita* fingerlings fed different experimental diets.

Diets	Variables					
	ALT		AST		LDH	
	Liver	Muscle	Liver	Muscle	Liver	Muscle
Control	2.11	9.34	14.06	15.31	3.25	2.04
RPI 25	2.87	8.92	17.72	15.42	3.71	1.78
RPI 50	1.99	8.63	15.50	12.86	2.63	1.88
RPI 75	2.04	9.06	17.00	14.37	3.07	1.49
RPI 100	1.93	9.96	16.44	13.96	2.76	1.31
SEM[a]	0.274	0.650	1.154	1.097	0.372	0.509
Overall	0.168	0.671	0.270	0.491	0.324	0.845
Linear	0.199	0.520	0.293	0.306	0.199	0.300
Quadratic	0.442	0.198	0.298	0.477	0.982	0.864

Estimated Marginal Means ($P < 0.05$).
Alanine transaminase (ALT): specific activities expressed as nanomoles of sodium pyruvate formed mg protein^{-1} min^{-1} at 37 °C; Aspartate transaminase (AST): specific activities expressed as nanomoles of oxaloacetate released min $^{-1}$ mg protein^{-1} at 37 °C. Lactate dehydrogenase (LDH): specific activity expressed as Units min^{-1} mg protein^{-1} at 37 °C.
[a] Standard error of the mean.

but showed no quadratic effects ($p > 0.05$). Highest triglyceride level was detected in the control group, which was found to follow linear and quadratic trends ($p < 0.05$). Significantly higher serum glucose level was recorded in RPI 50 and lowest in RPI 75 fed group ($p < 0.05$). The liver glycogen contents showed both linear and quadratic effects, with the highest value recorded in RPI 75 (50.26 ± 0.66) and RPI 100 (50.06 ± 1.77) groups, while the group fed RPI 50 (23.34 ± 2.01) registered the lowest value ($P < 0.05$) (Table 3).

4. Discussion

Improving the nutritional quality of plant protein ingredients for efficient utilization requires innovative approach for sustainable aquafeed production. Isolation of protein is one of such approach by separating the pure protein fractions from other non-proteinaceous components present in the ingredients including antinutritional factors (Mwachireya et al., 1999; Devappa and Swamylingappa, 2008; Saetae and Suntornsuk, 2010; Wanasundara, 2011), thus improve the digestibility and nutrient utilization. In the present study, the protein content of the isolated protein (RPI) from rubber kernel meal was found to be 908 g kg^{-1} with a concomittant reduction in the cyanide contents (64.3%), phytic acid (51.1%), tannin (92.4%) and insoluble carbohydrate. De-hulling the rubber seeds and subsequent treatment with alkali seems to help in reducing the tannin contents to a very low levels as recommended by Griffiths (1991). The cyanide content in RPI (27 mg kg^{-1}) was less than the value reported for detoxified rubber kernel meal (60.1 mg kg^{-1}) fed to *L. rohita* fingerling (Sharma et al., 2014). The protein content of RPI was similar to canola protein isolate (Mwachireya et al., 1999), but more than those reported by for rapeseed protein isolate (Nagel et al., 2012), pea protein isolate (Schulz et al., 2007), canola protein isolate (Slawski et al., 2013) and jatropha protein isolate (Kumar et al., 2012). Isolating the proteins from rubber kernel meal resulted in a significant increase in the protein content and reduction in the antinutritional factors as reported by other researchers in different studies (Devappa and Swamylingappa 2008; Saetae and Suntornsuk, 2010; Nagel et al., 2012; Kumar et al., 2012; Slawski et al., 2013). The higher *in vitro* digestibility of RPI recorded in this study may be due to the significant reduction in the soluble and indigestible carbohydrates, antinutritional factors, and more accessibility of amino acid peptide bonds by the digestive enzymes as reported by Devappa and Swamylingappa (2008). Protein isolate (RPI) obtained in this study exhibited lower *in vitro* digestibility values than casein (97%), but more than rapeseed (83%) and jatropha protein isolates (88.5–90.6%) (Savoie et al., 1988; Devappa and Swamylingappa, 2008).

Although, rubber seed meal has been reported to be a promising alternative protein source for aquafeeds production (Sharma et al., 2014; Suprayudi et al., 2015; Deng et al., 2015), but utilization by fish is limited due to the presence of ANFs, especially cyanide. According to Deng et al. (2015), feeding rubber seed meal above 30% to juvenile tilapia (*Oreochromis niloticus x O. aureus*) lead to growth depression, which was also supported by Alegbeleye et al. (2004). Contrarily, defatted rubber seed meal successfully replaced 50% of the protein in common carp (*Cyprinus carpio*) diets without adverse effects on the feed intake and growth (Suprayudi et al., 2015). The variation in their results may be due to the levels of ANFs, non-starch polysaccharide, and or species differences. Literature have shown that varying level of antimetabolites, indigestible carbohydrates, amino acid imbalances and other complexes may contribute to growth depression often observed when plant protein ingredients are fed at higher inclusion levels to fish, rather than pinpointing one single factor as the primary reason for the adverse effects noticed (Mambrini et al., 1999; Francis and Becker, 2001; Schulz et al., 2007; Slawski et al., 2013; Shamna et al., 2015). However, in the present study, no significant variation was observed in the growth performance of the fish fed RPI in replacement for SPI. This indicate that RPI was better utilized compared to detoxified rubber seed meal fed to rohu (Sharma et al., 2014), and other fish species (Suprayudi et al., 2015; Deng et al., 2015). The presence of ANFs in plant-based diets is one of the reason for diminishing feed intake, nutrient absorption and growth depression in fish due to unpleasant tastes, and decreased feed acceptability (Francis and Becker, 2001). Conversely, the inclusion of RPI in the diets of rohu as seen in this study did not cause any decrease in feed intake. Rather, the RPI 100 fed group recorded the highest feed intake, though not differ from other fed groups, indicating that diets palatability and acceptability were not affected. Our findings are in consonant with Slawski et al. (2013) and Kumar et al. (2012), who observed no significant difference in the feed intake of rainbow trout and common carp fed canola and jatropha protein isolates, respectively. Sharma et al. (2014) reported that feeding RKM diets containing a high level of cyanide (>39 mg kg^{-1}) to rohu led to a reduction in protein utilization and digestion processes, which resulted in poor growth performance. Similar result was observed (31.8 mg kg^{-1} diets) in the work of Deng et al. (2015). However, feeding RPI in the present study showed no adverse effect on the nutrient utilization, protein accretion, and digestion processes. This can be attributed to the low level of cyanide (1.27–5.05 mg kg^{-1}) (Table 2) in the diets of the present study, which was below the levels reported to cause no adverse effects in earlier studies. The lack of differences in the PER, FCR and PR indicate that the RPI was well digested, absorbed and utilized by the fish for muscle growth. Also, the insignificant differences in the feed intake signifies that RPI can be accepted by rohu without any palatability-mediated feed rejection.

HSI value can be correlated with the amount of fat or glycogen deposition (Gao et al., 2012; Debnath et al., 2007). In the present study, significantly higher HSI value was recorded in the control and RPI 50 fed groups compared to others, which indicate higher lipid deposition in the liver. Increased hepatic lipogenic enzymes activities has been reported in European seabass fed soybean protein concentrates or corn gluten meal, thereby, leads to higher whole body lipid composition (Dias 1999; Kaushik et al., 2004). This may be the reason for the higher HSI value recorded in control (0.88) and RPI 50 (0.76) fed groups. The trends in both the HSI value and whole body lipid contents follow a similar pattern ($y = 0.1216 \times - 0.0934$, $r^2 = 0.94$). This was further supported by Gao et al. (2012) who found a correlation between HSI value and amount of fat deposition in the body of red sea bream fed diets devoid of vitamin E. Similar results have also been reported in common carp fed plant protein based diets (Kumar et al., 2012). Paradoxically, our data revealed higher glycogen contents in fish fed RPI 75 and RPI 100. As observed in the work of Suprayudi et al. (2015), the groups of fish showing high HSI value recorded low lipid and high glycogen contents in the hepatopancreas, while the groups with lower HSI value showed opposite trend after fed rubber seed meal based diets. Our findings are in opposite to this, wherein we observed that the groups with higher HSI value recorded high lipid and low glycogen contents. This shows that an inverse relationship may exists between

lipid and glycogen deposition in fish, but further study is required in this aspect. The non-significant difference observed in the ISI value among the experimental groups, indicates that the physiological well-being of the digestive system was not compromised as a result of feeding RPI. These findings are in accord with Kumar et al. (2012) who observed a significantly higher ISI in fish fed plant protein (soybean and jatropha protein isolates) based diets compared with fishmeal fed group.

Apparent digestibility coefficients of protein and phosphorus in *L. rohita* fingerlings fed practical diets with varying levels of microbial phytase ranged between 73.90–82.13% and 52.08–65.39%, respectively (Baruah et al., 2007). However, higher value was recorded in the present study, with ADC of protein in RPI-fed groups ranging between 80.49–86.24%. Although RPI 50 and RPI 75 recorded the lowest values compared to other fed groups, but no significant impact was found on the protein retention and growth performance, which indicates that the fish were able to utilize the protein in the diets optimally. The ADCs of protein and dry matter recorded in the present study were higher than the values reported for juvenile turbot fed rapeseed protein isolates (Nagel et al., 2012), but similar to those reported in common carp fed defatted rubber seed meal (Suprayudi et al., 2015). No significant difference in ADC of lipid were recorded among the various groups, which is also similar to the findings of Kumar et al. (2012).

The various dietary treatments did not significantly influence whole body composition. The similarity observed in the protein content in whole body composition suggest that RPI-based diets had a balanced amino acid profile for optimum growth of *L. rohita* fingerlings. The lower ADC of phosphorus observed among the various groups did not cause any decline in the whole body ash, and this may be due to the low level of phytic acid or dicalcium phosphate incorporated in the diets as additional phosphorus source. Indian major carp has been reported to tolerate phytic acid level below 1% in the diets with no adverse effect on growth and body composition (Usmani and Jafri 2002; Alvi, 1994), and the levels in all the fed diets were lower than 1%. The insignificant difference in the whole body lipid content detected among the groups were inconsonant with those of Akinleye et al. (2012) and Slawski et al. (2013).

Improvement in the growth performance of animals is a function of the digestive enzyme influence on the digestion processes (Lemieux et al., 1999). In the present study, no significant variation was observed in the activities of digestive enzymes (protease and amylase), signifying that the dietary RPI did not elicit any inhibitory effects on the digestion processes. This may be ascribed to the low level of ANFs present in the diets. Kumar et al. (2012) reported that dietary inclusion of detoxified jatropha and soybean protein isolates did not alter the digestive enzymes activities in common carp. The authors attributed this to the absence of trypsin inhibitors, lectin and addition of phytase in the diets. Similarly, Luo et al. (2012) found no significant differences in the activities of protease and alpha-amylase enzymes in cobia fed rapeseed meal. The non-significant slight decreased in protease activity observed in RPI 50 and RPI 75 correlate with the ADC of protein recorded for this groups. Transaminase (AST and ALT) enzymes catabolize amino acids and transfer amino groups to α-keto acids. But when a diet is deficient in essential amino acids, the keto acids may be reduced, thereby resulting in a decreased activities of ALT and AST enzymes (Cheng et al., 2010). In the present study, the activities of both protein metabolism enzymes were found to be similar with the control, indicating that the dietary protein fed met the fish requirement and adequately utilized by the fish. Similar to these, Deng et al. (2015) found no significant differences in the hepatic ALT and AST activities of tilapia fed dietary RSM. On the other hand, Luo et al. (2012) reported that feeding juvenile cobia with increasing dietary rapeseed meal led to a reduction in the activities of ALT and AST enzymes in the liver. LDH enzyme is known to be active when there is an oxygen debt in the tissue, causing pyruvate to be converted to lactate in anaerobic glycolysis (Murray et al., 2000). Increased tissue level activity of this terminal enzyme was reported as the characteristic features of lactic acidosis resulting from the inhibitory effect of cyanide on aerobic metabolism (Speijers, 1993; Okolie and Osagie, 1999), thus, producing a state of histotoxic anoxia. This effects were evidence in cyanide-exposed rabbits and common carp (Okolie and Osagie, 1999; Sadati et al., 2013). Shamna et al. (2015) also observed an increased level activity of LDH enzymes in *Labeo rohita* fingerlings fed phorbol esters-containing jatropha protein concentrate. Nevertheless, such effects were not seen in the present study where LDH activity in both the muscle and liver were found to decrease with increasing RPI levels, suggesting that there is no shift in aerobic metabolism and the fish were not under hypoxic condition.

Plant products have been reported to have a hypocholesteromic effect in human, terrestrial animal, and fish (Lees et al., 1977; De Schrijver, 1990; Kaushik et al., 1995). The present study clearly showed that dietary RPI inclusion gradually reduce serum cholesterol levels in *L. rohita* fingerlings. The decreasing trend in cholesterol level with the increasing dietary RPI levels showed a second order polynomial relationship ($y = 0.0016x^2 - 0.3299x + 92.047$, $r^2 = 0.92$). Several authors have also reported the hypocholesteromic effects of plant-based protein in fish and attributed it to the presence of compound that causes increased excretion of bile salts or impede intestinal absorption of cholesterol (Kaushik et al., 2004; Lim and Lee, 2009; Deng et al., 2010; Akinleye et al., 2012). The decreased serum cholesterol level in RPI-fed groups compared to the control hypothetically suggest that rubber seeds may contain some secondary metabolites with hypocholesteromic properties than soybean. The reduction in serum triglyceride levels recorded in RPI fed groups were similar to the findings of Lim and Lee (2009) and Slawski et al. (2012). In contrast, Kumar et al. (2010) and Akinleye et al. (2012) observed increased triglyceride levels in fish fed plant protein based diets against fish meal (control), hence, the variation observed in comparison with our findings may be due to the differences in the major protein source used as control. Elevated blood glucose level has been reported in many fish species fed plant protein based diets due to the higher contents of carbohydrates, and the result obtained in the present study is in accord with other findings (Kikuchi, 1999; Kumar et al., 2010; Akinleye et al., 2012). Conversely, Slawski et al. (2012) detected no significant differences in the plasma glucose levels in rainbow trout fed rapeseed protein concentrate.

5. Conclusion

In conclusion, based on the results obtained in the present study rubber protein isolate could serve as a potential replacer for soybean protein isolate without any detrimental effects on feed palatability, acceptability, growth performance and nutrient utilization. However, it is important to state that this study is a preliminary investigation on the possibility of utilizing RPI as an alternative protein source in the diets of *Labeo rohita*. Hence, further study is recommended to understand its effect on haemato-immunological response of the fish before its inclusion in aquafeeds.

Conflict of interest

The authors have no conflict of interest.

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