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Effectiveness of Acidification and Phytase Pretreatment on Growth Performance, Muscle Proximate Composition and Nutrient Digestibility of Rohu (*Labeo rohita*, Hamilton 1822) Juveniles Fed Soybean Meal Based Diet

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Authors' Contribution
MA designed the experiment and supervised the work. NS and AH performed the experiment and collected the data. SZHS and MF statistically analyzed the data and wrote the manuscript.

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Citric acid, Mineral absorption, Plant meal based diet, Dry matter, Crude protein.

ABSTRACT

This research project studies the growth performance, muscle proximate composition and nutrient digestibility performance by rohu (*Labeo rohita*) juveniles fed citric acid (CA) acidified phytase (PHY) pretreated soybean meal (SBM) based diet. Basal SBM based diet was supplemented with two levels of CA (0 and 2%) and two levels of microbial PHY (0 and1000 FTUkg^{-1}) in factorial arrangement resulting in the formulation of four experimental diets. Chromic oxide (1%) was added as an inert marker in the diets to evaluate the nutrient digestibility. Important water quality parameters such as temperature, pH and dissolved oxygen were monitored and maintained within acceptable limits for fish during entire experiment. The findings of the experiment showed improved weight gain%, specific growth rate (SGR), feed conversion ratio (FCR), muscle composition and digestibilities of dry matter, crude protein and crude fat in *Labeo rohita* juveniles when fed CA supplemented diet. Similar observations were also recorded for the group fed on PHY supplemented diet. Citric acid addition in the diet also resulted in improved ($p<0.05$) digestibilities of Ca, Mg, P, Na, K, Cu, Zn, Fe and Mn. Similarly, PHY pretreatment had also resulted in enhanced mineral digestibility as compare to control group. However, both the supplements did not interact positively ($p>0.05$) for the observed responses except SGR, FCR and crude ash contents in muscles. It was evident from the present study that soybean meal based diet supplemented with CA and PHY performed better than without supplementation.

INTRODUCTION

Fishmeal is a high cost protein source and for successful development of aquaculture it has become necessary to replace the fishmeal by low cost plant protein sources (Debnath *et al*., 2005). Soybean meal (SBM) is a high-quality ingredient that is widely used in animal feed formulations because of its low cost, high level of crude protein and better amino acid profile as compared to other plant based meals (Cheng and Hardy, 2004). It was found that SBM can replace fishmeal without imparting any change in the protein level in *Labeo rohita* diet (Rahman *et al*., 2013).

However, SBM contains phytate (3.88 gkg^{-1}) which is an antinutritional factor. It is the main storage form of P in plants and nearly 60% of total P in soybean meal is bound to it (Cao *et al*., 2007). This phytate-P is not available to agastric or mono gastric fish species (Hussain *et al*., 2011). Phosphorus deficiency induce skeletal deformities in fish (Lall and Lewis, 2007) as well as undigested phytate-P can contribute to eutrophication of water bodies (Baruah *et al*., 2007a). Moreover, phytate reduces the digestibility of many important nutrient like protein, fat and mineral by making insoluble complexes with them (Nwanna *et al*., 2008).

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The use of organic acids to hydrolyse the phytate is one of the modern approaches being used in fish nutrition nowadays. Citric acid (CA) and its salt complexes have been widely used for the acidification of diet because of its unique flavour and high buffer capacity (Hossain *et al*., 2007). Citric acid dephosphorylate the phytate to release the bound P and other nutrients (Zyla *et al*., 1995). Sugiura *et al*. (2001) reported that addition of CA in fish diet improved the P and other mineral utilization. A recent study had shown that the addition of CA improved P and Ca contents in Beluga (*Huso huso*) fed SBM diets (Khajepour and Hosseini, 2012). Other studies have also reported improved fish growth by addition of organic acid in fish feed (Hossain *et al*., 2007; Ng *et al*., 2009; Liebert *et al*., 2010; Lim *et al*., 2010; Koh *et al*., 2016).

Another approach, which is being used to hydrolyse the phytate for fish nutrition, is the supplementation of phytase (PHY) in the feed. PHY is an enzyme which hydrolyses the phytate to release the bound P and other chelated nutrients for absorption (Kumar *et al*., 2011). Agastric or monogastric fish species lack this enzyme, however, its supplementation in feed resulted in enhanced bioavailability of plant P and other nutrients in fish (Cao *et al*., 2007). Significant improvements in growth as well as feed efficiency of rohu (Hussain *et al*., 2011), Nile tilapia (Trichet *et al*., 2014; Olusola and Nwanna, 2014), sea bass (Ganzon-Naret, 2013) and tra catfish (Hung *et al*., 2014) by PHY addition in feed has been reported recently. Also, increased availability of nutrients including minerals (Cu, Ca, Mg, Fe and Zn) in rainbow trout has been reported by adding PHY in the diet (Sugiura *et al*., 2001).

PHY is a pH dependent enzyme and work optimally at lower pH (2.5 and from 5.0 to 5.5). Citric acid inclusion in the diet lower the gut pH which may favor the action of PHY. Hence it is hypothesized that, efficacy of PHY can be enhanced by adding the CA in the diet. An increased growth performance and feed efficiency was observed in rohu, when both the supplements were added to plant meal based diet (Baruah *et al*., 2007a). Improved growth performance and muscles lipid contents as a result of combine supplementation of CA and PHY has been reported recently in *Cyprinus carpio* (Khajepour *et al*., 2012). Several authors (Bolling *et al*., 2000, 2001; Brenes *et al*., 2003) have found that CA alone or in combination with PHY increased the phytate hydrolysis. Baruah *et al.* (2005) observed a significant relationship between CA and PHY for bone ash and mineral contents in *L. rohita* juveniles.

Many studies have been reported in fish nutrition regarding the supplementation of CA and PHY independently or in combination. In combine supplementation studies, CA was added in the diet before pelleting while PHY was sprayed on dried pellets (Baruah *et al*., 2005, 2007a, b). However, up to our knowledge, there is not a single study in which PHY was incubated (pretreatment) in the presence of CA in the diet so that CA can provide optimum conditions for PHY activity. Hence, present study was designed to investigate the effects of simultaneous pretreatment of CA and PHY on growth performance, muscle proximate analysis and nutrient digestibility performance of *L. rohita* juveniles.

Table I.- Ingredient composition of test diets.

Ingredients	SBM 1	SBM 2	SBM 3	SBM 4
SBM	65	65	65	65
Wheat flour	15	13	14.95	12.95
Rice polish	9	9	9	9
Fish meal	5	5	5	5
Soybean oil	3	3	3	3
Vitamin premix[a]	1	1	1	1
Mineral mixture[b]	1	1	1	1
Chromic oxide	1	1	1	1
CA	0	2	0	2
PHY[c]	0	0	0.05	0.05
Total	100	100	100	100

[a]Each kg of vitamin premix contains; Vitamin A, 15 MIU; Vitamin D_3, 3 MIU; Vitamin E, 6000 IU; Vitamin K, 4000 mg; Vitamin B_1, 5000 mg; Vitamin B_2, 6000 mg; Vitamin B6, 4000 mg; B_9, 750 mg; Vitamin B_{12}, 9000 ug; Calcium pantothenate, 10000mg; Vitamin C, 15000mg; Nicotinic acid, 25000mg. [b]Each kg of mineral mixture contains; Ca (Calcium), 155 gm. P (Phosphorous), 135gm. Mg (Magnesium), 55gm. Na (Sodium), 45gm. Zn (Zinc), 3000 mg. Mn (Manganese), 2000 mg. Fe (Iron), 1000 mg. Cu (Copper), 600 mg; Co (Cobalt), 40 mg; I (Iodine), 40mg; Se (Selenium), 3mg. [c]The 0.05 g of PHY provides 1000 FTU, where, the FTU is one phytase activity unit that liberates 1 µmol of inorganic orthophosphate/min from 5.1mmol/L substrate (sodium phosphate) at 5.5 pH and 37°C temperature (Engelen *et al*., 1994).

MATERIALS AND METHODS

Four SBM based experimental diets were made (Table I). SBM1 was experimental diet without CA and PHY supplementation, SBM2 was supplemented with 2% CA while SBM3 had 1000 $FTUkg^{-1}$ PHY only. SBM4 was supplemented with both of these supplements (2% CA+ 1000 $FTUkg^{-1}$ PHY). Feed ingredients were procured from market and ground to 0.05 mm particle size. Method of pretreatment of CA and PHY was as follows. Paste of ground ingredients along with CA and PHY was made by adding distilled water (1.5 times ingredient volume). Paste was incubated for 15.5 h at 40°C and was further oven dried at 60°C for 12.5 h. This dried dough was again ground to fine powder and vitamins and minerals mixtures were added to it, which were further mixed in electric mixture with gradual addition of soybean oil (Nwanna *et al*., 2008). Procedure of pretreatment was same for all the experimental diets except that SBM1 was incubated without any supplement and SBM2 and SBM3 were pretreated with CA and PHY respectively while SBM4 was pretreated simultaneously with both of the additives (CA and PHY). Required moisture for pelleting was gained by adding 15% water into above mixture and was processed through hand pelletizer for pellets (3 mm) formation. Pellets were dried up to 5% moisture and stored at -20°C throughout the feeding trial. Experiment was run under 2×2 factorial experiment under completely randomized design with three replicates per treatment. Proximate and mineral composition of diet is given in Table II.

Table II.- Proximate and mineral composition of test diets.

Nutrient	SBM 1	SBM 2	SBM 3	SBM 4
DM (%)	95.51±0.05	96.00±0.62	96.19±0.51	95.57±0.01
CP (%)	35.70±0.91	35.20±0.67	35.90±0.61	35.70±1.09
CF (%)	10.82±0.32	10.95±0.24	11.65±0.66	11.64±0.64
Ca (mg/kg)	16.34±0.40	15.87±0.25	15.97±0.23	16.43±0.09
Mg (mg/kg)	6.22±0.65	6.54±0.04	5.90±0.12	6.14±0.27
Cu (ug/kg)	19.66±0.80	20.19±0.37	19.16±0.45	19.52±0.55
Zn (ug/kg)	72.53±2.18	73.06±1.58	73.45±1.11	72.50±0.98
Mn (ug/kg)	38.54±0.96	38.28±0.65	38.19±0.79	39.12±0.40
P (mg/kg)	14.11±0.32	13.37±0.66	13.23±0.67	14.05±0.54
Na (mg/kg)	7.51±0.04	7.90±0.21	8.11±0.39	7.97±0.56
K (mg/kg)	9.53±0.95	10.24±0.35	10.79±0.54	9.98±0.72
Fe (ug/kg)	0.45±0.00	0.45±0.00	0.45±0.00	0.45±0.00

The data are means of two replicates. DM, dry matter; CP, crude protein; CF, crude fiber.

Feeding experiment was performed in Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad, Pakistan. Juveniles of *L. rohita* were obtained from Government Fish Seed Hatchery, Faisalabad, Pakistan and acclimatized to laboratory conditions before initiation of feeding trial. Fish were freed from ectoparasites by giving 5g NaCl L^{-1} bath and stored into 70 L water capacity tanks with basal diet feeding once a day for acclimation (Allan and Rowland, 1992). After acclimation 120 fish of average 3.16±0.027 g weight were shifted into 12 experimental tanks such that every tank contained 10 fish. Each experimental diet was fed to duplicate tanks twice a day. After the feeding session of three hours the fish from each experimental tank were moved to separate clean water, the experimental tanks were washed completely to remove the particles of diet, refilled with fresh water and fish were shifted back to experimental tanks. Again, after the interval of two hours the feces were collected from the fecal collection tube of each tank having two subsequent valves. Care was taken to avoid breaking the thin fecal strings in order to minimize the nutrient leaching. Fecal material of each replicated treatment was dried in oven, ground and stored for chemical analysis. Temperature (24.9-28.7°C), pH (7.4-8.6) and dissolved oxygen (5.8-7.3 mgL^{-1}) were monitored and maintained to the standard conditions of rohu culture with the help of thermometer, Jenway pH meter (model 3510) and D.O. meter (model 970), respectively. Aeration was provided round the clock to all tanks throughout the study. The feeding as well as digestibility trail last for 60 days. Initial weight was measured at the initiation of trial and then weight gain was recorded on weekly basis. Growth performance was calculated by formulae used by Mohseni *et al*. (2009).

Fish from each experimental tank were chemically analyzed for muscle proximate composition. After 24 h starvation fish were dipped into 3000 mg L^{-1} clove oil solution for 40-60s and were sacrificed by a sharp blow in head following Khajepour *et al*. (2012). Muscle samples of fish were taken, sealed in plastic bags and stored at -20°C until further analyses. Proximate composition of diet, feces and muscles were determined by following standard methods of AOAC (1995). Crude protein (N×6.25) contents of samples were determined by micro Kjeldahl apparatus after acid digestion, crude fat by following petroleum ether extraction method through soxtec HT2 1045 system, dry matter contents by oven drying at 105°C up to a constant weight. Muscle sample was ashed via 6 h incineration at 550°C in electric furnace (Eyela-TMF 3100) until stable weight. Gross energy of diet and feces was determined using adiabatic oxygen bomb calorimeter (Parr Instrument Co., Moline. USA). For mineral estimation, the test diets and feces samples were digested in a boiling nitric acid and perchloric acid mixture (2:1). After appropriate dilution, Ca, Mg, Fe, Cu, Mn and Zn contents were estimated by atomic absorption spectrophotometer (Hitachi Polarized Zeeman AAS, Z-8200, Japan). While P contents were analyzed colorimetrically on UV/VIS spectrophotometer (U-2001, Hitachi) at 720 nm wavelength. Sodium and potassium were analyzed by using flamephotometer (Jenway PFP-7, UK) (AOAC, 1995). The chromic oxide contents were also determined on UV/VIS spectrophotometer (U-2001, Hitachi) at 350 nm wavelength after acid digestion by following the method described by Zhou *et al*. (2004).

Apparent nutrient digestibility coefficients (ADC) of experimental diets were calculated by following formula reported in NRC (1993).

$$ADC (\%) = 100 - 100 \times \frac{\text{Percent marker in diet} \times \text{Percent nutrient in feces}}{\text{Percent marker in feces} \times \text{Percent nutrient in diet}}$$

Results were analysed using two-way analysis of variance (Steel *et al*., 1996) and considered significant at $p<0.05$ (Snedecor and Conhran, 1991). As only two levels of each additive (0 and 2% for CA and 0 and 1000 FTUkg^{-1} for PHY) were used, the significant or non-significant response of these factors and their interaction for observed responses can be clearly confirmed by the *p*-value of two way analysis of variance. Therefore, no post hoc test was applied. CoStat computer package (Version 6.303, PMB 320, Monterey, CA, 93940 USA) was used for statistical analyses.

RESULTS

Effects of CA and PHY supplementations on growth and feed performances is given in Table III. CA supplementation ($p<0.05$) increased the weight gain% and specific growth rate (SGR), while decreased ($p<0.05$) the feed conversion ratio (FCR). Similar response of growth performance was recorded by juveniles when fed PHY pre-treated SBM based diet. Both supplements interacted ($p<0.05$) positively for increased SGR and decreased FCR, while no effect of their interaction was observed for weight gain%. Maximum increase in SGR was observed in the group of fish feeding both PHY and CA (SBM4), next to it was CA supplemented diet (SBM2), then PHY supplemented diet (SBM3) and minimum increase in SGR was observed in fish group feeding on diet without any supplement (SBM1).

Effects of CA and PHY supplementations on muscle proximate composition (dry matter, crude protein, crude fat and crude ash) of fingerlings is presented in Table IV. Muscle proximate composition was ($p<0.05$) affected by CA and PHY supplementations. Dry matter, crude protein and crude ash were ($p<0.05$) increased while crude fat contents were decreased ($p<0.05$) in response to CA and PHY supplementations. However, results showed no significant interaction between these supplements for improving the muscle proximate composition except for crude ash, where interaction of CA and PHY was positive.

Table III.- Growth performance of *L. rohita* fed CA and PHY supplemented SBM based diets

PHY (FTU/kg)	0		1000		p values			
CA (%)	0	2	0	2	PSE	PHY	CA	PHY×CA
Test diets	SBM1	SBM2	SBM3	SBM4				
Initial weight (g)	3.13	3.18	3.2	3.15				
Final weight (g)	11.35[c]	14.62[b]	14.62[b]	17.57[a]	0.02	<0.05	<0.05	<0.05
Weight gain %[a]	261.95	359.87	357.48	458.00	2.05	<0.05	<0.05	0.477
Specific growth rate[b]	1.42	1.69	1.68	1.91	0.01	<0.05	<0.05	<0.05
Feed conversion ratio[c]	1.11	0.99	0.98	0.99	0.00	<0.05	<0.05	<0.05
Survival rate (%)	100	100	100	100	0.00			

The data are means of two replicates. PSE (pooled SE), √MSE/n (where MSE, mean-squared error). [a]Weight gain%=(Final weight – Initial weight / Initial weight) x 100. [b]SGR=ln (Final body weight – Initial body weight) / day x 100. [c]FCR= Total dry feed intake (g) / Wet weight gain (g).

Table IV.- Muscle proximate composition of *L. rohita* fed CA and PHY supplemented SBM based diets.

PHY (FTU/kg)	0		1000		p values			
CA (%)	0	2	0	2	PSE	PHY	CA	PHY×CA
Test diets	SBM1	SBM2	SBM3	SBM4				
Dry matter (g/kg)	264.03	275.12	279.86	288.71	1.03	<0.05	<0.05	0.3079
Crude protein (g/kg)	151.18	165.00	168.99	184.62	0.66	<0.05	<0.05	0.2071
Crude fat (g/kg)	61.04	54.76	52.91	44.64	0.89	<0.05	<0.05	0.2944
Crude ash (g/kg)	3.19	3.95	3.95	4.98	0.04	<0.05	<0.05	<0.05

The data are means of two replicates. PSE (pooled SE), √MSE/n (where MSE= mean-squared error).

Table V.- Nutrient digestibility of *L. rohita* fed CA and PHY supplemented SBM based diets.

PHY (FTU/kg)	0		1000		p values			
CA (%)	0	2	0	2	PSE	PHY	CA	PHY×CA
Test diets	SBM1	SBM2	SBM3	SBM4				
Dry matter (%)	64.10	74.87	73.06	82.21	0.75	<0.05	<0.05	0.31
Crude protein (%)	60.65	72.20	69.69	80.13	0.72	<0.05	<0.05	0.47
Crude fat (%)	49.56	68.10	68.11	78.98	1.63	<0.05	<0.05	0.47
Ca (%)	49.39	61.24	60.53	77.46	1.12	<0.05	<0.05	0.54
Mg (%)	48.18	66.20	61.58	81.07	1.48	<0.05	<0.05	0.63
Cu (%)	41.83	58.02	53.58	72.23	1.54	<0.05	<0.05	0.44
Zn (%)	48.50	63.44	59.88	71.05	1.33	<0.05	<0.05	0.20
Mn (%)	54.16	67.87	62.87	76.85	0.49	<0.05	<0.05	0.80
P (%)	37.51	49.88	49.44	65.35	1.50	<0.05	<0.05	0.27
Na (%)	47.18	61.45	64.45	76.71	1.77	<0.05	<0.05	0.59
K (%)	50.21	65.47	65.98	82.67	0.91	<0.05	<0.05	0.59
Fe (%)	36.73	57.78	60.05	77.46	0.72	<0.05	<0.05	0.31

The data are means of two replicates. PSE (pooled SE), √MSE/n (where MSE, mean-squared error).

In Table V a significant ($p<0.05$) increase in digestibilities of dry matter, crude protein and crude fat by *L. rohita* juveniles was observed with 2% CA inclusion in SBM based diet comparative to control diet (SBM1). Significant ($p<0.05$) enhancement in dry matter, crude protein and crude fat digestibility was also recorded by PHY inclusion in the diet. It was also observed that simultaneous addition of CA and PHY in SBM based diet did not show any significant ($p>0.05$) interaction for digestibility of these nutritional attributes in *L. rohita* juveniles. Digestibilities of Ca Mg Cu, Zn, Mn, P, Na, K and Fe were ($p<0.05$) enhanced by CA (2%) and PHY (1000 FTUkg^{-1}) supplementations as compare to control group in the SBM based diet (Table V). Nevertheless, no interaction between the supplements were observed to improve the mineral digestibility.

DISCUSSION

In the present study, CA supplementation in the SBM based diet had resulted in improved ($p<0.05$) growth and feed performance in *L. rohita* juveniles. This improved growth may be attributed to the fact that CA being an organic acid lowers the intestinal pH which enhances the phytate-P solubility and increases the phosphorous absorption in the small intestine (Cross *et al*., 1990). Breakdown of phytate complexes had also resulted in the release of other chelated nutrients and minerals like proteins, Ca^{+2}, Mg^{+2}, Zn^{+2} and Fe^{+2} which lead to the improved growth performance of fish (Baruah *et al*., 2005; Vielma *et al*., 1999). Growth rate was also increased due to the physiological fact that during high feed intake and when the animals are young, or when the feeds are high in crude protein, free hydrochloric acid levels in the stomach are reduced which have a negative impact on pepsin activation as well as on pancreatic enzymes secretion. So, acidifier supplementation resolve this problem and lead to improved growth (Eidelsburger, 1997). Similar to our results, improved growth performance against dietary CA acidification was also recorded in rainbow trout, *Oncorhynchus mykiss* (Pandey and Satoh, 2008) and red sea bream, *Pagrus major* (Sarker *et al*., 2007).

From the results obtained, increased growth rate while decreased FCR were recorded with PHY supplementation to SBM based diet. PHY might have hydrolysed the phytate resulting in release of bound P and other chelated nutrients which lead to improved growth performance (Hussain *et al*., 2017; Shah *et al*., 2016; Kumar *et al*., 2012; Cao *et al*., 2007). Present results are strongly supported by Wang *et al*. (2009) who also observed improved growth performance in rainbow trout, *Onchorynchus mykiss* fed on PHY pre-treated plant based diet. Increased growth performance against PHY supplemented diet has also been observed in catfish (Nwanna *et al*., 2005; Kim and Hung, 2007; Hung *et al*., 2014), tilapia (Cao *et al*., 2008; Tahoun *et al*., 2009), common carp (Sardar *et al*., 2007; Phromkunthong *et al*., 2010), gibel carp (Liu *et al*., 2012) and rohu (Baruah *et al*., 2007a; Hussain *et al*., 2011).

To continue the comparison, a significant ($p<0.05$) positive interaction between PHY and CA was recorded for SGR and FCR; however, this interaction remained non-significant for weight gain%. Positive interaction may be attributed to the fact that CA may have provided the favourable environment for phytase to perform optimally by lowering the gut pH. Improved growth performance as a result of combined effect of CA and PHY has also been reported in *Cyprinus carpio* by Khajepour *et al*. (2012). In contrast to our results, in a recent study Zhu *et al*. (2014) reported non-significant interaction between CA and PHY for growth performance of yellow catfish. This discrepancy in results may be due to the differences in experimental conditions including water temperature and pH, amount of phytate-P in diet, feed processing methods, PHY treatment techniques and physiological conditions in the gut of different fish species (Liebert and Portz, 2005).

Following with the discussion, citric acid acidification had ($p<0.05$) enhanced the muscle dry matter, crude protein, crude ash while decreased the crude fat contents. Citric acid might reduce the intestinal pH resulting in the solubility of phytate-nutrient complex which lead to increased deposition of these nutrients in body including muscles. Our results are in close agreement with Khajepour and Hosseini (2012) who observed increased crude protein and crude ash contents in muscles of beluga, *Huso huso* against 2% and 3% CA supplementation in the diet.

PHY supplementation improved ($p<0.05$) the muscle proximate composition of *L. rohita* as well. Due to phytate hydrolysing ability of PHY, it releases the nutrients and makes them more available to fish. Studies had suggested that PHY supplementation enhances protein utilization in fish species fed on plant based diets (Storebakken *et al*., 1998; Sugiura *et al*., 2001). In agreement to our study, Sardar *et al*. (2007) also reported increased crude protein and decreased crude fat in whole body of common carp as a result of PHY addition in the feed. Increased crude ash in the body by PHY supplementation was also reported by Biswas *et al*. (2007) for red sea bream (*Pagrus major)*.

In this experiment, a non-significant interaction between both the supplements was observed to improve nutrient profile of fish muscles. However, improved muscle proximate composition of common carp, *Cyprinus carpio* was obtained by Khajepour *et al*. (2012) by feeding 3% CA and 500 U/kg PHY in SBM based diet.

Khajepour and Hosseini (2012) also recorded similar results for crude protein and dry matter digestibilities by adding 3% CA in SBM based diet of beluga, *Huso huso*. Furthermore, improved protein digestibility by supplementing 0.2% and 0.4% formic acid was also observed in rainbow trout (Luckstadt, 2008). In our experiments, a significant ($p<0.05$) increase in dry matter, crude protein and crude fat digestibilities by *L. rohita* juveniles was observed with CA inclusion in SBM based diet.

In the present study, digestibilities of P, Ca, Mg, Na, K, Cu, Zn, Mn, and Fe were ($p<0.05$) increased by CA supplementation. Our experiment results are in agreement with many previous studies. Sugiura *et al*. (1998) reported an increased P availability from 68.3% (control diet) to 87.45% by supplementing 5% CA in the diet of rainbow trout. Similarly, increased mineral digestibility by CA (3%) supplementation was also observed in *L. rohita* juveniles (Baruah *et al*., 2007b). Sarker *et al*. (2007) reported similar results with CA supplementation in plant protein based diet. They observed increased ($p<0.05$) absorption and retention of mineral at 5% CA level in the diet of red sea bream, *Pagrus major*.

Increased ($p<0.05$) digestibilities of dry matter, crude protein and crude fat has been observed in the present study in PHY supplemented diets as compare to control group. Improved dry matter digestibility was also reported in rainbow trout (*Onchorhynchus mykiss)* (Cheng and Hardy, 2004) and Korean rock fish (*Sebastes schlegeli*) (Yoo *et al*., 2005) in response to 500 and 1000 Ukg^{-1} PHY supplementation respectively. Furuya *et al*. (2001) also observed an increased crude protein digestibility at 700 $FTUkg^{-1}$ PHY level in the diet of Nile tilapia. Similarly, Ashraf and Goda (2007) observed higher apparent digestibility coefficient (88.3%) for crude fat in Nile tilapia fed SBM based diet supplemented with PHY at the level of 1000 $FTUkg^{-1}$. Hussain *et al*. (2011) observed significantly higher values of crude fat digestibility at 750 $FTUkg^{-1}$ PHY level in *L. rohita* fingerlings.

PHY supplementation, in this experiment, had showed significant ($p<0.05$) positive influence on mineral digestibility. Baruah *et al*. (2007b) also observed improved absorption of P, Mn, Na, K and Fe by feeding 500 $FTUkg^{-1}$ PHY to *L. rohita* juveniles. Our results are in agreement with other studies which reported positive effects of PHY in *L. rohita* (Baruah *et al*., 2005), *Cyprinus carpio* (Schafer *et al*., 1995), *Ictalurus punctatus* (Jackson *et al*., 1996), *Morone saxatilis* (Hughes and Soares, 1998; Papatryphon and Soares, 2001), *Salmo salar* (Sajjadi and Carter, 2004) and other fish species (Storebakken *et al*., 1998; Liebert and Portz, 2005; Yan *et al*., 2002; Debnath *et al*., 2005; Papatryphon *et al*., 1999).

Addition of organic acid in the diet reduces the dietary pH as well as the pH in the intestinal lumen, which is likely to enhance the PHY activity (Erdman, 1979). However, in the present study, a non-significant ($p>0.05$) interaction was recorded between both the supplements to improve the digestibilities of dry matter, crude protein, crude fat and mineral. Similar to our results, a non-significant ($p>0.05$) interaction between both the supplements for dry matter digestibility was also observed in *L. rohita* (Baruah *et al*., 2007b) and *Cyprinus carpio* (Phromkunthong *et al*., 2010). Besides, this work did not show a significant interaction between both of the additives for mineral bioavailability as reported in *L. rohita* (Shah *et al*., 2015; Akram *et al*., 2016) and *Cyprinus carpio* (Phromkunthong *et al*., 2010).

CONCLUSION

In conclusion, present study evidenced that the experimental diets in which soybean meal was supplemented with PHY and CA performed better than the diets without supplements. The dietary pre-treatment with PHY and CA not only enhanced growth performance, but also improved the muscle proximate composition and nutrient digestibility in *L. rohita* juveniles. However, the interaction between dietary supplements was non-significant for most of the studied parameters except FCR, SGR and muscle ash contents. The interaction study between PHY and CA with more graded levels of supplements need to conducted in future. Moreover, there is also a need to study the effect of PHY, CA pre-treatment on digestive enzyme activities of fish, either any of the supplement or their interaction helps to improve the activities of digestive enzymes.

Statement of conflict of interest
Authors have declared no conflict of interest.

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