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Combined Effect of Acidification and Phytase Supplementation on Calcium and Phosphorus Digestibility and Body Composition of Rohu (*Labeo rohita*)

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ABSTRACT

Rohu fish is tastiest fish among carps but its production is less than other species due to its slow growth and high mortality due to unavailability of suitable diet especially at juveniles stage. This study focus on the healthy production with low cost by using sunflower meal to some extent replace fish meal. In this 3× 3 factorial and completely randomized design study 324 fish were used. The acidification levels were (0, 1.5 and 3%) whereas the phytase levels were (0, 500 and 1000 FTU). The addition of 3 % citric acid and 1000FTU phytase produced significant interaction effect on calcium and phosphorus digestibility and different body contents of *L. rohita.* Phytase and citric acid increased the digestibility of calcium and phosphorus with improvement in body dry matter, protein, fat, energy and ash contents of *L. rohita.* Acidification and phytase supplementation showed significant ($P < 0.05$) interaction on body composition and mineral utilization in rohu. Citric acid and phytase supplementation improves the diet by increasing the availability of minerals and quality of fish by positively affecting the body composition of fish. 3% citric acid and 1000FTU phytase proves to be interacted positively. Further study is required to find the optimum levels of phytase and citric acid in this regard.

INTRODUCTION

Feed production for aquaculture is expected to be increased from 45 to 50, 65 and 87 million metric ton from 2015, 2020 and 2025, respectively. Major culture-able species includes carps, catfish, tilapia, shrimp, salmon and other marine fish (Tacon and Metian, 2015). Many aquaculturist are focusing towards reduction in fish meal inclusion in diet with enzyme application as done in poultry. So they try to increase the phosphorus availability with other nutrients and minerals by using phytase (Vandenberg *et al*., 2011; Suprayudi *et al*., 2012). It can be said that the continuous change in fish feed formulation may include the increasing levels of plants ingredients with additives (enzymes and organic acids) for better nutritional quality of diet (Bedford and Partridge, 2010). Application of phytase relies on the specific activity in the gastric tract of animals and in fish species it vary according to the presence or absence of stomach (Lemos and Tacon, 2015; Rust, 2002). The reported pH for phytase activity remain between 4.0-6.0 (Greiner and Konietzny, 2010) which is a limitation in its use in agastric species like carp and shrimps (Dall *et al*., 1990; Kumar *et al*., 2012).

The effect of acidification is considerably studied in different animals. The citric acid supplementation can improve the P availability and minerals in fish (Sugiura and Hardy, 2000). Citric acid supplementation decreases the use of inorganic minerals in diet so plant meal based citric acid supplemented diet lower not only the phosphorus supplementation but also the phosphorus excretion (Hernandez *et al*., 2013). Other research results showed that acidification of diets can increase the availability of phosphorus from plants ingredients, making the replacement of fish meal possible and decreasing the phosphate supplementation (Khajepour and Hosseini, 2012).

Phytase and citric acid improved not only growth but bone mineralization and immune response with improvement in intestinal working (Emami *et al*., 2013). Phytase (500 FTU/kg) with citric acid (3%) have been reported to increase sodium, potassium, calcium, iron and manganese in bone (Baruah *et al*., 2005; Afzal *et al*., 2016) as well as weight gain, protein efficiency ratio and whole body ash (Baruah *et al*., 2007a) in *Labeo rohita* juveniles. The interactive mechanism of citric acid and phytase released minerals (calcium and phosphorus) in fish from phytate- calcium or phosphorus complex so that phytic

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acid become exposed to phytase for hydrolysis. Citric acid was given importance in the land-dwelling animal's studies (Liu *et al*., 2014). It is well known that citric acid can enhance the uptake of phosphorus, zinc and calcium in salmonids (Sugiura *et al*., 1998). Nevertheless, there is limited work on the effect of phytase and citric acid on fish. Therefore, in the present study, we used phytase with citric acid to explore the interactions between low fish meal diet, phytase and citric acid on *Labeo rohita*.

MATERIALS AND METHODS

Experimental design and diet

A 3 × 3 factorial experiment was designed with three citric acid levels (0, 1.5 and 3%) and three phytase levels (0, 500 and 1000 FTU/kg) to study their main and interaction effects on calcium and phosphorus digestibility and body composition of fish. The ingredients and diets chemical composition is shown in Tables I and II.

All the ingredients of diet with different levels of citric acid were mixed in a mixer, moisture was added by adding distilled water and pellets were made using extruder. Phytase was supplemented by spraying method (Jackson *et al*., 1996) after pellet formation of diet. The pellets were then dried at room temperature and refrigerated at 4°C until the start of feeding trial. This experiment was a completely randomized design with triplicates of each treatment.

Table I.- Formulation of the experimental diets.

Ingredients	C_0P_0	C_0P_{500}	C_0P_{1000}	$C_{1.5}P_0$	$C_{1.5}P_{500}$	$C_{1.5}P_{1000}$	C_3P_0	C_3P_{500}	C_3P_{1000}
Sunflower meal[a]	15	15	15	15	15	15	15	15	15
Fish meal[a]	52	52	52	52	52	52	52	52	52
Wheat flour[a]	10	10	10	10	10	10	10	10	10
Rice polish[a]	16	16	16	16	16	16	16	16	16
fish oil	3	3	3	3	3	3	3	3	3
Vitamin premix[b]	1	1	1	1	1	1	1	1	1
Mineral premix[b]	1	1	1	1	1	1	1	1	1
Ascorbic acid	1	1	1	1	1	1	1	1	1
Chromic oxide	1	1	1	1	1	1	1	1	1
Citric acid	0	0	0	1.5	1.5	1.5	3	3	3

[a] fish meal (% dry matter): crude protein 23.4, crude lipid 0.9, ash 13.6; sunflower meal (% dry matter): crude protein 6, crude lipid2.2, ash1.05; wheat flour (% dry matter): crude protein 1.2, crude lipid 0.25, ash 0.17; rice polish (%dry matter): crude protein 1.76, crude lipid 2.1, ash 1.6. [b] vitamin premix: same as Hussain *et al*. (2015b).

Table II.- Proximate and mineral composition of the experimental diets.

Diets	C_0P_0	C_0P_{500}	C_0P_{1000}	$C_{1.5}P_0$	$C_{1.5}P_{500}$	$C_{1.5}P_{1000}$	C_3P_0	C_3P_{500}	C_3P_{1000}
Dry matter (%)	94.04	94.78	95.16	94.25	95.27	95.64	94.53	95.44	95.84
Crude protein (%)	32.12	32.22	32.43	32.22	32.45	32.46	32.24	32.36	32.38
Crude fat (%)	10.11	11.08	11.57	10.92	11.05	11.81	10.92	11.78	12.04
Gross energy (kcal/g)	2.64	2.66	2.64	2.67	2.62	2.64	2.63	2.69	2.63
P (%)	2.60	2.64	2.65	2.63	2.92	3.06	3.15	3.27	3.48
Ca (%)	3.27	3.54	3.8	3.68	3.82	3.91	3.81	3.89	4.21
Mg (%)	0.82	0.85	0.92	0.85	0.92	0.99	0.92	1.03	1.07
K (%)	0.60	0.62	0.60	0.61	0.84	0.86	0.98	1.00	0.98
Na (%)	0.62	0.64	0.63	0.64	0.93	0.95	0.99	0.99	0.98
Mn (%)	0.0035	0.0038	0.0042	0.0043	0.0045	0.0048	0.0044	0.0051	0.0054
Zn (%)	0.0094	0.0098	0.0114	0.0118	0.0122	0.0129	0.0124	0.0132	0.0148
Cu (%)	0.0011	0.0012	0.0014	0.0013	0.0015	0.0016	0.0013	0.0015	0.0016
Fe (%)	0.024	0.030	0.032	0.025	0.031	0.035	0.025	0.034	0.036

Fish and feeding trial

To start this experiment, 400 *Labeo rohita* fish were taken from the fish seed hatchery Faisalabad (Punjab, Pakistan) and supplied to fish nutrition laboratory, Department of Zoology, Wild Life and Fisheries, University of Agriculture, Faisalabad, Pakistan. Formerly all fish were acclimatized to laboratory conditions for fifteen days and fed with the basal experimental diet (Table I). All fish were starved for 24 h before the start of feeding trial. Entire 324 fish with same magnitude (3.4 g) were disseminated in 27 steel tanks (70 L) with 12 fish in each tank. Triplicate tanks (v shape at base) were used for each treatment attached as flow through system. Water temperature 26.5 ± 0.3°C, dissolved oxygen 6.7±0.05mg/L, pH 7.4±0.04 and electric conductivity remain 1.32-1.53 dS/m. throughout the experiment, fish were fed the experimental diet by hand till apparent satiation at 08:00 am for two months. Uneaten feed was collected after an hour of feeding.

Sample collection and analysis

Initial fish weight noted in the start of the research experiment. After the completion of feeding trial, the fish were weighed to determine the growth parameters, and chemical composition of diets and body was determined by following method: Dry matter was determined by constant heating at 105°C for 6 h and crude protein was determined by Kjeldahl method (AOAC, 1998). Gross energy was determined by oxygen bomb calorimeter and Crude fat was determined by petroleum ether extraction for 12 h in a Soxhlet (AOAC, 1998). Minerals were determined after digesting them in a nitric acid and perchloric acid mixture (2:1) according to AOAC (1998) and using Atomic Absorption Spectrophotometer (Hitachi Polarized Atomic Absorption Spectrometer, Z-8200) as recommended by manufacturers.

Calculation and statistical analysis

Apparent digestibility coefficients of calcium and phosphorus were calculated by using following equation:

$$\text{Apparent digestibility of nutrients (\%)} = 100 - 100 \times \left[\frac{\text{percent chromic oxide in diet} \times \text{percent nutrient in feces}}{\text{percent chromic oxide in feces} \times \text{percent nutrient in diet}}\right]$$

Mean values of three replicate are reported ± standard error of mean. After confirming the homogeneity of variance and normality, data was subjected to two way ANOVA using citric acid and phytase levels and differences were considered significant at $P < 0.05$. Data was also analysed using one way ANOVA followed by Tukey test using Costate (6.303, PMB320, Monterey, CA, 93940 USA).

Table III.- Calcium and phosphorus apparent digestibility coefficient.

Diets	C_0P_0	C_0P_{500}	C_0P_{1000}	$C_{1.5}P_0$	$C_{1.5}P_{500}$	$C_{1.5}P_{1000}$	C_3P_0	C_3P_{500}	C_3P_{1000}
Calcium (%)	53.38± 0.443^{e}	58.95± 0.159^{d}	63.37± 1.016^{bc}	60.03± 0.267^{d}	64.38± 0.147^{bc}	63.83± 0.361^{bc}	62.24± 0.124^{c}	64.63± 0.103^{b}	70.14± 0.467^{a}
Phosphorus (%)	53.20± 0.22^{f}	56.54± 0.09^{e}	58.92± 1.12^{e}	58.20± 0.34^{e}	66.76± 0.22^{d}	67.95± 0.35^{cd}	70.19± 0.43^{c}	73.35± 0.45^{b}	78.62± 0.25^{a}

Table IV.- Body composition of *L. rohita* fed with graded levels of citric acid and phytase supplemented diet.

Diets	C_0P_0	C_0P_{500}	C_0P_{1000}	$C_{1.5}P_0$	$C_{1.5}P_{500}$	$C_{1.5}P_{1000}$	C_3P_0	C_3P_{500}	C_3P_{1000}	C.A	PHY	C.A× PHY
Dry matter	21.16± 0.008h	22.04± 0.011f	22.26± 0.006e	21.22± 0.008gh	22.34± 0.011d	24.92± 0.017b	21.28± 0.014g	22.71± 0.014c	25.93± 0.011a	<0.05	<0.05	<0.05
Crude protein	16.84± 0.015i	17.25± 0.017g	17.66± 0.025f	16.94± 0.025h	18.14± 0.02c	19.25± 0.011b	17.82± 0.008e	17.94± 0.025d	19.55± 0.017a	<0.05	<0.05	<0.05
Crude fat	3.12± 0.005e	3.446± 0.014c	3.25± 0.023d	2.873± 0.008f	2.836± 0.012f	3.846± 0.012b	2.366± 0.008g	3.236± 0.02d	4.283± 0.003a	<0.05	<0.05	<0.05
Gross energy (Kcal/g)	1.56± 0.43^{h}	1.60± 1.63^{f}	1.64± 2.02^{d}	1.58± 1.35^{g}	1.62± 1.83^{e}	1.68± 1.87^{b}	1.58± 2.28^{g}	1.66± 2.28^{c}	1.79± 1.95^{a}	<0.05	<0.05	<0.05
Crude ash	1.453± 0.003e	1.63± 0.011c	1.563± 0.008d	1.436± 0.012e	1.426± 0.008e	1.886± 0.003b	1.163± 0.008f	1.613± 0.008c	2.133± 0.003a	<0.05	<0.05	<0.05

RESULTS

Apparent digestibility coefficient of calcium and phosphorus

The maximum calcium digestibility was 70.14±0.467% of C_3P_{1000} while minimum was 53.38±0.443 % of C_0P_0. The analysis of variance of digestibility of calcium was shown in Table III. The digestibility of calcium of test diets remain significantly different from each other (Table III). The citric acid and phytase significantly affected the digestibility of calcium. The results also conform the synergistic effect of citric acid and phytase on calcium digestibility.

The maximum phosphorus digestibility was 78.62±0.25% of C_3P_{1000} while minimum was 53.20±0.22 % of C_0P_0. The phosphorus digestibility was also significantly affected by citric acid and phytase as in the case of calcium. The analysis of variance of digestibility of phosphorus was shown in Table III. The phosphorus digestibility of all test diets were significantly different from each other (Table III).

Body composition

The proximate compositions of the whole body of *L. rohita* was shown in Table IV. The results present significant difference among dietary treatments (Table IV). Citric acid and phytase affected the body composition of fish and there was also interaction effect of both on all the parameters studied (ash, protein, fat, energy and dry matter).

DISCUSSION

In the present study citric acid and phytase significantly affected the digestibility of nutrients in *L. rohita* diets. Acidification of diet with 3% citric acid significantly improved the apparent digestibility of phosphorus in *L. rohita* diets. Comparable improvements were observed in Beluga (*Husso huso*) at 3% citric acid (Khajepour and Hosseini, 2010, 2012), *L. rohita* (Baruah *et al*., 2005), *Oncorhynchus mykiss* at 5% and 1% citric acid, respectively (Sugiura *et al*., 2001; Hernandez *et al*., 2012), tiger shrimp at 2% acidifications (Ng *et al*., 2015), white shrimp at 4% acidification (Romano *et al*., 2015) in pigs at 1% of citric acid (Boling *et al*., 2000). Acidification may decrease the discharge of phosphorus in feces which improve the environment of aquatic organisms (Hernandez *et al*., 2012; Sarker *et al*., 2007). Minerals and nutrient's digestibility and absorption depend upon the acidifier levels (Partanen and Mroz, 1999; Hedayati *et al*., 2013).

The higher apparent digestibility of phosphorus was observed at 1000 FTU/kg in the present study. Similarly significant ($P<0.05$) results of phytase were observed by Baruah *et al*. (2007b) and Roy *et al*. (2014) in *L. rohita*, Schafer *et al*. (1995) in *C. carpio*, Papatryphon and Soares (2001) in striped bass, Yoo *et al*. (2005) in Korean rockfish. Storebakken *et al*. (1998) in Atlantic salmon observed increase in apparent digestibility coefficient (20 to 30 %) of phosphorus with the increase of plant meal by the addition of phytase. Phytate phosphorus remain unavailable to fish owing to the absence of enzyme in stomach of fish (NRC, 1983) as Phytate is the principal anti-nutritional factor in diets based on crops which binds minerals (Satoh *et al*., 1989). Enzymatic breakdown of phytate by phytase may increase the retention and availability of phosphorus (Cao *et al*., 2007; Morales *et al*., 2014) and may decrease the discharge of phosphorus in feces (Yan and Reigh, 2002; Zhu *et al*., 2014) by increasing utilization of phosphorus (Liu *et al*., 2012; Jackson *et al*., 1996; Sugiura *et al*., 2001).

There was a positive interaction of citric acid and phytase in the present study and higher phosphorus digestibility was observed at 3% citric acid and 1000 FTU/kg. Sugiura *et al*. (2001) reported significant increment in the apparent absorption of phosphorus by the citric acid (5%) and phytase supplementation in the diet of rainbow trout, *Oncorhynchus mykiss* and similar effect of citric acid and phytase on phosphorus was observed in birds (Hariharan and Gangadevi, 2015). Citric acid remain important not only in terrestrial animal studies (Liu *et al*., 2014) but also in aquatic animals like fish for enhancing the uptake of calcium, phosphorus and zinc (Sugiura *et al*., 1998). Higher apparent digestibility coefficient of calcium was observed in the present work. Acidification of diet has been reported in animal feed to chelate calcium and phosphorus and increases their solubility, availability and utilization in rainbow trout (Vielma *et al*., 1999).

Phytase can improve the digestibility coefficient of calcium in fish but feed ingredients also influences the activity of phytase (Bedford, 2000). Phytase (20,000 FTU/kg) improved the availability of minerals in pigs (Zeng *et al*., 2015). Apparent digestibility coefficient of calcium and phosphorus was improved by 4000 FTU/kg of phytase in *Oncorhynchus mykiss* (Morales *et al*., 2015). So the supplementation of phytase in feed may increase the bio-availability of minerals (Vielma *et al*., 2004; Debnath *et al*., 2005a).

The dry matter content of *L. rohita* was increased in the present study and higher dry matter content was observed in fish fed on diet acidified by 3% citric acid. Khaled (2015) and Cuvin-Aralar *et al*. (2011) observed no negative effect of acidification on body composition of Nile tilapia (*Oreochromis niloticus*). No effect of citric acid (3%) for moisture in muscles was observed in common carp (*Cyprinus carpio*) by Khajepour *et al*.

(2012) and in Beluga (*Huso huso*) by Khajepour *et al*. (2011). In the present study there was increase in body dry matter of *L. rohita* with the increasing level of phytase and higher dry matter content in body was observed at 1000 FTU/kg phytase. Similar results were observed by Sardar *et al*. (2007) in common carp (*Cyprinus carpio*) but Hassan *et al*. (2013) observed no effect of phytase on the dry matter content. These differences in results may be due to difference in experimental conditions, composition of diets, source of phytase and species of fish.

There was significant interaction of phytase and citric acid in the present study with partial replacement of fish meal with sunflower meal in *Labeo rohita* diet. Citric acid 3% and phytase 1000FTU/kg was considered best in the present study with positive effect on protein contents of fish. Similar results were obtained by Shah *et al*. (2016) in *Labeo rohita.* But Nezhad *et al*. (2008) stated that citric acid may not affect the ability of phytase in chicks. It was suggested that this might be due to the none influencing behavior of citric acid on phytase activity to low P diets, or citric acid (organic acid) might had speedily metabolized in the body and negligibly effected the pH of intestine.

The results of the present study showed that acidification have significant effect on crude lipid contents of body composition of *L. rohita.* 3% citric acid in diet of fish positively affected the crude fat contents of fish whereas 1.5% citric acid decreased the body lipid contents in partially replaced diets. An increasing trend in body lipid was observed at 1.5% acidified diet in Nile Tilapia (*Oreochromis niloticus*) (Hassaan *et al*., 2014). Khaled (2015) and Cuvin-Aralar *et al*. (2011) observed negative effect of acidification on body composition of Nile tilapia with non- significant crude lipid contents. Similar results were observed in poultry (Park *et al*., 2009) and aquaculture (Ng *et al*., 2011).

There was an increasing trend of lipid contents in body of *L. rohita* with the increasing supplementation level of phytase in partially replaced diets. Phytase 1000 FTU/kg significantly influenced the crude lipid contents of fish body in the present research work.

Similar results were obtained in *O. niloticus* by Hassan *et al*. (2013), in common carp (*Cyprinus carpio*) by Kumar *et al*. (2011), Atlantic salmon (*Salmo salar*) by Sajjadi and Carter (2004a), *P. pangasius* by Debnath *et al*. (2005a, 2007) and in pigs by Shelton *et al*. (2003) and Brady *et al*. (2003). The complex of phytate with lipid and other nutrients lower down its availability (Debnath *et al*., 2005a) as observed by Usmani and Jafri (2002). In the present study, results showed that phytase 1000 FTU/kg made available the fat and other nutrients from phytic acid to fish which increases their level in body. Baruah *et al*. (2007a, b, c) also reported same findings in *L. rohita.*

Khajepour and Hosseini (2010) observed that 2 and 3% of citric acid can increase ash content in muscles of beluga. Other studies by Sugiura *et al*. (2001) on rainbow trout, Baruah *et al*. (2007a), on *Labeo rohita* and Hossain *et al*. (2007) on red sea bream supported these results. Acidification of diet by citric acid can significantly increase iron in whole body of fish (Vielma *et al*. 1999). Acidification of diet chelates minerals in rainbow trout and increases their solubility and mineral utilization is improved (Vielma *et al*. 1999). Two factors affecting the results are; 1) acidification and solubilization and 2) chelation of cations. Citric acid crosses the brush border in intestine by mechanism of Na transport system (Wolffram, 1990, 1992).

Dietary phytase supplementation increased ash contents (Zhu *et al*., 2014), alike supportive results were observed in rainbow trout (Sugiura *et al*., 2001), *P. pangasius* (Debnath *et al*., 2005b), *L. rohita* (Baruah *et al*., 2007b), pig (Jendza *et al*., 2005; Veum *et al*., 2006), and poultry (Deepa *et al*., 2011). The increment in ash in body by phytase reveals that phytase had released minerals during hydrolysis of phytate. Phytase can hydrolyze phytic acid which increases minerals contents and result into increment in the body ash content (Masumoto *et al*., 2001; Liebert and Portz, 2005; Debnath *et al*., 2005a).

Statement of conflict of interest

Authors have declared no conflict of interest.

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