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Enhancing the mineral retention in *Labeo rohita* juveniles fed citric acid and phytase supplemented soybean meal based diet

Khadija Akram[1], Muhammad Afzal[1], Syed Zakir Hussain Shah[*1,2], Mahroze Fatima[1,3], Zainab Akram[1], Hafiz Fakhar Hayat[4]

[1]*Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad, Pakistan*

[2]*Department of Zoology, University of Sargodha, Sargodha*

[3]*Department of Zoology, Government College Women University, Faisalabad, Pakistan*

[4]*Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan*

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Abstract

The aim of this study was to evaluate the effect of pretreatment of citric acid (CA), phytase (PHY) and their interaction on major mineral (P, Ca, Mg, Na and K) retention and excretion in *Labeo rohita* juveniles fed soybean meal based diet. Four experimental diets were designed by supplementing CA (%) and PHY (FTU/kg) at the levels of 0,0, 2,0, 0,1000 and 2,1000 respectively. Juveniles (initial weight 3.15±0.03 g) were fed at 2 % of their live body weight for 2 months. Three replicates were allocated for each test diet and fifteen fish were kept in each replicate. At the end of feeding trial, fish were sacrificed and digested in nitric acid and perchloric acid for mineral contents determination. Results revealed that CA supplementation significantly ($p<0.05$) increased the mineral retention in *L. rohita* juveniles. Similarly, the mineral retention was also significantly ($p<0.05$) enhanced by PHY pre-treatment in whole body of juveniles. Moreover, both supplements (CA and PHY) showed significant ($p<0.05$) interaction for P and Ca retention in juveniles. However, CA significantly ($p<0.05$) decreased the excretion of the observed mineral in whole body of juveniles. Likewise, by the addition of phytase, less excretion was also recorded in the observed mineral except Mg. Both supplements (CA and PHY) interacted significantly ($p<0.05$) to reduce the excretion of observed mineral except K in *L. rohita* juveniles. In conclusion, increased retention and less excretion of observed mineral was recorded in CA and PHY pretreated soybean meal based diet in *L. rohita* juveniles.

* **Corresponding Author:** Syed Zakir Hussain Shah ✉ zakiruaf@gmail.com

Introduction

Aquaculture is the fastest growing food producing industry (FAO, 2000) that plays an important role to fulfill the requirement and demand for fish consumption. Its production depends upon species, stocking density and feed formulation (Wu, 1995). Fish require high protein level in their diets that is mainly provided by fishmeal.

It contains essential nutrients such as amino acids, fatty acids, vitamins and minerals (Zhou *et al.*, 2004). However, increasing demand, high prices unstable supply (Lunger *et al.*, 2007) and high phosphorus contents which is the main cause of eutrophication of rivers, lakes and reserviors (Correll, 1999) makes it compulsory to search for alternative protein sources (Pham *et al.*, 2008).

Plant protein sources are the most appropriate candidates for replacement of fishmeal due to their steady supply, well balanced amino acid profile, low cost (Storebakken *et al.*, 2000) and less phosphorus contents (Cao *et al.*, 2007). Nevertheless, anti-nutritional factors are present in plant protein sources (Cao *et al.*, 2007) that cause hindrance in digestion of nutrients (Olli *et al.*, 1995; Francis *et al.*, 2001).

Phytate, one of the anti-nutritional factors, contains 50-80% phosphorus (Harland and Morris, 1995) which is not available for agastric or mono-gastric fishes (NRC, 1993). It is a negatively charged molecule and chelates positively charged compounds in the gut including minerals, amino acids and fatty acids (Vohra and Satanarayana, 2003) therefore, reduces their availability to fish.

The negative impacts of phytate can be neutralized by the addition of phytase to the diet (Simons *et al.*, 1990;Rodehutscord and Pfeffer, 1995). Phytase supplementation increases the concentration of minerals such as Mg^{2+}, K^{+}, Ca^{2+}, Mn^{2+} and Zn^{2+} in fish body (Vielma *et al.*, 1998) by hydrolyzing the phytate mineral complexes. The improved digestibility of dry matter (Papatryphon *et al.*, 1999) and crude protein (Storebakken *et al.*, 1998) was also reported by phytase supplementation in fish diet (Kornegay, 1995).

Citric acid (CA), an organic acid, has the capability to replace the fishmeal up to 70% with low phosphorus containing plant protein sources (Sarker *et al.*, 2012b). By lowering the gastric pH, CA increases the phytate solubility (Jongbloed, 1987) and nutrient absorption (Boling-Frankenbach *et al.*, 2001). It also chelates positively charged ions and enhances their availability (Ravindran and Kornegay, 1993). Furthermore, it has high gross energy values (Freitag and Luckstadt, 2007). Besides of its independent effects, CA favours the activity of phytase as phytase performs better at lower pH (Simons *et al.*, 1990). Supplementation of CA (3 %) in a PHY (500 FTU/kg) treated diet showed significant interaction in improving the body mineralization in *L. rohita* juveniles (Baruah*et al.*, 2005).

The aim of present study was to determine the independent as well as combine effects of phytase and citric acid supplementations in improving major mineral retention in *Labeo rohita* juveniles fed soybean meal based diet.

Materials and methods

The present research work was carried out to study the efficacy of citric acid and phytase in soybean meal based diet to improve major mineral retention in rohu (*Labeo rohita*) juveniles. The experiment was conducted in the Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad.

Fish and experimental conditions

Labeo rohita juveniles of same size (initial weight 3 g) and age were brought from Government Fish Seed Hatchery, Faisalabad.

They were treated with 5 g/LNaCl to ensure the removal of ectoparasites and to prevent fungal infections. They were placed in cemented tanks (200 L water capacity) to acclimatize the fish for 2 weeks. Basal diet was given to fingerlings once a day to satiation level (Allan and Rowland, 1992). Before beginning the trial, they were shifted to V-shaped tanks having capacity of 70 L.

Each tank was provided with 15 fish. Three replicates were taken for each diet. The experiment was run for a period of 2 months.

The tanks were well aerated through capillary system to maintain oxygen level during the study period. Temperature, pH and dissolved oxygen were monitored by using thermometer, pH meter (Jenway, model 3510) and D.O. meter (Jenway, model 970) respectively, throughout the feeding trial.

Feed ingredients and experimental diets

Feed ingredients were procured from commercial feed mill and were ground and sieved to require size. These ingredients were examined for chemical composition by following the standard methods of AOAC (1995) before making the experimental diets. Chemical analysis was performed to evaluate ingredients and feed samples. Moisture was assessed by drying at 105°C for 12 h. Crude protein was analyzed using the Kjeldahl method and crude lipid by petroleum ether extraction using soxlet apparatus. Ash contents were determined by ignition at 650°C in muffle furnace (Eyela-TMF 3100) until constant weight was acquired and gross energy was assessed by using bomb calorimeter. The composition of experimental diets is shown in Table 1.

Soybean meal based diet was used to formulate 4 experimental diets named as D1, D2, D3 and D4, containing two levels of phytase (PHY; 0 FTU/kg and 1000 FTU/kg) and two levels of citric acid (CA; 0 % and 2 %) in 2×2 factorial experiment under Complete Randomized Design (CRD). D1 contained no supplementation of PHY (FTU/kg) and CA (%) and kept as control group; D2 contained 2% CA and 0 FTU/kg PHY supplementation; D3 was supplemented with 1000 FTU/kg PHY and 0% citric acid and D4 was provided with both supplements (1000 FTU/kg PHY and 2% CA). Both supplements (CA and PHY) in the diet were incubated with other ingredients through a pre-treatment method (Nwanna *et al.*, 2008).

In pre-treatment method, paste was prepared by mixing 1 kg of the ground ingredients (fishmeal, soybean meal, rice polish and wheat flour) and 1.5 L of distilled water.

The paste was incubated for 15.5 h at 40°C to provide optimum condition for phytase activity. Paste was dried in oven at 60°C for 12.5 h. Before mixing with the other ingredients, the dried paste was converted into powder form (Nwanna *et al.,* 2008). Vitamin premix and mineral mixture were added in powder form and during electric mixing soybean oil was added. Dough was formed by using 10-15 % distilled water. The pellets were formed through hand machine and were kept safe in freezer at -18°C throughout the feeding trial.

Feeding protocol and sample collection

Labeo rohita juveniles were fed according to the 2% of their live body weight. After three hours of feeding session, tanks were washed and great care was taken to remove the uneaten particles of diets from the tanks. These tanks were refilled with water and fish were shifted back in the tanks. At the end of trial, the fish were deprived of food for 24 h. Fish were sacrificed by using clove solution (3000 mg/L for 40-60s) and then executed by a sharp blow on the head. Fish samples were collected and ground into powder form for further analysis.

Mineral analysis

Samples were digested by using nitric acid and perchloric acid in 3:1 ratio. Sample were diluted upto appropriate volume. Ca and Mg analysis was done by using Atomic Absorption Spectrophotometer (Hitachi Polarized Zeeman AAS, Z-8200, Japan). Sodium (Na) and potassium (K) were estimated by using Flame photometer (Jenway PFP-7, UK). Phosphorus (P) was examined by UV/VIS spectrophotometer by using molybedate reagent at 720 nm absorbance.

Statistical analysis

Two-way analysis of variance was applied for the statistical analysis of major mineral retention and excretion data (Steel *et al.,* 1996).Statistical analyses were performed using CoStat computer package (Version 6.303, PMB 320, Monterey, CA, 93940 USA).

Results

Effect of CA and PHY supplementation on major mineral retention (%) is given in Table 2. A significant increase in P, Ca and Mg retention was recorded in CA supplemented groups as compare to control group. A similar increase in the retention of the minerals was also observed in groups having PHY treated diets. Moreover, similar observation of improved mineral retention in response to the addition of both supplements were also recorded in the case of Na and K retention. However, simultaneous pre-treatment of both the supplements showed significant interaction only for P and Ca, while Mg, Na and K remained unaffected.

Table 1. Composition (%) of experimental diets.

Ingredients	D1	D2	D3	D4
Soybean meal	65	65	65	65
Wheat flour	15	13	15	13
Rice polish	10	10	10	10
Fish meal	5	5	5	5
Soybean oil	3	3	3	3
Vitamin premix*	1	1	1	1
Mineral mixture**	1	1	1	1
CA (%)	0	2	0	2
PHY (FTU/kg)	0	0	1000	1000
Total	100	100	100	100

Data for major mineral excretion(kg/t production) is reported in Table 3. P and Ca excretion values were significantly decreased in CA supplemented diets in comparison with control group. Similarly, the addition of PHY caused significant decrease in excretion values of these mineral except Mg. Moreover, Na and K excretion values were also significantly reduced by the pretreatments of CA and PHY. Significant interaction of CA and PHY were recorded for P, Ca, Mg and Na while K remained unaffected by their interaction.

Table 2. Effect of phytase and citric acid on major mineral retention (%).

Diet	CA level (%)	PHY (FTU/kg)	P retention (%)	Ca retention (%)	Mg retention (%)	Na retention (%)	K retention (%)
D1	0	0	33.69	30.88	5.47	15.13	41.37
D2	2	0	52.17	40.11	7.96	21.89	50.88
D3	0	1000	54.08	40.7	9.67	21.8	47.59
D4	2	1000	60.62	43.74	11.67	28.18	57.46
PSE*			1.2852	0.37795	0.2352	0.6485	1.1724
ANOVA							
Source of variation					P-value		
PHY			.0000***	.0000***	.0000***	.0000***	.0000***
CA			.0000***	.0000***	.0000***	.0000***	.0006***
PHY × CA			.0017**	.0000 ***	.3243ns	.7730ns	.8804 ns

*PSE= pool standard error =√MSE/n (where MSE= mean-squared error)

Discussion

Among major minerals (P, Ca, Mg, Na and K), specifically P is an imperative source of pollution in freshwaters. Therefore, environmental pollution can be reduced by decreasing the dietary mineral levels and fecal mineral discharges from aquaculture facilities. Phytate, an anti-nutritional factor in plant meal based diets, form complexes with these minerals and make them unavailable to the fish, resulting in higher excretion of these minerals in fish farm effluents. In the present study, improved mineral retention and their reduced excretion was recorded by phytase supplementation in soybean meal based diet. Enzymatic hydrolysis of phytate may improve the mineral absorption which lead to improved mineral retention and reduced excretion (Wang *et al.,* 2009). Similarly, less excretion of P, Ca and protein was observed in rainbow trout (*Oncorhynchus mykiss*) by PHY addition in diet (Wang *et al.*, 2009). A reduced amount of fecal P was released in the environment when fish fed with diet containing phytase treatedsoy-protein concentrate based diets to Atlantic salmon, *Salmo salar,* in comparison with that fish fed either fish meal or soy concentrate diets (Storebakken *et al.*, 1998). PHY supplementation in diets having high levels of plant protein significantly enhanced the assimilation of P and reduced the discharge of P from feces in Atlantic salmon, *Salmo salar* (Sajjadi and Carter, 2004). By adding PHY in diets, less mineral excretion was recorded for other mono-gastric animals like broilers (Demirel *et al.*, 2012). Thus, the phytase supplementation is considered to be highly significant in fish diets.

Table 3. Effect of phytase and citric acid on major mineral excretion (kg/t production).

Diet	CA level (%)	PHY (FTU/kg)	P excretion (kg/t production)	Ca excretion (kg/tproduction)	Mg excretion (kg/t production)	Na excretion (kg/t production)	K excretion (kg/t production)
D1	0	0	8.3972	10.1307	5.2787	5.7181	5.0136
D2	2	0	4.9359	7.3243	4.6409	4.7562	3.8852
D3	0	1000	4.6416	7.2254	4.0693	4.8409	4.3213
D4	2	1000	4.527	7.5507	4.436	4.6816	3.4726
PSE			0.2577	0.1405	0.1853	0.1737	0.1828
ANOVA							
Source of variation			P-value				
PHY			.0001***	.0000***	.4856ns	.0121*	.0006***
CA			.0000***	.0000***	.0051**	.0255*	.0165*
PHY × CA			.0002***	.0000 ***	.0267*	.0497*	.4665 ns

CA pretreatment in soybean meal based diet, in the present study, resulted in increased mineral retention and decreased excretion in the body of *L. rohita*.

The increase of P, Ca, Mg, Na and K with CA supplementation in fish body revealed that utilization rate of P and other elements can be considerably enhanced by the breakdown of phytate. CA may decrease the intestinal pH which lead to increased solubility of phytate mineral complexes. It also acts as a catalyst in the absorption of P in the small intestine (Cross *et al.*, 1989). Improved fish growth, increased P retention and reduced P loading was observed in rainbow trout by adding 1% CA to low fishmeal based diets (Hernandez *et al.*, 2013).

Increased P and N retention was observed in red sea bream fed the diet containing 1% CA (Sarker *et al.*, 2007). Enhanced P retention by CA supplementation was also observed in broilers (Demirel *et al.*, 2012).

The simultaneous effect of CA and PHY showed a synergistic increase in P and Ca retention. Moreover, similar synergistic reduction in the mineral excretion was also registered in the present study. The addition of CA in the diet decreased the intestinal pH and provided a suitable environment for PHY to perform its activity which may lead to synergistic effect observed in the current study (Phromkunthong *et al.,* 2010). In concurrent with our study, Sugiura *et al.* (2001) also reported enhanced mineral absorption in rainbow trout, *Oncorhynchus mykiss* fed CA acidified diet and PHY treated plant based diet. Similar results were observed for poultry by the interaction of CA and PHY (Demirel *et al*., 2012).

Conclusion

Conclusively, CA and PHY pretreated soybean meal based diet showed increased retention and less excretion of major mineral (P, Ca, Mg, Na and K) in *L. rohita* juveniles.

Phytase was added at the expense of wheat flour. *Each Kg of Vitamin premix contains Vitamin A 15 M.I.U.; Vitamin D3 3 M. I. U.; Nicotinic acid 25000mg; Vitamin B1 5000 mg; Vitamin E 6000 IU; Vitamin B2 6000 mg; Vitamin K3 4000 mg; Vitamin B6 4000 mg; Folic acid 750 mg; Vitamin B12 9000 mcg; Vitamin C 15000mg; Calcium pantothenate 10000mg. **Each Kg mineral granules contains Cu (Copper) 600m; Co (Cobalt) 40mg; Fe (Iron) 1000 mg; I (Iodine) 40mg; Se (Selenium) 3mg Zn (Zinc) 3000 mg; Mn (Manganese) 2000mg.

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