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Role of Citric acid and Phytase supplementation in improving trace Mineral Retention in Rohu (*Labeo rohita*) juveniles fed Soybean meal based diet

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ABSTRACT

A 2×2 factorial experiment was designed to assess the efficacy of pretreatment with citric acid (CA), phytase (PHY) and their interaction on trace mineral (Cu, Zn, Mn and Fe) retention and excretion in rohu, *Labeo rohita* juveniles fed soybean meal based diet. Treatments, used for the study, consisted of four experimental diets, supplemented with CA (%) and PHY (FTU/kg) at the levels of 0,0; 2,0; 0,1000 and 2,1000, respectively. For each experimental diet, two replicates were allocated and in each replicate fifteen fish were stocked. Diets were fed to *L. rohita* (initial weight 3.15+0.03 g) juveniles at 2% of their body weight for 8 weeks. Results showed the significant (p<0.05) increase in the retention of trace mineral by CA supplementation. Similarly, PHY pre-treatment also improved (p<0.05) the trace mineral deposition in whole body of juveniles. Moreover, significant interactions were observed between CA and PHY to increase the trace mineral retention (Zn, Mn and Fe) in juveniles. Nevertheless, less excretion of these observed mineral was recorded in all the supplemented groups as compared to control. In conclusion, pretreatment of SBM based diet by CA and PHY increased the availability and utilization of trace mineral resulting in higher retention and lower excretion of these minerals in *L. rohita* juveniles.

Key Words: Phytase pretreatment, dietary acidification, Rohu, Trace mineral, Retention, Excretion,

INTRODUCTION

Fishmeal is considered an important source of protein in fish feed formulations as it contains essential nutrients (Zhou *et al*., 2004). However, owing to its increasing demand, high price, restricted supply (Lunger *et al*., 2007) and high phosphorus contents (Correll, 1999), it is necessary to replace it by alternative protein sources (Toko *et al*., 2008). Alternatively, plant protein sources are considered most suitable because of their amino acid profile, reasonable price, steady supply, high protein and low phosphorus contents (Hardy, 1999, Storebakken *et al*., 2000, Ai & Xie, 2005). Soybean meal (SBM), one of the plant protein sources, has high protein content and good amino acid profile. It is consistently available and reported to be palatable to most of the fish species (Lim & Akiyama, 1992). However, SBM, like the other plant protein sources contains anti-nutritional factors like phytate (Usmani & Jafri, 2002, Baruah *et al*., 2004).

Phytate is the major phosphorus (P) storage compound in plant seeds as 80 % of total P is present in it (Harland & Morris, 1995). It also acts as a strong chelator of positively charged metal ions as it contains negatively charged phosphate groups (Erdman, 1979, Vohra & Satanarayana, 2003). It also combines with dietary protein, vitamins, form insoluble complexes then decreases digestibility and activity of these nutrients (Liu *et al.,* 1998, Sugiura *et al*., 2001). Phytate nutrient complexes cause eutrophication on their discharge into natural water bodies (Liebert & Portz, 2005). To improve the nutritional value of plant protein sources and to avoid the aquatic pollution different approaches are being used in fish nutrition (Simons *et al*., 1990, Rodehutscord & Pfeffer, 1995).

Phytase, a natural enzyme of phytate, is added to the diet, to improve the nutritive value (Forster *et al*., 1999) of plant protein sources. Improved growth, feed performance and nutrient and mineral bioavailability have been observed in *L. rohita* fed on different phytase treated plant meal based diets (Hussain *et al*., 2011a, 2011b, 2011c, 2011d, 2011e).

Another approach which is being used to break this phytate is the supplementation of organic acids in the diet. Citric acid (CA), one of the organic acid, is extensively used for diet acidification as it has high buffering capacity and unique flavor (Hossain *et al.*, 2007). It enhances the solubility of phytate nutrient complexes and increases the absorption of nutrients (Zyla *et al*., 1995, Wood & Serfaty-Lacrosinere, 1992). It acts as a strong chelator of positively charged ions and increases their bioavailability (Wood & Serfaty-Lacrosinere, 1992).

Author's Contribution: Z.A., Did experiments; **M.A., & S.Z.H.S.**, Designed, planned and supervised research work; **M.F.**, Helped in experimental work; **K.A.**, Analyzed data statistically; **S.M.S**., Wrote manuscript; **M.S.**, Helped in manuscript writing.
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The aim of the present study was to determine the effect of citric acid and phytase supplementation on the retention and excretion of trace mineral in rohu (*Labeo rohita*) juveniles fed soybean meal based diet.

MATERIALS AND METHODS

The experiment was performed in the Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad, Pakistan.

Fish and experimental condition

Before the onset of experiment, *Labeo rohita* juveniles were procured from Government Fish Seed Hatchery, Faisalabad and were dipped in 5 g/L NaCl solution to remove all kinds of ectoparasites and fungal toxicity. Fish were placed in cemented tanks (1000 L water capacity) for two weeks for acclimatization to indoor conditions. Basal diet was given to fish 6 days a week to apparent satiation level during this period (Allan & Rowland, 1992). For experimental feeding trial, fifteen fish were stocked in each V shaped tanks (70 L) with nearly same initial weight (3.15±0.03 g). Two replicates were allotted to each test diet. The feeding trial continued for about two months. All tanks were provided with continuous aeration through capillary system to provide dissolved oxygen (DO) during the experimental trial. Temperature, pH and DO were monitored throughout the trial and kept in the range of *L. rohita* culture.

Feed Ingredients and Experimental Diets

Prior to experimental diet preparation, good quality feed ingredients were procured from commercial feed mill and were ground and sieved. Standard methods were used to analyze the ingredients and experimental diets (AOAC, 1995). Moisture was determined by oven through drying at 105°C for 12 h. Kjeldahl apparatus was used to determine crude protein and crude fat was estimated by petroleum ether extraction method using soxtec system. Ash was obtained when feed samples were ignited at 600°C for 12 h in muffle furnace. The composition of experimental diets is shown in the table 1 while proximate analysis is given in table 2.

Four soybean meal based experimental diets were formulated named as D1, D2, D3 and D4 by adding two levels of phytase (PHY; 0 FTU/kg and 1000 FTU/kg) and two levels of citric acid (CA; 0 % and 2 %) in a 2 × 2 factorial experiment under completely randomized design (CRD). D1 contained no supplementation of PHY (FTU/kg) and CA (%); D2 contained 2% CA and 0 FTU/kg PHY; D3 contained 1000 FTU/kg PHY and 0% citric acid and D4 contained both 1000 FTU/kg PHY and 2% CA. These supplements (PHY and CA) were added in the diet by incubating them with feed ingredients through a pretreatment method (Nwanna *et al.,* 2008). The method of pretreatment was as follows: For the pretreatment of ingredients, paste was formed by adding 1 kg of the ground ingredients (soybean meal, wheat flour, rice polish and fish meal) and 1.5 L of distilled water. The paste was kept at 40°C for 15.5 h and later on was dried at 60°C for 12.5 h. However, the paste was again changed into powdery form after drying. Vitamin premix and mineral mixture were added in this powdery stuff and mixed in electric mixture. Soybean oil was also added during mixing. Distilled water (10-15%) was added to this mixture to form a dough. This dough was used to form pellets through hand pelletizer. After air drying the pellets, these were crumbled and screened up to required size. Pellets were kept in freezer at -18°C till the completion of feeding trial.

Feeding Protocol and Sample Collection

Feed was given to *Labeo rohita* juveniles up to their apparent satiation. After the feeding session of three hours, feed was removed from the tanks by manual siphoning and water was replaced with filtered fresh water. At the end of two months feeding duration fish were starved for one day, anesthetized by giving a dose of 3000 mg/L clove oil, and sacrificed by a sharp blow on head (Khajepour *et al.,* 2012). Samples were collected and stored in freezer until analysis.

Mineral Analysis

Cu, Fe, Mn and Zn were estimated by atomic absorption spectrophotometer after acid digestion. Samples were subjected to wet digestion by using nitric acid and perchloric acid in ratio 3:1. Samples were diluted up to appropriate volume.

Calculation of Mineral Retention and Excretion

$$Retention\,(\%) = \frac{Final\ nutrient\ content - Initial\ nutrient\ content}{Nutrient\ intake} \times 100$$

$$Excretion\left(\frac{kg}{t}production\right) = \frac{FCR \times \text{Nutrient in diet (kg)} - Nutrient\ retained\ in\ fish}{Production\,(kg)} \times 1000$$

Required data for the estimation of retention (%) and excretion (kg/t production) were collected during the trial (unpublished), however, in the present manuscript, resultant data of retention (%) and excretion (kg/t production) is given only.

Statistical Analysis

Finally, two-way analysis of variance was applied to analyze trace mineral retention and excretion data (Steel *et al.,* 1996). 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 confirmed by the *p*-value of two-way analysis of variance.

RESULTS

Effect of CA, PHY and their interaction on trace mineral retention in *L. rohita* juveniles fed SBM meal based diet is given in Table 3. Results showed improved ($p<0.05$) mineral deposition in CA treated diets as compare to control group. Similarly, PHY supplementation also resulted in enhanced ($p<0.05$) mineral retention in the body of juveniles. Moreover, CA showed significant ($p<0.05$) interaction with PHY to improve the retention of these trace elements except Cu.

Trace mineral excretion (FTU/kg) data is reported in Table 4. Results showed that CA significantly ($p<0.05$) reduced the excretion (kg/t production) of Cu, Fe and Mn in *L. rohita* juveniles. Similarly, supplementation of PHY to the diet also resulted in a significant ($p<0.05$) decrease in the excretion of these minerals. However, Zn excretion remained unaffected ($p>0.05$) by the addition of both the supplements. Moreover, significant interactions were observed between CA and PHY to reduce all of the trace mineral excretion.

DISCUSSION

Minerals are the primary nutrients that cause pollution in freshwater bodies. Therefore, it is necessary to control environmental pollution by reducing dietary mineral levels and fecal mineral discharge. Phytate is considered as a major issue in plant based feed formulation due to its anti-nutritional behaviour because it strongly chelates with divalent mineral such as Cu^{2+}, Fe^{2+}, Zn^{2+} and Mn^{2+} and make them unavailable to fish by reducing their digestibility. In the present study, PHY pretreatment was found effective in improving trace mineral retention and reducing the excretion of these mineral in the feces of juveniles fed SBM based diet. This may be due to the enzymatic hydrolysis of phytate resulting in the release of chelated trace elements which lead to improved mineral retention and less excretion in rainbow trout (Wang *et al*., 2009). Similar to our results, addition of dietary PHY reduced the excretion of P, Ca and protein in rainbow trout (*Oncorhynchus mykiss*) fed SBM based diet (Wang *et al*., 2009). Similarly, Storebakken *et al*., (1998) also reported that Atlantic salmon, *Salmo salar* fed with PHY supplemented diet released less fecal P in freshwater bodies than fish fed without PHY supplemented diet. The addition of PHY in diets containing high levels of plant protein improved the digestibility of P and decreased P discharge in Atlantic salmon (Sajjadi & Carter, 2004). Reduced mineral excretion was also reported in broilers fed PHY supplemented diets (Demirel *et al.,* 2012).

In the current study, dietary CA resulted in higher retention of trace elements in the body of juveniles. Meantime, mineral excretion in fish of this group was significantly lower than of fish fed control diet in *L. rohita* juveniles. CA might had hydrolyzed the phytate mineral complexes which lead to improved retention and less excretion of these minerals (Cross *et al*., 1990). Increased P retention and decreased P loading by adding 1% CA in low-fish meal based diet were also observed in rainbow trout (Hernandez *et al*, 2013). Similar results were observed in broilers by CA addition to the diet (Demirel *et al.*, 2012).

In the present experiment, a significant ($p<0.05$) synergism between CA and PHY was observed to improve mineralization in *L. rohita* juveniles. Possibly, gastric acidification due to CA might reduce the gastric emptying rate that provided more time to PHY to hydrolyze the phytate (Jongbloed *et al*., 1987). Moreover, CA might favor PHY action by lowering the gastric pH as PHY perform optimally at low pH (Simons *et al*., 1990, Phromkunthong *et al*., 2010). Similar to our results, Sugiura *et al*., (2001) also observed increased minerals absorption in rainbow trout fed acidified and PHY treated plant based diets. In poultry, improved P retention and decreased excretion was also reported in response to combine supplementations with CA and PHY (Demirel *et al.,* 2012).

In conclusion, pretreatment of SBM based diet by CA and PHY increased the availability and utilization of trace minerals resulting in higher retention and lower excretion of these minerals in *L. rohita* juveniles.

Table I: 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

*****Each Kg of Vitamin premix contains Vitamin A, 15 MIU; Vitamin D3, 3 MIU; Nicotinic acid, 25000 mg; 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, 15000 mg; Calcium pantothenate, 10000 mg.

**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

***PHY was added at the expense of wheat flour.

Table II: Proximate analysis of experimental diets

Nutrient	D1	D2	D3	D4
Dry matter (%)	95.51±0.05	96.00±0.62	96.19±0.51	95.57±0.01
Crude protein (%)	35.70±0.91	35.20±0.67	35.90±0.61	35.70±1.09
Crude fat (%)	10.82±0.32	10.95±0.24	11.65±0.66	11.64±0.64

Data are means of three replicates

Table III: Retention (%) of trace elements in *L. rohita* fingerlings fed experimental diets

Diet	CA level (%)	PHY (FTU/kg)	Cu retention	Zn retention	Mn retention	Fe retention
D1	0	0	63.63	22.56	2.41	4.68
D2	2	0	80.44	22.65	3.36	6.2
D3	0	1000	83.46	22.74	3.49	6.24
D4	2	1000	94.24	24.46	4.07	7.01
PSE			1.449	0.2857	0.395	0.0138
ANOVA			***p*-value**			
	PHY		0.0000***	0.0078**	0.0000***	0.0000***
	CA		0.0000***	0.0049**	0.0000***	0.0000***
	PHY × CA		0.0712 ns	0.0033**	0.0017**	0.0000***

Data are means of three replicates. PSE = pooled SE = √MSE/n (where MSE= mean-squared error). P<0.05

Table IV: Excretion (kg/t production) of trace elements in *L. rohita* fingerlings fed experimental diets

Diet	CA level (%)	PHY (FTU/kg)	Cu excretion	Zn excretion	Mn excretion	Fe excretion
D1	0	0	0.0064	0.0503	0.0337	0.2966
D2	2	0	0.003	0.0435	0.0284	0.2487
D3	0	1000	0.0024	0.0432	0.0281	0.2481
D4	2	1000	0.0009	0.0471	0.0306	0.2628
PSE			0.00032	0.000786	0.000343	0.03125
ANOVA			***p*-value**			
PHY			0.0000***	0.0558 ns	0.0010**	0.0000***
CA			0.0000***	0.0911 ns	0.0044**	0.0000***
PHY × CA			0.0185*	0.0001 ***	0.0000***	0.0000***

Data are means of three replicates. PSE = pooled SE = √MSE/n (where MSE= mean-squared error). P<0.05

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