Nutrient digestibility performance by rohu (*Labeo rohita*) juveniles fed acidified and phytase pre-treated sunflower meal-based diet

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**ABSTRACT**
The present research work was conducted to investigate the effects of microbial phytase and citric acid supplantations on nutrient digestibility performance by rohu (*Labeo rohita*) juveniles fed a sunflower meal (SFM)-based diet. The basal diet was supplemented with two levels of phytase (0 and 1000 FTU/kg) and each level of this phytase-supplemented diet was further supplemented with two levels of citric acid (0% and 2%). Chromic oxide (1%), as an inert marker, was added to the diet to determine nutrient digestibility. Results showed that digestibility of dry matter, crude protein and ether extract was significantly (*p < .05*) enhanced by citric acid supplementation. Similarly, phytase pretreatment also resulted in improved (*p < .05*) digestibility of dry matter, crude protein and ether extract. Also, citric acid and phytase supplementation improved (*p < .05*) the digestibility of P, Na, K, Ca, Mg, Cu, Zn, Mn and Fe. Nevertheless, the addition of both supplements (citric acid and phytase) simultaneously did not produce any interaction for the digestibility of these minerals. Hence, it is concluded that phytase (1000 FTU/kg) and citric acid (2%) supplementation to an SFM meal-based diet improved the nutrient digestibility in *L. rohita* juveniles.

1. Introduction

Fishmeal is a preferable source of nutrients in the aquaculture feed industry. It has many essential nutrients such as vitamins, essential fatty acids, minerals, amino acids and growth factors (Zhou et al. 2004). However, its increasing demand has resulted in high cost and less availability. Secondly, it has a large amount of indigestible phosphorus, which, on excretion, causes water pollution. Furthermore, it is contaminated with different heavy metals and pesticides which lower the feed digestibility. These concerns have led to the search for alternative protein sources to fishmeal in aquaculture (NRC 1981).

Plant sources are considered the best alternative to fishmeal as they have comparably fewer quantities of phosphorus than fishmeal (Wenblad et al. 2013). Among many plant protein meals, sunflower meal (SFM) is considered the most promising alternative because of low cost and easy availability. It ensures efficient use of nutrients by fish because it is free from harmful and growth-depressing entities (Rehman et al. 2013). However, the main problem in the use of the SFM meal is that it has some anti-nutritional factors, among which phytate or phytic acid is most prominent. Phytate has deleterious effects on the physiology and morphology of the digestive tract, thus affecting the overall fish growth (Baruah et al. 2004). It binds about 80% of the total phosphorus present in plants and makes it unavailable to fish (NRC 1993). It also binds other minerals such as Zn, Mg and Ca, Fe, Cu and Mn and reduces their availability (Denstadi et al. 2006).

To overcome this problem, phytase, an enzyme, is being used in fish feed formulations. Chemically, it is known as myo-inositol hexaphosphate phosphohydrolase and is used to degrade the phytate present in plant sources, releasing the bound nutrients. It can also inhibit the chelation of various cations with phytate (Ravindran et al. 1995). Dietary inclusion of phytase in fish feed increases the digestibility of plant proteins. Improved growth and nutrient digestibility performances were observed in response to phytase supplementation in rohu, *Labeo rohita* (Baruah et al. 2007a), Korean rockfish, *Sebastes schlegeli* (Yoo et al. 2005), Japanese flounder, *Paralichthys olivaceus*, (Masumoto et al. 2001) and rainbow trout, *Onchorhynchus mykiss* (Cheng & Hardy 2002). It also played an important role in reducing the aquatic pollution by enhancing the phosphorus retention and reducing its discharge (Baruah et al. 2004).

Another approach being applied to fish nutrition to break the phytate is the inclusion of organic acids in the diet. Jongbloed (1987) observed the increased phytate solubility and phosphorus absorption in fish by supplementation of organic acids in the diet. Among different organic acids, citric acid is being widely used in animal nutrition. Zyla et al. (1995) reported its phytate dephosphorylating activity in *vitro*. In addition, supplemental organic acids also act as chelating agents by binding various cations along the intestine, thus increasing the mineral absorption through the intestine (Ravindran & Kornegay 1993). Khajepour and Hosseini (2012) observed that application of citric acid in feed significantly improved the mineral utilization in *Huso huso* fingerlings. Sarker et al. (2005) observed improved growth and nutrient retention with reduced feed conversion ratio, and N and P loading in *Pagrus major* fed citric acid acidified diet.
Phytase is a pH-dependent enzyme having optimal performance at two pH levels, that is, 2.5 and 5.0–5.5 (Simons et al. 1990). However, *L. rohita* being an agastric fish has higher pH (above 6). Citric acid inclusion in a phytase pre-treated diet may enhance the phytase activity for two reasons. First, by reducing the gastric pH, citric acid provides a favourable environment for phytase activity (Ravindran & Kornegay 1993). Second, dietary acidification slows down the gastric emptying rate (Jongbloed 1987), which provides more time for phytase activity. Previous studies showed synergism between citric acid and phytase to improve phosphorus digestibility in *L. rohita* (Baruah et al. 2007b) and Cyprinus carpio (Phromkunthong et al. 2010).

So the main purpose of our research was to investigate the combined effects of citric acid and phytase on the nutrients’ digestibility of rohu (*L. rohita*) fingerlings fed an acidified phytase pre-treated SFM-based diet. This research-based information may prove very useful for the formulation of cost-effective and environmentally friendly fish feed.

### 2. Materials and methods

The present trial was conducted at Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad. The main effects and interaction effect of two supplements, that is, phytase (0 and 1000 FTU/kg) and citric acid (0% and 2%), were investigated in a $2^2$ factorial arrangement with three replicates.

#### 2.1. Experimental fish

*L. rohita* juveniles were obtained from Government Fish Seed Hatchery, Faisalabad, and randomly allotted into V-shaped tanks (70 L water capacity) with the stocking density of 15 fish each. For two weeks, the fish were allowed to adapt to the new experimental conditions until starting of trial which lasted for 90 days. The juveniles were healthy and kept free of parasites and fungal infection during the experimental session.

#### 2.2. Water quality

All tanks were regularly aerated and water quality was monitored and maintained by using physical equipment, including thermometer for temperature, D.O. meter (Jenway 970) for dissolved oxygen and pH meter (Jenway 3510) for pH measurements.

#### 2.3. Experimental diets

Feed ingredients were bought from a commercial feed mill and ground to the required particle size. An electric mixer was used to mix all dry ingredients for 10–20 min and soybean oil was added gradually while mixing. Chromic oxide was added in the diets at a 1% concentration level as an inert marker to estimate the digestibility. Four SFM-based experimental diets were formulated by supplementing two levels of phytase (0 and 1000 FTU/kg) and two levels of citric acid (0% and 2%) in a factorial arrangement. The ingredients’ composition and proximate analysis of diets are given in Tables 1 and 2, respectively. Phytase was supplemented by a pretreatment method described by Nwanna et al. (2008) with some modifications. Ingredients’ mixture (1 kg) was mixed with distilled water (1.5 L) to form a paste which was incubated with phytase and citric acid for 15.5 h at 40°C. After incubation, the paste was oven-dried for 12.5 h at 60°C. After drying, the dough was again blended into powdery form before mixing with chromic oxide, minerals’ mixture and vitamin premix. Diets were pelleted by hand machine and dried pellets were stored at $-20^\circ$C. Fish were fed their prescribed diets at a feeding rate of 2% of their live wet weight during the whole experimental session. Faecal material was daily collected carefully and oven-dried before the analysis.

#### 2.4. Chemical analysis

The samples of feed ingredients, faeces and test diets were homogenized and analysed according to AOAC (1995): samples were digested in nitric acid and perchloric acid (3:1) mixture for mineral estimation (AOAC 1995). After appropriate dilution, minerals contents were analysed using atomic absorption spectrophotometer (Hitachi Polarized Atomic Absorption Spectrometer, Z-8200). For proximate analysis: moisture was analysed by drying in an oven for 12 h at 105°C; crude protein ($N \times 6.25$) was determined by micro-Kjeldahl apparatus; crude fat by petroleum ether extraction method through Soxtec HT2 1045 system and gross energy was determined by oxygen bomb calorimeter (Parr Instrument Co., Moline, USA). Phosphorus and chromic oxide contents were determined calorimetrically at 720 and 350 nm absorbance, respectively, using a UV/VIS spectrophotometer (U-2001, Hitachi).

### Table 1. Ingredients’ composition (%) of SFM-based diet.

| Ingredient         | SFM1 (%) | SFM2 (%) | SFM3 (%) | SFM4 (%) |
|--------------------|----------|----------|----------|----------|
| Sunflower meal     | 65       | 65       | 65       | 65       |
| Wheat flour        | 15       | 15       | 15       | 15       |
| Rice polish        | 9        | 9        | 9        | 9        |
| Fishmeal           | 5        | 5        | 5        | 5        |
| Soybean oil        | 3        | 3        | 3        | 3        |
| Vitamin premix     | 1        | 1        | 1        | 1        |
| Minerals premix    | 1        | 1        | 1        | 1        |
| Chromic oxide      | 1        | 1        | 1        | 1        |
| Total              | 100      | 100      | 100      | 100      |

### Table 2. Proximate composition and minerals’ contents of SFM-based diets.

| Diets  | SFM1 | SFM2 | SFM3 | SFM4 |
|--------|------|------|------|------|
| CA (%) | 0    | 2    | 0    | 2    |
| PHY (FTU/kg) | 0    | 0    | 1000 | 1000 |
| DM (%) | 95.79 ± 0.04 | 97.51 ± 0.05 | 97.94 ± 0.20 | 97.67 ± 0.10 |
| CP (%) | 33.93 ± 0.73 | 34.62 ± 0.84 | 34.06 ± 0.52 | 34.44 ± 1.0 |
| EE (%) | 10.81 ± 0.41 | 11.17 ± 0.06 | 11.11 ± 0.25 | 11.26 ± 0.33 |
| P (mg/g) | 15.34 ± 0.42 | 15.95 ± 0.26 | 15.60 ± 0.69 | 15.74 ± 0.38 |
| Na (mg/g) | 7.23 ± 0.33 | 7.46 ± 0.11 | 7.43 ± 0.47 | 7.83 ± 0.25 |
| K (mg/g) | 9.95 ± 0.28 | 9.84 ± 0.37 | 9.81 ± 0.25 | 10.08 ± 0.24 |
| Ca (mg/g) | 17.76 ± 0.45 | 17.98 ± 0.34 | 18.37 ± 0.56 | 17.95 ± 0.38 |
| Mg (mg/g) | 8.506 ± 0.06 | 8.073 ± 0.21 | 8.07 ± 0.28 | 8.5 ± 0.15 |
| Cu (µg/g) | 23.09 ± 0.58 | 23.59 ± 0.58 | 22.73 ± 0.58 | 23.63 ± 0.94 |
| Zn (µg/g) | 66.19 ± 0.91 | 66.09 ± 0.46 | 66.74 ± 1.00 | 66.14 ± 0.79 |
| Mn (µg/g) | 43.49 ± 0.13 | 45.75 ± 0.23 | 45.47 ± 0.190 | 45.96 ± 0.07 |
| Fe (µg/g) | 0.523 ± 0.00 | 0.523 ± 0.00 | 0.524 ± 0.00 | 0.524 ± 0.00 |

SFM, Sunflower meal.
Apparent nutrient digestibility coefficients (ADC) of experimental diets were calculated by the formula according to NRC (1993):

\[
\text{ADC}\% = 100 - \frac{\% \text{ marker in diet} \times \% \text{ nutrient in feces}}{\% \text{ nutrient in diet} \times \% \text{ marker in feces}}
\]

\[2.5. \text{Statistical analysis}\]

All data were subjected to two-way analysis of variance. The differences among means were compared by Tukey’s test considering significance at \( p < .05 \) (Snedecor & Cochran 1991). All statistical analyses were done using CoStat computer package (Version 6.303, PMB 320, Monterey, CA, 93940 USA).

\[3. \text{Results}\]

The analysed composition of nutrients and minerals of faeces is shown in Table 3. Supplementation of citric acid reduced \((p < .05)\) the nutrient and mineral contents in the fish faeces which showed enhanced absorption of these nutrients in the body. Similarly, pretreatment of diets with phytase also resulted in less \((p < .05)\) excretion of these nutrients and minerals compared to the control group. However, synergism among both supplements to reduce the excretion were recorded only for crude protein, P, K, Ca, Mg, Cu, Zn and Fe.

Results from digestibility data are presented in Table 4. Dietary supplementation of citric acid enhanced the digestibility of dry matter, crude protein and ether extract by 16%, 23% and 50%, respectively, compared to control diet. Similarly, phytase supplementation had also resulted in improved digestibility of dry matter, crude protein and ether extract by 18%, 24% and 66%, respectively, in comparison with the group having no supplementation. However, both supplements showed non-significant interaction for these nutritional attributes. Incorporation of citric acid in the diet had significantly \((p < .05)\) increased the minerals’ digestibility including P, Na, K, Ca, Mg, Cu, Zn, Mn and Fe by 34%, 31.6%, 34.5%, 20.4%, 21.2%, 14.2%, 12.9%, 31.2% and 47.6%, respectively, as compared to the control group. Phytase supplementation also showed enhanced absorption of P, Na, K, Ca, Mg, Cu, Zn, Mn and Fe by 32%, 30.3%, 29.9%, 21.7%, 20%, 9.6%, 11.6%, 28.4% and 47.3%, respectively, when compared with the control group. Nevertheless, the combination of both supplements did not show any significant interaction for the absorption of these minerals.

### Table 3. Proximate composition and minerals contents of faeces of *L. rohita* juveniles fed SFM-based diets.

| Diet | SFM1 | SFM2 | SFM3 | SFM4 | p-value |
|------|------|------|------|------|---------|
| Citric acid (%) | 0 | 2 | 0 | 2 | |
| Phytase (FTU/kg) | 0 | 0 | 1000 | 1000 | |
| DM (%) | 42.51a | 33.81b | 31.31c | 20.72d | 0.542 | <.05 | <.05 | ns |
| CP (%) | 17.09a | 13.2b | 12.44c | 7.19d | 0.158 | <.05 | <.05 | <.05 |
| EE (%) | 6.92a | 4.83b | 3.85c | 2.99d | 0.126 | <.05 | <.05 | <.05 |
| P (mg/g) | 8.51a | 6.19b | 5.97c | 4.01d | 0.025 | <.05 | <.05 | <.05 |
| Na (mg/g) | 3.82a | 2.73b | 2.66b | 1.53c | 0.018 | <.05 | <.05 | ns |
| K (mg/g) | 6.29a | 4.84b | 4.85b | 3.92c | 0.015 | <.05 | <.05 | <.05 |
| Ca (mg/g) | 7.98a | 5.94b | 5.68b | 3.34c | 0.097 | <.05 | <.05 | <.05 |
| Mg (mg/g) | 4.143a | 3.01b | 2.95c | 1.833d | 0.011 | <.05 | <.05 | <.05 |
| Cu (µg/g) | 11.85a | 10.56b | 10.37c | 8.99d | 0.013 | <.05 | <.05 | <.05 |
| Zn (µg/g) | 30.29a | 25.73b | 25.52b | 19.84c | 0.137 | <.05 | <.05 | <.05 |
| Mn (µg/g) | 0.608a | 0.451b | 0.452b | 0.302c | 0.210 | <.05 | <.05 | ns |
| Fe (µg/g) | 0.317a | 0.205b | 0.198b | 0.11d | 0.000 | <.05 | <.05 | <.05 |

SFM, sunflower meal.

Note: Mean values within row having different superscripts are significantly different at \( p < .05 \). Data are means of three replicates. PSE = pooled SE = \( \sqrt{\text{MSE}/n} \) (where MSE = mean-squared error).

### Table 4. Apparent digestibility of nutrients and minerals (%) in *L. rohita* juveniles fed SFM-based diet.

| Diet | SFM1 | SFM2 | SFM3 | SFM4 | p-value |
|------|------|------|------|------|---------|
| Citric acid (%) | 0 | 2 | 0 | 2 | |
| Phytase (FTU/kg) | 0 | 0 | 1000 | 1000 | |
| DM (%) | 59.813c | 69.111b | 70.343b | 81.322a | 0.562 | <.05 | <.05 | ns |
| CP (%) | 53.515c | 66.015b | 66.133b | 81.602a | 0.72 | <.05 | <.05 | ns |
| EE (%) | 40.912d | 61.376c | 67.835b | 76.553a | 1.481 | <.05 | <.05 | <.05 |
| P (%) | 48.791d | 65.478b | 64.456b | 77.474a | 0.983 | <.05 | <.05 | ns |
| Na (%) | 51.122c | 67.318b | 66.663b | 82.737a | 0.971 | <.05 | <.05 | <.05 |
| Ca (%) | 41.646c | 56.049b | 54.108b | 65.677a | 1.327 | <.05 | <.05 | <.05 |
| Mg (%) | 58.551c | 70.537b | 61.270b | 83.579a | 0.809 | <.05 | <.05 | <.05 |
| Cu (%) | 55.058c | 66.736b | 66.072b | 80.993a | 0.797 | <.05 | <.05 | ns |
| Zn (%) | 52.617d | 60.109b | 57.680b | 66.375a | 1.196 | <.05 | <.05 | <.05 |
| Mn (%) | 57.774c | 65.259b | 64.530b | 73.669a | 0.658 | <.05 | <.05 | ns |
| Fe (%) | 45.268c | 59.391b | 58.165b | 71.790a | 1.136 | <.05 | <.05 | ns |

SFM, Sunflower meal.

Note: Mean values within row having different superscripts are significantly different at \( p < .05 \). Data are means of three replicates. PSE = pooled SE = \( \sqrt{\text{MSE}/n} \) (where MSE = mean-squared error).

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4. Discussion

In the present study, the analysed values showed that citric acid supplementation significantly \( (p < .05) \) increased the dry matter, crude protein and ether extract digestibility as compared to the control diet. It may be due to the fact that citric acid solubilizes the phytate-nutrient complexes resulting in the availability of chelated nutrients for fish consumption (Jongbloed 1987). It also provides low pH for the optimum activity of digestive enzymes, thus improving digestibility. Similarly, improved digestibility of crude protein and dry matter was also recorded in beluga, *H. huso* by feeding citric acid acidified diet (Khajepour & Hosseini 2012). Improved protein digestibility was also observed in rainbow trout fed formic acid containing diet (Luckstadt 2008).

The present study also showed that dietary phytase inclusion increased \( (p < .05) \) the digestibility of dry matter, crude protein and ether extract. Phytase is highly specific to hydrolyse, the indigestible phytate that is present in plant protein sources. It ultimately reduces the chelation power of phytate for various nutrients, leading to increased digestibility of these nutritional attributes. Baruah et al. (2007b) reported significant \( (p < .05) \) increase in apparent digestibility coefficient of dry matter by feeding 500 U/kg phytase-supplemented diet to *L. rohita*. Phytase addition at 700 FTU/kg level also increased the digestibility of crude protein in Nile tilapia (*Oreochromis niloticus*) (Furuya et al. 2001). Portz and Liebert (2004) while working with Nile tilapia reported improvement in crude fat digestibility for diet supplemented with phytase at 1000 and 2000 FTU/kg levels. However, combined supplementation of citric acid and phytase did not produce a significant \( (p > .05) \) interaction in improving digestibility of dry matter, crude protein and ether extract. Similar to our results, Baruah et al. (2007b) also reported a non-significant interaction between phytase and citric acid in improving dry matter digestibility in *L. rohita* fingerlings.

In the present study, dietary inclusion of citric acid (2%) significantly enhanced the digestibility of minerals. Citric acid has been reported to increase the phosphorus bioavailability by dephosphorylation of phytate in vitro (Zyla et al. 1995; Baruah et al. 2004). Similar to our findings, increased phosphorus absorption in red sea bream was also observed by supplementing 1% (Hossain et al. 2007) and 3% (Sarker et al. 2005) citric acid. Increased P digestibility was also recorded by Baruah et al. (2007b) in *L. rohita* juveniles by feeding citric acid acidified diet.

Dietary supplementation of phytase (1000 FTU/kg), in the present study, had also resulted in improved absorption of minerals. Phytase supplementation probably broke down the phytate, liberating the chelated minerals and resulting in increased absorption. Improved minerals’ absorption was also recorded in Japanese flounder (Masumoto et al. 2001), rainbow trout (Sugiura et al. 2001), Nile tilapia (Liebert & Portz 2005) and rohu (Baruah et al. 2007a) in response to phytase supplementation.

In the current study, combined supplementation of citric acid and microbial phytase had not shown any significant interaction in improving the digestibility of minerals, including P, Na, K, Ca, Mg, Cu, Zn, Mn and Fe. In contrast to our results, Phromkunthong et al. (2010) observed synergism of both supplements to improve P digestibility and bone mineralization in *C. carpio*. In another study by Baruah et al. (2005), it was found that citric acid and phytase significantly interacted to increase minerals’ utilization in *L. rohita* juveniles fed soybean meal-based diets. Sugiura et al. (2001) also found a significant increase in the apparent absorption of magnesium and phosphorus by the simultaneous addition of citric acid and phytase in the diet of rainbow trout, *O. mykiss*. Discrepancies in the results may be due to the differences in the phytase treatment methods, diet composition and species.

Disclosure statement

No potential conflict of interest was reported by the authors.

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