Cholecalciferol and Formic Acid Synergistically Enhance Digestive Enzymes Activity and Bone Mineralization in *Ctenopharyngodon idella*

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**ABSTRACT**

This study was planned to determine intestinal digestive enzymes activity and bone mineralization of *Ctenopharyngodon idella*. Four type food were prepared by using formic acid (%) and vitamin D₃ (IU/Kg) in experiment viz, FD1, FD2, FD3 and FD4, respectively. Fish fed with formic acid showed considerable increase in calcium, magnesium potassium, however vitamin D₃ considerably increase iron and manganese contents (µg/g) in the bones of *C. idella*. Amylase and protease activity was maximum in fish intestine fed with formic acid followed by formic acid × vitamin D₃, vitamin D₃ = control diet. Activity of lipase enzyme was reduced by formic acid supplementation as well as vitamin D₃. The observed formic acid and vitamin D₃ (interactions) were synergistically bone mineralization of phosphorus, sodium, copper and also showed positive result for the activity of amylase, protease and lipase. Finally, formic acid and vitamin D₃ improves bone mineralization and digestive enzymes activity in the *C. idella*.

Fish meat is highly palatable, delicious and providing high quality proteins as well as source of vitamins and minerals (Shaikh *et al*., 2011). Many prebiotics and supplements are used in aquaculture farming which progresses the growth and fish survival rate (Shaikh *et al*., 2011). Prebiotics which are used in fish diet has been banned throughout the world for the well-being of humans. Many investigators are paying attention towards the use of supplements which increases the survival and growth rate in aquaculture industry (Luckstadt, 2006). Fish supplements contain amino acids, fatty acids and various minerals which are used in fish diet (Zhou *et al*., 2004). But due to its high demand and cost, it is compulsory to find alternate sources of protein (Toko *et al*., 2008; Correll, 1999). Instead of it, plant based proteins such as cereals are recommended as fish meal which are cost effective as well as low phosphorus content (Dalsgaard *et al*., 2009). The presence of phytate in fish meal reduces the digestibility as it contain indigestible carbohydrates and various anti-nutritional factors (Laining *et al*., 2010). However, fish feed supplemented with organic acid hydrolyzes phytate and enhances its solubility and absorption of minerals (Hossain *et al*., 2007; Zyla *et al*., 1995; Wood and Serfaty-Lacrosinere, 1992).

Fish meal supplemented with organic acid has following advantages: it reduces the microbial activity in the gut of fish and also inhibited the toxicity; it increases the absorption of nutrients by the proliferation of mucosal epithelium of intestine; it reduces the release of phosphorus ultimately decreases the water pollution, and increases the mineral (phosphorous, magnesium and calcium) absorption in fish as investigated by Baruah *et al.* (2008). Formic acid increases the absorption of minerals in fish (Vielma and Lall, 1997). Organic compounds including vitamins, minerals play important part in fish growth and fitness.
As these are essential compounds so must be consumed by fish meal. Vitamin D is a fat-soluble vitamin having steroid-based chemistry. Naturally, there are two main sources of vitamin D, ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3) (Anthony, 1979). The primary role of cholecalciferol is the uptake and utilization of calcium and phosphorus as well as it maintains the normal concentration of calcium and phosphorus in blood to enhance the bone development as well as calcification (Li, 1994).

The aim of present research work was to find out the effect of dietary formic acid and vitamin D3 supplementation on intestinal digestive enzyme activities and bone mineralization of *Ctenopharyngodon idella*.

**Materials and methods**

The present research work was conducted in Fisheries research Farmstead, University of Agriculture, Faisalabad. Four earthen ponds (19m×6m×1.5 m) were covered well with mesh net to avoid numerous undesired animals to enter in the specific ponds. Ponds were filled by tube well water. *C. idella* (grass carp) (n=25) was added to each pond after measuring the weight and length. In each earthen pond, inorganic compost containing urea and superphosphate was added on weekly basis for one month. During this one month duration, no fertilizer was added to ponds. Fish were then nourished with about 2 % body weight of fishes twice a day. In each pond, water level was kept constant throughout the study period. After duration of fourteen days, sampling was done and feed ratio was set accordingly. Finally, after feeding duration of 90 days, the fishes were harvested.

Four different experimental diets (FD1, FD2, FD3 and FD4) comprising formic acid (%) and vitamin D3 (IU/Kg) by following the levels 0, 0; 2, 0; 0, 500 and 500, 2, respectively. The time period for the whole experiment was about three months. For the formulation of experimental diets, instruments like, cereal grinding machine (FFC-45, JIMO, China) was used for formulation of pellets for pellet construction, and also the experimental extruder (Model SYSLG30-IV) was used for formulation of pellets (3.5 mm). Pellets were dried with about 10% of moisture content and stored at 20°C during the whole nourishing trial and then packed in plastic bags.

For bone mineralization, bone samples were digested in boiling nitric acid and perchloric acid mixture (3:1) for the estimation of minerals. Calcium, magnesium, zinc, iron, copper and manganese were measured by atomic absorption spectrophotometer (Hitachi Polarized Zeeman AAS, Z-8200, Japan) after appropriate dilution. Phosphorus content was analyzed at 720 nm wavelength using UV/VIS spectrophotometer (U-2001, Hitachi). Flame-photometer (Jenway PEP-7, UK) (AOAC, 1995) was used for sodium and potassium estimation.

### Table I. Diet formulation n for experimental treatments.

| Ingredient         | %       |
|--------------------|---------|
| Fish meal          | 20      |
| Soybean meal       | 25      |
| Sunflower meal     | 20      |
| Rice polish        | 15      |
| Wheat flour        | 10      |
| Fish oil           | 7       |
| Minerals mixture * | 2       |
| Vitamin mixture ** | 1       |

*Each kg of mineral mixture contains; Ca (Calcium) 155 gm; P (Phosphorus) 135gm; Mg (Magnesium) 55mg; 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; K (Potassium) 2000 mg. **Each g of vitamin mixture contains; Vitamin A (Retinoic acid) 5.0mg; Vitamin B1 (Thiamine) 0.5mg; Vitamin B2 (Riboflavin) 3.0mg; Vitamin B3 (Niacin) 5.0mg; Vitamin B6 (Pyridoxine) 1.0mg; Vitamin B7 (Biotin) 0.05mg; Vitamin B9 (Folic acid) 0.18mg; Vitamin B12 (Cobalamine) 0.002mg; Vitamin C (Ascorbic acid) 5.0mg; Vitamin E (Vitamin E) 6.0mg; Cellulose 874.26mg; Choline 100mg.*

For digestive enzymes assays, eight fishes were randomly collected, anaesthetized and dissected from each replicate for the collection of intestines. The tissue homogenizer (Wisd HG-15D, DAIHAN Scientific) was used for homogenization of sample in cold sucrose (0.25 M) solution. The homogenate were centrifuged at 5,000 × g for 15 min in a cooling centrifuge (5°C), supernatant was collected, frozen in sample vials, and stored at -20°C until use. The dinitrosalicylic acid (DNS) methods were used for the estimation of amylase activity (Rick and Stegbauer, 1974). In reaction mixture starch (0.5Ml) and enzyme extract (0.5ml) were incubated at 37°C for half an hour. For stopping reaction dinitrosalicylic acid was added and placed in boiling water for five minutes. After cooling, distilled water was added for dilution and absorbance were taken at 540nm. Activity were observed by malsee standard curve. Kunitz (1947) describes the casein digestion method for analyzing the protease activity. In reaction mixture casein as substrate (1%), phosphate buffer (pH 7.5) and tissue homogenate were added and kept at 37°C for fifteen minutes. Reaction was stopped after addition of trichloroacetic acid (5%). The reaction mixture was filtered and absorbance taken at 280nm.
The activity of lipase was analysed by using p-nitrophenylpalmitate as the substrate (Mahadik et al., 2002). In reaction mixture p-nitrophenylpalmitate solution (0.9 mL), phosphate buffer (0.5 M) and enzyme extract were incubated at 37°C for 30 min. p-nitrophenol extract values were calculated at 410 nm expressed as μmol/min (one unit of activity).

Two-way analysis of variance under RCBD was applied for data analysis (Steel et al., 1996). For comparison of means Student-Newman-Keuls test was used for determining significant differences (Snedecor and Cochrane, 1991). For analyzing statistical data CoStat computer package (Version 6.303, PMB320 Monterey, CA, 93940 USA) was used.

Results

The fish group fed with formic acid significant showed increase in calcium, magnesium and potassium, however vitamin D3 significantly increased the iron and manganese contents (μg/g) in the bones of C. idella fingerlings (Table II). Table III shows the intestinal digestive enzyme activities of C. idella fingerlings. The amylase and protease activities were maximum in intestine of fish fed with formic acid followed by formic acid: vitamin D3 (500:2), vitamin D3 (control diet). The activity of lipase enzyme was reduced followed by fornic acid: vitamin D3 (500:2), vitamin D3 were maximum in intestine of fish fed with formic acid of C. idella. The observed formic acid and vitamin D3 (interactions) significantly increased the iron and manganese absorption during the development of bones of C. idella. The phytate hydrolysis alters the physiological condition which enhances the utilization of minerals (Sugiura et al., 2006). Similar results were reported by Khajepour and Hosseini (2010)

who described that phosphorous and calcium content were increased in muscle tissue by the supplementation of 2% and 3% citric acid in the diet of beluga. In our research work the vitamin D3 supplementation significantly affects the iron and manganese in bones of C. idella. Likewise, Zhu et al. (2015) stated that addition of vitamin D3 in fish diet considerably enhanced the retention of sodium, potassium, phosphorous, calcium, magnesium and zinc in whole body of yellow catfish. Connell and Gatlin (1994) described that blue tilapia fed with dietary vitamin D3 considerably condensed the mineralization in hard tissues as compared to soft tissue, because dietary vitamin D had partial participation in calcium metabolism and mineralization of the hard tissues of tilapia.

Discussion

In the present research work, the supplementation of formic acid exhibited noteworthy effect on calcium, potassium and manganese absorption during the development of bones of C. idella. The phytate hydrolysis alters the physiological condition which enhances the utilization of minerals (Sugiura et al., 2006). Similar results were reported by Khajepour and Hosseini (2010) who described that phosphorous and calcium content were increased in muscle tissue by the supplementation of 2% and 3% citric acid in the diet of beluga. In our research work the vitamin D3 supplementation significantly affected the iron and manganese in bones of C. idella. Likewise, Zhu et al. (2015) stated that addition of vitamin D3 in fish diet considerably enhanced the retention of sodium, potassium, phosphorous, calcium, magnesium and zinc in whole body of yellow catfish. Connell and Gatlin (1994) described that blue tilapia fed with dietary vitamin D3 considerably condensed the mineralization in hard tissues as compared to soft tissue, because dietary vitamin D had partial participation in calcium metabolism and mineralization of the hard tissues of tilapia.

Table II. Minerals content (μg/g) in the bones of C. idella.

| Diet | FA (%) | VD3 (IU/kg) | Phosphorus | Calcium | Magnesium | Sodium | Potassium | Zinc | Copper | Iron | Manganese |
|------|--------|-------------|------------|----------|-----------|--------|-----------|------|--------|------|-----------|
| FD1  | 0      | 0           | 12.51±0.03d| 15.29±0.01d| 0.34±0.02d| 1.76±0.02d| 0.19±0.02d| 113.90±0.07d| 13.76±0.02d| 113.90±0.07d| 13.76±0.02d|
| FD2  | 2      | 0           | 12.77±0.03c| 15.35±0.03c| 0.39±0.02c| 1.86±0.04c| 0.28±0.01c| 114.08±0.04a| 14.14±0.04a| 114.08±0.04a| 14.14±0.04a|
| FD3  | 0      | 500         | 13.09±0.04b| 15.73±0.03b| 0.44±0.02b| 2.07±0.02b| 0.36±0.01b| 113.91±0.04c| 14.2±0.04c| 113.91±0.04c| 14.2±0.04c|
| FD4  | 500    | 2           | 13.13±0.04a| 15.76±0.03a| 0.49±0.03a| 2.11±0.03a| 0.47±0.02a| 113.97±0.03b| 14.3±0.04b| 113.97±0.03b| 14.3±0.04b|

Values sharing similar letters in a column are non-significant at P<0.05. FA, Formic acid; VD3, Vitamin D3.

Table III. Digestive enzymes activities (units/mg protein) in the intestine of C. idella.

| Diet | FA (%) | VD3 (IU/kg) | Amylase | Protease | Lipase |
|------|--------|-------------|---------|----------|--------|
| FD1  | 0      | 0           | 2.65±0.01c| 0.92±0.01c| 0.34±0.01a|
| FD2  | 2      | 0           | 2.84±0.01a| 1.01±0.01a| 0.32±0.01b|
| FD3  | 0      | 500         | 2.65±0.01c| 0.95±0.01b| 0.30±0.01c|
| FD4  | 500    | 2           | 2.75±0.00b| 0.95±0.01b| 0.30±0.01c|

Values sharing similar letters in a column are non-significant at P<0.05. FA, Formic acid; VD3, Vitamin D3.

In the current research work, it was concluded that the activity of amylase, lipase and protease considerably improved in the intestine of C. idella fed with formic acid supplemented diets. The activity of enzymes was perhaps due to release of trace element by supplementation of organic acid in fish diet (Shah et al., 2015). The results are in line with those of Shah et al. (2015) who stated that supplementation of 3% citric acid in the diet of Labeo rohita fingerlings improved the activities of protease, lipase and amylase. Likewise, the tilapia fed with 1% citric acid supplemented diet improved the activity of amylase. When citric acid was added in diet, metal ion present in diet increases the activity of amylase in intestine of tilapia (Li et al., 2009). Furthermore, according to Agouz et al.
supplementation of organic salts (malic+oxalic acids blend and Na-acetate+Ca-lactate blend) in the diet of Nile tilapia considerably increased the activity of protease enzyme.

The conclusion is dietary formic acid and vitamin D₃ improves the bone mineralization and digestive enzymes activity in the *C. idella* juveniles.

Statement of conflict of interest

The authors have declared no conflict of interest.

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