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Short Communication

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

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Authors' Contribution
MA designed the experiment. LS executed the research. SZHS did statistical analysis. MF helped in compiling the data. HN, SUR and QL helped in writing the article.

Key words
***C. idella*, Formic acid, Vitamin D3, Bone mineralization, Digestive enzymes**

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 D_3considerably 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 fornic acid × vitamin D3, vitamin D3 = control diet. Activity of lipase enzyme was reduced by formic acid supplementation as well as vitamin D_3. The observed formic acid and vitamin D_3 (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 D_3improves 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

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mineral (phosphorous, megnisum 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 D_3 (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 the purpose of screening (0.05 mm) as well as grinding the diet ingredients. After electrically grinding the ingredients, mixture of mineral samples of fish oil and vitamins were added. For better texturing of dough, good quantity of watter was used 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 contents 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. Calicum, 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 (5ºC), supernatant was collected, frozen in sample vials, and stored at -20ºC 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 37ºC 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 maltose standard curve. Kunitz (1947) describes the casein digestion method for analyzing the protease activity.

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

Diet	FA (%)	VD_3 (IU/kg)	Phosphorus	Calcium	Magnesium	Sodium	Potassium	Zinc	Copper	Iron	Menganese
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	36.83±0.02d	30.59±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	37.04±0.02c	30.74±0.02c
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	37.2±0.02b	30.98±0.02b
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	37.3±0.03a	31.14±0.02a

Values sharing similar letters in a column are non-significant at P<0.05. FA, Formic acid; VD_3,Vitamin D_3

In reaction mixture casein as substrate (1%), phosphate buffer (pH 7.5) and tissue homogenate were added and kept at 37ºC for fifteen minutes. Reaction was stopped after addition of trichloroacetic acid (5%). The reaction mixture was filtered and obsorbance 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 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 Cochran, 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 D_3 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 fornic acid: vitamin D3 (500:2), vitamin D3 (control diet). The activity of lipase enzyme was reduced by supplementation of formic acid as well as vitamin D_3. The observed formic acid and vitamin D_3 (interactions) were synergistically enhanced bone mineralization of phosphorous (13.13±0.04a), sodium (2.11±0.03a), copper (14.3±0.04) and also showed enhanced results for the activity of amylase (2.75±0.00), protease(0.95±0.01) and lipase (0.30±0.01).

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

Diet	FA (%)	VD_3 (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; VD_3,Vitamin D_3.

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*. (2015) 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 D_3 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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