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Abstract

Inclusion of high levels of plant protein ingredients in fish feeds induces the presence of undesirable compounds such as Anti-Nutritional Factors, including non-starch polysaccharides and phytates. The present study evaluated the effect of partial replacement of dietary soybean meal by a high protein distillers dried grains (HP-DDG) a co-product of corn based ethanol production. We evaluated HP-DDG in experimental diets with a supplemented commercial phytase on growth performance, physiological parameters and histological changes of the intestine and liver of European sea bass, *Dicentrarchus labrax* fingerlings. The experiment was conducted for six weeks. A total of 240 *D. labrax* fingerlings was randomly divided to four experimental treatments (each in triplicates groups) and fed to apparent the satiation six days a week for a six weeks' period. Four dietary treatments: containing 0, 30, 40 and 50% HP-DDG supplemented with enzyme phytase 0.5g kg^{-1} diet respectively, were tested. The results showed that growth performance and feed utilization efficiency of sea bass was significantly higher ($P \leq 0.05$) with increasing levels of HP-DDG-and phytase supplementation. Superior phosphorous utilization was also observed with respect to whole body retention for each incremental level of HP-DDG inclusion. Hematology and serum biochemistry (hemoglobin (Hb), red blood cells (RBCs), white blood cells (WBCs) and humoral immune parameters including total protein, globulin, cholesterol, lysozyme activity and total antioxidant capacity (TAC) were improved ($P \leq 0.05$) in fish fed diet with increased levels of HP-DDG and supplemented phytase. The findings suggest that the use of HP-DDG supplemented with phytase in sea bass diets enhanced growth, physiological and immunological responses, and evidenced a cost benefit advantage for European sea bass production compared to the use of a diet without either HP-DDG or phytase incorporation.

Keywords: HP-DDG, phytase, growth, P utilization, liver & intestine histology, hematology

Introduction

Sea bass, Dicentrarchus labrax is the most economically relevant marine fish species produced in Egypt, representing 2% of the total marine production in the country (GAFRD, 2018).Unlike, European seabass production, where fries are obtained from large scale commercial hatcheries, Egyptian production still rely on wild collected fries. Thus, intensive aquaculture is of great importance, as their production represents about 60% of animal protein used for human consumption, but as rapidly expanding sector, it places enormous pressure on the aquaculture industry to find sustainable and cost-effective ingredients in fish diets (Tacon & Metian, 2015; Goda et al., 2020) Plant ingredients are now mainly used to reduce costs and improve sustainability of ingredients used in feeds for high valuable carnivorous species like sea bass and sea bream (Sparus aurata).However, plant ingredients have some restrictions, including competitive demand with human consumption and international market availability with escalating prices and questionable sustainability with respect to environmental stewardship (Matos et al., 2017). Thus, not surprisingly, the manufacturing process wastes have gained considerable interest as direct protein sources in fish feeds. High Protein Distillers Dried Grains (HP-DDG) is a co-product from the fermentation of milled corn and distillation of bioethanol in the increasing bio-refinery markets. HP-DDG is relatively low in lysine but nonetheless contains high crude protein content (43 to 49%). This makes HP-DDG a good candidate for aquafeeds when formulated with complementary protein concentrates. However, Anti-Nutritional Factors (ANFs) as NSP's (Non-Starch Polysaccharides) in HP-DDG may diminish or even inhibit the digestibility of organic matter, energy and protein due to their higher viscosity in the intestinal tract. The addition of exogenous enzymes could mitigate the negative impacts of ANFs and represent an innovative strategy to improve nutrient availability of plant-based diets (Castillo & Gatlin, 2015; Dalsgaard et al., 2016; Hassaan et al 2020). Previous research succeeded to prove the usefulness of using phytase to enhance phosphorus availability (Kumar et al., 2012). The use of exogenous enzymes is a common procedure to improve ANFs effectively and enhance nutrient digestibility in swine and poultry (Bedford & Cowieson, 2012), and its use has also been reported to increase the bioavailability of amino acids and nutrient utilization in aquaculture diets (Castillo & Gatlin, 2015). The anti-nutritive effects of phytate are highly influential on dietary amino

acid and energy digestibility, raising the value of phytase to the end user beyond being just a contributor to phosphorus (and calcium) nutrition. The information on HP-DDG efficiency in combination with exogenous enzymes is still scarce since many potential enzymes are available including those based on SSF (solid state fermentation products) as mentioned by Bowyer et al. (2020). An identical HP-DDG product was significantly improved in diets for sea bass as reported recently by Goda et al. (2020) augmented with a commercial protease. This success required validation of phytase under similar conditions for sea bass. Therefore, the present study aimed to evaluate the effects of including a commercial phytase supplemented HP-DDG as a relatively new ingredient source on the growth performance, feed utilization efficiency, humoral immune parameters, liver and intestinal morphology in European sea bass, *Dicentrarchus labrax* and to assess its economic relevance.

Materials and methods

Feed preparation

Four isonitrogenous and isocaloric experimental diets were formulated (Table 1). The control diet ($C_{0\%}$) had no high protein distillers dried grains **(**HP-DDG). Tested diets were formulated to contain 30%, 40%, and 50% of HP-DDG added at the expense of the soybean content. All the diets were equally supplemented supplemented with 0.5 g/kg of Phytase (Axtra PHY$^{@}$). The patented phytase, isolated from *Buttiauxella*, Danisco Animal Nutrition, Dupont Industrial Biosciences, Marlborough, UK. HP-DDG and phytase in the present study are considered as a single ingredient complex. HP-DDG is one of the most competitive sources of protein due to its lower moisture content, higher levels of lysine (3%) and longer shelf life. HP-DDG (a co-product of bioethanol distillation from corn fermentation with high levels of residual gluten protein after yeast separation and recovery) was supplied from POET Nutrition Dakota Gold; POET Nutrition, LLC 4506 N. Lewis Ave, Sioux Falls, SD 57104 (USA).

The chemical composition of HP-DDG used in the feed formulation was crude protein 47%, crude fat 4%, crude fiber 4%, Ash 7% and moisture 7%.

Feeding protocol

During the 42 -day experimental period, all fish were fed with their respective diets at 5% of body weight d^{-1} for 6 days $week^{-1}$. Every 14 days, fish were weighed and the daily ration was adjusted accordingly. The daily ration was divided into three equal amounts and offered three times a day (09:00, 12:00 and 15:00 h). Experimental diets were individually prepared by mixing the dry ingredients with 200 ml of water per kg diet. Two grams of commercial phytase enzyme contain the enzymatic activity of 2000,000 Units was dissolved into the 200 mL water at 37 °C (Yoo et al., 2005). Commercial phytase enzyme product (Axtra® PHY) was purchased from Gloray Vet COMPANY, USA.

The solution was incubated for 24 hours at room temperature according to the method of von Danwitz et al. (2016) prior to its addition to the experimental diets. The mixture was blended, turned into a paste and pelleted by passing the blended mixture through a laboratory pellet machine with a 1mm diameter matrix. The resulting wet pellets were dried at room temperature for two days and then stored in plastic bags and kept refrigerated (-2°C) until use**.**

Fish and experimental facilities

European sea bass, *D.labrax* fingerlings with an average initial body weight of 7.5 ± 0.5 g $fish^{-1}$ were obtained from a commercial fish farm “El-Shref farm", Wady Marriott, Alexandria” Egypt and acclimated to the experimental conditions for 15 days. During this period, fish were fed a standard commercial diet (Biomar) (45% protein). Then, fish were randomly distributed into twelve glass aquaria measuring (70 × 40 × 30 cm each) representing four treatments (each in triplicate) at a stocking density of 20 fish per aquaria.

On a daily basis, 50% of the water volume of each tank was exchanged to maintain adequate water quality. Environmental parameters throughout the experiment were; salinity (37 ppt), temperature (18 ± 1 °C), and pH (7.0 ± 0.50) under a photoperiod regime of 12:12 hr (light: dark. The experimental protocols were all approved by the local Institutional Animal Care Committee (IACC) meeting ethical standards and legislation and statutes for animal studies.

Growth Indices

The mean final body weight (FBW) in experimental treatment was determined by dividing the total fish weight in each aquarium by the number of fish. Weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), protein productive value (PPV), fat retention (FR), energy retention (ER), Phosphorus retention, economical conversion rate (ECR) and survival (%) were calculated using the following equations:

WG = final body weight (g) - initial body weight (g).

SGR = 100× [(ln final body weight (g) - ln initial body weight (g))/ duration of feeding (day)]

FCR = feed intake (g)/weight gain (g).

PER = weight gain (g)/protein intake (g).

PPV = (protein gain (g)/protein intake (g)) × 100.

FR = (fat gain (g)/fat intake (g)) ×100.

ER = (energy gain (kJ)/energy intake (kJ)) ×100.

ECR = cost of diet ($ kg^{-1}) x Feed Conversion Ratio (FCR)

Survival (%) = 100 × (initial number of the fish/final number of fish).

Phosphorous Retention (PR %) was calculated according to Morales et al. (2018):

$PR=100 \times (BW_{final} \times P_{final} - BW_{initial} \times P_{initial})/(\text{Feed intake} \times P\%_{diet})$

Where: $P\%_{diet}$ is the content of phosphorous in the diet; $P_{initial}$ and P_{final} represent the initial and final concentration of phosphorous in fish.

Diet and feed analysis

Diets were analysed as described by Davies et al., (2019) in accordance with standard proximate composition AOAC (2000) methods for crude protein N*6.25, crude lipid, ash, crude fibre and moisture/Dry Matter DM.

Phosphorous analysis

250 mg of sample were accurately weighed into glass tubes. After that, 5 mL of the digestion solution (a

mixture of nitric and perchloric acid at the ratio of 2:1, 3:1, or 4:1 v/v) were added. The tubes were then heated at 200°C until the solution became translucent and a brownish smoke stopped being released, which indicated the complete digestion of the organic matter. Digested samples were quantitatively transferred to 50 mL volumetric flasks using ash-free quantitative filter paper (Whatman No. 41, Whatman International Ltd, Springfield, Kent, England, UK). The volume of the solutions was made up to 50 mL using deionized water. Aliquots of the solutions were transferred to polyethylene flasks and kept cool (4°C).

Total phosphorous (P) in feeds and fish was determined according to the following principle. When ammonium molybdate solution is added to a solution of phosphate containing concentrated H_2SO_4 it produces a yellow crystalline precipitation of ammonium phospho-molybdate. Phospho-molybdate reacts with amino-naphthol-sulphonic acid and produces a molybdenum complex which forms an intense blue- coloured solution. A standard curve was produced from KH_2PO_4 solution and the absorbance intensity of the colour of the reaction mixture is measured by colorimeter at 570nm. The colour generated from suitably diluted extracts from individual samples of digested fish diets and fish was measured against the standard curve according to the method of Palma et al., (2015)

Blood Samples and Haematological Analysis

Blood samples were collected at the end of the experiment. From each of the dietary treatments, five fish were used for hematological indices analysis and five for plasma content analysis. The fish were anesthetized with Tricaine Methanesulfonate (MS-222) and the blood samples were taken by puncturing the caudal vessels. Blood samples were collected into two tubes, one containing heparin as anticoagulant agent for haematological assessment and the other was anticoagulant free for biochemical estimation. The haematological parameters are expressed in international units (SI). The total red and white blood cell counts (RBC; 10^6 mm^{-3} and WBC; 10^3 mm^{-3}, respectively) were obtained by using a standard Neubauer-hemocytometer chamber using Shaw's solution as diluting fluid (Stoskopf, 1993). Hemoglobin

(Hb; g dL^{-1}) was determined colorimetrically using commercial kits (Diamond, Egypt) according to the cyan- methemoglobin procedure (Drabkin, 1945). Hematocrit (Hct) were determined by using microhematocrit-heparinized capillary tubes and a microhematocrite centrifuge (10000 g for 5 min) Levels of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and *alkaline phosphatase* (ALP) were estimated according to the method described by Reitman & Frankel (1957).

Biochemical and immune parameters

The total protein (g dL^{-1}) was determined in plasma samples of fish from the different experimental groups by the Biuret method according to Doumas et al. (1981). Albumin (g dL^{-1}) was determined by the bromocresol green method (Reinhold, 1953) and globulin (g dL^{-1}) was calculated as the difference between total protein and albumin, and cholesterol was measured by a commercial kit (Pasteur, Lab, France, Egypt) (Yousefi et al. 2011). Triglycerides were determined according to MGowan et al., (1983). Lysozyme activity (U mg^{-1} protein) in serum was determined according to the method of Ellis (1990) based on the lysis of the lysozyme sensitive gram-positive bacterium *Micrococcus lysodeikticus* (Sigma, St. Louis, MO). Lysozyme acts upon susceptible bacteria by combining with and breaking down a mucopolysaccharide. This mucopolysaccharide, which is the lysozyme substrate, has been shown to be situated in the bacterial cell wall (Salton, 1952) and can be characterised chemically.

Total antioxidant capacity (TAC) level was estimated spectrophotometrically at 552 nm following the method with Tween 80 oxidation (Galaktionova et al. 1998). Briefly, 0.2 ml of tissue homogenate was added to 2 ml of 1% Tween 80. Instead of the sample, the blank assay included 0.2 ml of distilled water. The mixture was incubated for 48 hours at 37 ºC. After cooling, 1 ml of 40 % TCA was added. The mixture was centrifuged at 3,000 g for 10 min. After centrifugation, 2 ml of supernatant and 2 ml of 0.25% TBA reagent were mixed in. The mixture was heated in a boiling water bath at 100 ºC for 15 minutes. The TAC level was expressed in (%).

Histological examination

196 Four fish from each replicate of D. labrax were randomly collected and dissected for tissue removal.
197 The distal section of the intestine were removed, thoroughly washed with a physiological saline (0.9%
198 Nacl) solution and fixed in Bouin's fluid. The material was dehydrated, cleared and finally embedded in
199 paraffin wax. Serial sections were cut to the thickness of 5-6µm. The sections were stained with
200 haematoxylin counterstained with eosin and mounted in DPX (Yano, 1988). The sections were examined
201 with an Olympus light microscope and photographed with a digital camera as required. The histological
202 examination was carried out according to Culling (1983).

203 **Economic evaluation**

204 The economic evaluation was calculated according to the following equations (Salama et al., 2010).
205 Feed cost per kg fresh fish (LE) = Cost / kg diet (LE) * consumed feed to produce 1 kg fish .
206 Relative feed cost/kg fresh fish = Values of feed cost/kg fresh fish / the minimum value of the same
207 parameter
208 Feed cost / 1 kg gain (LE) = Feed intake per kg gain (FCR) * cost/kg diet (LE).
209 ECR = cost of diet ($ kg^{-1}) x Feed conversion ratio (FCR)

210 **Statistical analysis**

211 One-way ANOVA and Duncan's multiple rank test (Duncan, 1955) were used to test were
212 calculated to test effects with a probability of $P \leq 0.05$ that were considered significant. The data from
213 the experiments were statistically analyzed using GLM (general linear model) procedure according to
214 Statistical Analysis System (SAS, Institute 2003, SAS User's Guide: Statistics. SA Institute, Cary, NC.
215). However, data is presented untransformed to facilitate comparisons.

216 **Results**

217 **Growth performance and feed utilization**

218 Growth parameters are presented in (Table 2). At the end of the experiment, fish fed diets with either 40%
219 HP-DDG or 50 % HP-DDG supplemented with phytase, grew significantly more ($P<0.05$) with improved growth
220 parameters (higher final body weight, weight gain, specific growth rate and better feed conversion ratio) than those

fed either the control or 30% HP-DDG supplemented with phytase diets. As for the phosphorus retention, fish fed 50% HP-DDG supplemented with phytase recorded the superior values (P≤0.05), while the control group recorded the lowest value (Table 3). Significantly higher whole body P level was noticed in sea bass with the incremental inclusion of HP-DDG with phytase and this reflected dietary P levels. The best relative feed cost/kg per fresh fish was recorded for fish fed diet containing 50% HP-DDG supplemented with phytase, in opposition to fish fed the control diet (Table 8).

Blood parameters

Fish fed 50% HP-DDG recorded significantly lower values (P≤0.05) of ALT, AST, and ALP, while fish fed the control diet recorded significantly higher values (P≤0.05) in all blood parameters (Table 3). Moreover, fish fed 50% HP-DDG recorded significantly higher (P≤0.05) triglyceride values than fish fed the remaining dietary treatments. Fish fed either 30% or 50% HP-DDG supplemented with phytase recorded the highest (P≤0.05) values of RBCs, WBCs and PCV, while fish fed the control diet recorded the lowest value (P≤0.05) of WBCs, RBCs, Hb and PCV (Table 4). In addition, compared to the control diet, fish fed diets 50% HP-DDG supplemented with phytase recorded significantly higher values (P ≤ 0.05) of total protein, albumin and globulin (Table 5). In terms of immune parameters, it was observed that fish fed diets with increasing inclusion rates of HP-DDG with supplemented phytase, evidenced a significant (P≤0.05) and correspondent increase of cholesterol, lysozyme and TAC levels compared to fish fed the control diet (Table 6).

Histological studies

The histological changes of intestinal and hepatic tissue were assessed by light microscopy. Observations revealed that intestine and liver of fish fed the control diet showed normal, intact intestinal wall, intestinal villi as well as goblet cells and distribution ($D_{0\%}$, Figure 1a). Conversely, fish fed 30% HP-DDG supplemented with phytase showed moderate improvement in length and width of intestinal villi as well as goblet cells (D30%, Figure 1b); fish fed 40 and 50% HP-DDG showed an improvement

in length and width of intestinal villi as well as an increase in the number of goblet cells (D40%, Figure 1c and D50%, Figure 1d). The histopathological analysis also revealed a significant increase in villus length, villus width and area of absorption in fish fed diets containing HP-DDG with phytase (Figure 2). Sea bass fed the control diet showed normal organization of the hepatic cell and blood capillaries (Figure 3a). Hepatopancreas of European sea bass, *D. labrax*, fingerlings fed (30% HP-DDG with phytase) showed activation of melano-macrophage centers and normal pancreatic acini and normal hepatocytes (Figure 3b), in addition with increasing dietary HP-DDG supplemented with phytase up to 50% necrosis in pancreatic tissue (blue arrows) with activation of melano-macrophage centers (black arrows) (Figure 3c & d), was also observed.

Discussion

The addition of HP-DDG with a supplementation of phytase resulted in an improved growth and feed efficiency sea bass under experimental conditions. The current results are in accordance with previous studies on different species including *L. rohita* (Bano and Afzal, 2017), hybrid grouper (Anthonius et al., 2018), and Nile tilapia (Abo Norag et al., 2018). Furthermore, Goda et al. (2019) reported that fed HP-DDG enriched with phytase resulted in higher growth rate of *D. labrax*. Ranjan, et al. (2017) found that 0.01 % phytase supplementation in basal diet significantly improved ($P \leq 0.05$) the weight gain, SGR and FCR of *L. rohita*. However, the current results are inconsistent with the findings of Hu et al. (2016) and Yigit et al. (2018) who reported no differences ($P \geq 0.05$) in WG, FCR and gut health respectively, Nile tilapia and rainbow trout fed diets supplemented with dietary microbial phytase. The discrepancies between these studies may be due to several factors such as the dosage and phytate sources, types of feed ingredients, fish species and the pH of the stomach (Dersjant-Li et al., 2015; Yigit et al., 2018). It should be noted that in the current investigation our source of phytase was a novel product, Axtra® PHY that offers unprecedented phytate degradation and phosphorus digestibility when compared with E. coliphytases that are more commonly employed in the industry. It has exceptionally

rapid activity in the stomach. The activity of Axtra® PHY, a *Buttiauxella* phytase, at pH 4.0 is almost double that at pH 5.5, the level at which all commercial phytases have their activity standardized, and much higher than other phytases. Axtra® PHY also improves sodium - and therefore also protein, glucose and nutrient - absorption from the gut, with positive effects on growth performance (Danisco Technical Report).

The present results could be attributed to several factors i) the inclusion of phytase eliminates the negative impact of phytate to reduce the availability of minerals particularly calcium, magnesium, iron, and zinc (Shah et al., 2016) and negatively affect the absorption of lipids and proteins (Jacob 2015) thus, the addition of the phytase in the diets enhances mineral availability and utilization of dietary energy and amino acids (Bowyer et al., 2020; Sharawy et al., 2020); ii) inclusion of phytase modulates the gut microbiota by hydrolysis of the phytate and may thus positively influence the intestinal health (Rachmawati et al., 2017); iii) the exogenous phytase improves nutrient digestibility and consequent availability by destruction of insoluble cell wall complexes and subsequent release of low-molecular-weight carbohydrates as sources of available energy for growth (Jacob, 2015); v) Phytase is also capable of converting the inactive form trypsinogen into the active form trypsin which degrades protein and oligopeptides into amino acids that consequently improves overall protein utilization (Haghbayan and Mehdi 2015).

The superior values of hematology and immune parameters where observed in fish fed the diet containing 50% HP-DDG, which is in agreement with the results obtained by Peatman and Beck (2016) who found that channel catfish fed with phytase supplemented diets significantly elevate RBC's, WBCs, PCV% and Hb levels. Also, the most elevated ALT, AST, ALP activities decrease with increasing HP-DDG dietary inclusion. The present results are also consistent with Shelby et al. (2007) who found that Nile tilapia fed DDGS diets showed enhanced immune system, liver function and disease resistance. Furthermore, Ghaly et al. (2017) revealed that for broiler chickens fed dietary DDGS supplemented with

different levels of Avizyme enzymes, there was a significant (P≤0.05) decrease in the values of ALT and AST activity in blood of birds. In the present study, enhancement of the immune response of sea bass fed diets contain HP-DDG supplemented with phytase could be due to i) the presence of significant amounts of biologically active compounds (mannans, β-glucans and nucleotides) derived from yeast, which comprises about 10 percent of total DDGS mass (Shurson, 2018; Kim et al., 2008).

In terms of Phosphorus (P) retention, the present study showed that increased phosphorus retention was detected in sea bass fed diet levels up to 50% HP-DDG with phytase compared to the control group (Table 7). This is in agreement with the findings of von Danwitz et al., (2016) who found the lowest phosphorus retention in fish fed a diet without phytase supplementation (46.5%) and increased by addition of 1000 FTU or 2000 FTU phytase to 52.2% and 67.2%. Furthermore, Totok Yudhiyanto et al. (2017) found that phosphorus retention increases with increasing phytase supplemtation when added to the diet of Asian Seabass, *Lates calcarifer*.

Light microscopy revealed a normal and healthy morphology of the intestinal tract of European sea bass fed HP-DDG enriched with phytase. The fish intestine enterocytes displayed healthy brush borders and no signs of damage. The present results are consistent with Adeoyea et al., 2016 who found that inclusion of exogenous enzymes in tilapia diets improved (P≤0.05) the intestinal morphology, goblet cells abundance and microvilli diameter surface than those fed the control diet. These results are also in line with those of Adeoyea et al. (2016). In terms of liver histology, sea bass fed diets supplemented with phytase showed a marked decrease in the hepatic and pancreatic lesions (Figure 3 a, b and c). The present findings are consistent with the studies of Abo Norag et al., 2018, who recorded a noticeable decrease in the hepatic and pancreatic lesions of fish fed diets supplemented with phytase in experiments with tilapia.

The current results show that the best relative feed cost per kg fish gain was observed in fish fed 50% HP-DDG supplemented with Phytase (Table 8). These are consistent with Nehad et al., (2019) who found that the feed cost to produce one kg fish gain was reduced by inclusion of phytase in the diet by

41.35%. In addition, Khan et al., (2006) stated that enzyme supplementation is a more realistic and cost-effective strategy to achieve maximum profitability.

In conclusion, using phytase in aquafeeds improves growth performance and minimizes the environmental impact of fish production when incorporating higher levels of vegetable protein sources in the diet for E. sea bass. The effective use of a phytase supplement in feed formulations can have a significant positive impact in terms of improving performance, reducing costs and boosting production efficiencies. In this example, our work demonstrates the potential of HP-DDG as a novel and sustainable ingredient with unique characteristics and versatility in compound feed formulations in a marine species such as sea bass. The combination of HP-DDG with phytase is synergistic and provides added value to the plant ingredient. More research is warranted to explore the optimum rate of enzyme feed application with considerations to thermal stability in extruded diets, post pelleting spraying technology and the different temperature and feeding strategies of various fish species. It will be imperative to examine the range of options best suited to the different production stages where diet formulations can be adjusted to incorporate novel feed ingredients like HP-DDGS especially in grower diets for larger fish approaching harvest weights where economic considerations prevail.

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Table (1): The composition (g/kg) and chemical analysis (% on dry matter basis) of the experimental diets (HP-DDG + phytase).

Ingredient	Control	30%+ Phytase	40%+ Phytase	50%+ Phytase
Fish meal 68 %	300	300	300	300
Soy bean meal 47%	375	262.5	225	187.5
Corn gluten 60%	90	89.5	89.5	89.5
Rice bran 12%	65	50	50	50
Wheat middlings13%	69.5	83.8	84.8	85.8
HP-DDG^{+}	0	112.5	150	187.5
Soy bean oil	41	41	40	40
Fish oil	48	49	49	48
Dicalcium phosphate	8	8	8	8
Vitamin/Mineral Premix	2	2	2	2
Vitamin C	0.2	0.2	0.2	0.2
Antytocsec	1	1	1	1
Phytase*	0.5	0.5	0.5	0.5
Total	1000	1000	1000	1000
Dry matter (DM)	93.75	93.77	93.8	93.6
Crude protein (CP)	44.88	44.4	44.97	45.2
Ether extract (EE)	13.1	15.2	14.6	15.0
Nitrogen free extract	25.7	25.2	26.5	27.6
Crude fiber (CF)	4.32	4.3	3.4	3.1
Ash	12	9.8	10	11
Gross energy (GE: MJ/Kg DM)	20.93	21.56	21.52	21.87

Gross energy (GE) = (CP×5.6) + (EE ×9.44) + (NFE× 4.1) Kcal/ 100g (NRC, 1993)

*Axtra® PHY (Danisco Animal Nutrition, Dupont Industrial Biosciences, Marlborough, UK + POET Nutrition Dakota Gold; POET Nutrition, LLC 4506 N. Lewis Ave, Sioux Falls, SD (USA)

Table (2): Growth performance and feed efficiency of European sea bass, *Dicentrarchus labrax* fed various levels of high protein distillers dried grains (HP-DDG) supplemented with phytase

Diets	IBW (g fish^{-1})	FBW (g fish^{-1})	WG (g fish^{-1})	FCR (Feed: gain)	Feed intake (g fish^{-1})	SGR (%/d)
Control	7.50±0.04	15.51±0.21^{b}	8.1±0.12^{c}	1.85±0.05^{a}	15.05±0.77^{a}	1.11±0.05^{b}
Diet (30%) + Phytase	7.50±0.06	17.60±0.21^{b}	9.80±0.15^{b}	1.40±0.05^{b}	13.95±0.40^{b}	1.37±0.01^{b}
Diet (40%) + Phytase	7.60±0.01	17.80±0.15^{ab}	9.95±0.15^{ab}	1.43±0.03^{b}	13.25±0.17^{b}	1.45±0.02^{ab}
Diet (50%) + Phytase	7.57±0.03	18.92±0.21^{a}	10.73±0.20^{a}	1.20±0.03^{b}	13.10±0.06^{b}	1.58±0.02^{a}

Values are mean ±SD of triplicate analyses. Means in the same column bearing different superscript differ significantly ($P \leq 0.05$). HP-DDG, high protein distillers dried grains; IW, initial weight; FBW, Final body weight; BWG, body weight gain ; FCR, feed conversion ratio and SGR specific growth rate.

Table (3) Effect of various dietary levels of high protein distillers dried grains (HP-DDG) supplemented with phytase on triglyceride levels, and AST, ALT and ALP activity of European sea bass, *Dicentrarchus labrax* fingerlings.

Diets	**Triglyceride** (mg dL^{-1})	**AST** (IU/L)	**ALT** (IU/L)	**ALP** (IU/L)
Control	95.88±1.22^{c}	39.77±0.36^{a}	36.87±0.60^{a}	18.48±0.05^{ab}
Diet (30%) + Phytase	140.35±2.20^{b}	36.64±1.50^{ab}	28.89±1.62^{b}	18.37±0.01^{ab}
Diet (40%) + phytase	171.70±2.23^{b}	31.49±1.26^{b}	23.73±0.40^{b}	18.39±0.12^{ab}
Diet (50%) + phytase	188.38±3.10^{a}	29.33±1.91^{c}	21.96±1.41^{c}	17.78±0.45^{b}

Values are mean ± SD of triplicate analyses. Means in the same column bearing different superscript differ significantly ($P \leq 0.05$). ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate transaminase.

Table (4) Effect of various dietary levels of high protein distillers dried grains (HP-DDG) supplemented with phytase on hematological parameters of European sea bass, *Dicentrarchus labrax* fingerlings.

Diets	WBCs ($10^3 mm^{-3}$)	RBCs ($10^6 mm^{-3}$)	Hb (g dL^{-1})	PCV (%)
Control	21.030 ± 0.140^{d}	1.360 ± 0.020^{d}	7.675 ± 0.055^{d}	18.590 ± 0.070^{c}
Diet (30%) + phytase	22.700 ± 0.520^{c}	1.500 ± 0.030^{c}	8.015 ± 0.105^{b}	$19.905 \pm 0.125b^{c}$
Diet (40%) + phytase	25.225 ± 0.055^{ab}	1.815 ± 0.035^{ab}	8.800 ± 0.340^{ab}	21.215 ± 1.055^{ab}
Diet (50%) + phytase	26.400 ± 0.040^{a}	1.915 ± 0.025^{a}	9.275 ± 0.015^{a}	22.400 ± 0.010^{a}

Values are mean ±SD of triplicate analyses. Means in the same column bearing different superscript differ significantly ($P \leq 0.05$). HP-DDG, high protein distillers dried grains; WBCs, white blood cells; RBCs, red blood cells; Hb, Hemoglobin; PCV%, Packed cell volume.

Table (5) Effect of various dietary levels of high protein distillers dried grains (HP-DDG) supplemented with phytase on biochemical parameters of European sea bass, *Dicentrarchus labrax* fingerlings.

Diets	Total protein ($g\ dL^{-1}$)	Albumin ($g\ dL^{-1}$)	Globulin ($g\ dL^{-1}$)
Control	3.360 ± 0.020^{d}	2.160 ± 0.010^{b}	1.200 ± 0.030^{d}
Diet (30%) + phytase	3.405 ± 0.015^{cd}	2.125 ± 0.005^{b}	1.280 ± 0.020^{d}
Diet (40%) + phytase	3.535 ± 0.055^{b}	1.895 ± 0.025^{c}	1.640 ± 0.030^{b}
Diet (50%) + phytase	3.785 ± 0.055^{a}	2.285 ± 0.045^{a}	1.940 ± 0.060^{a}

Values are mean ±SD of triplicate analyses. Means in the same column bearing different superscript differ significantly ($P \leq 0.05$). HP-DDG, high protein distillers dried grains.

Table (6) Effect of various dietary levels of high protein distillers dried grains (HP-DDG) supplanted with phytase on immune parameters of European sea bass, *Dicentrarchus labrax* fingerlings.

Diets	**Cholesterol (mgdl)**	**Lysozyme (U mg^{-1} protein)**	**TAC (%)**
Control	140.115±2.575^{d}	1.785±0.085^{d}	7.1195±0.035^{c}
Diet (30%) + phytase	159.885±3.655^{c}	2.315±0.045^{c}	8.100±0.130^{b}
Diet (40%) + phytase	175.230±2.020^{b}	2.810±0.060^{b}	9.555±0.185^{a}
Diet (50%) + phytase	191.695±2.555^{a}	3.190±0.070^{a}	9.855±0.265^{a}

Values are mean ±SD of triplicate analyses. Means in the same column bearing different superscript differ significantly ($P \leq 0.05$). HP-DDG, high protein distillers dried grains.

Table (7) Effect of various dietary levels of high protein distillers dried grains (HP-DDG) supplemented with phytase on dietary phosphorus utilization of European sea bass, *Dicentrarchus labrax* fingerlings (P values on a DM basis)

Diets	**P in Diet (g /Kg)**	**P (Initial fish) (g/Kg)**	**P (final fish) (g/Kg)**	**P (feed intake) (g/Kg)**	**P (fish gain) (g/kg)**	**P retention (%)***
Control	6.42±50^{c}	3.78±0.11	15.31±1.31^{c}	46.24±1.13^{c}	11.55±1.27^{c}	24.98±0.44^{c}
Diet (30%) + Phytase	7.38±12^{bc}	3.78±0.11	22.79±0.02^{b}	55.49±0.60^{b}	19.00±0.57^{b}	34.24±0.71^{b}
Diet (40%) + phytase	9.94±60^{b}	3.80±0.11	26.21±0.04^{ab}	53.85±0.56^{b}	22.41±0.56^{b}	41.61±1.24^{b}
Diet (50%) + phytase	11.25±25^{a}	3.78±0.11	35.85±0.04^{a}	62.95±0.34^{a}	32.06±0.31^{a}	50.93±1.12 ^{a}

Values are mean ±SD of triplicate analyses. Means in the same column bearing different superscript differ significantly ($P \leq 0.05$). HP-DDG, high protein distillers dried grains.

*Phosphorus retention (%) $=100 \times (BW_{final} \times P_{final} - BW_{initial} \times P_{initial})/(\text{Feed intake} \times P\%_{diet})$. Where: $P\%_{diet}$ is the content of phosphorous in the diet; $P_{initial}$ and P_{final} represent the initial and final concentration of phosphorous in fish.

Table (8): Cost of feed required for producing one Kg gain when seabass were fed various levels of high protein distillers dried grains (HP-DDG) supplemented with phytase on European sea bass (*Dicentrarchus labrax*).

Diets	Feed cost per Kg ($[a])	FCR (Feed : gain)	ECR ($[a])	Cost / Kg fresh fish ($[a])	Relative feed cost/Kg
Control	0.86	1.58	1.36	0.66	1.31
Diet (30%) + phytase	0.93	1.40	1.33	0.62	119
Diet (40%) + phytase	0.95	1.43	1.32	0.62	119
Diet (50%) + phytase	1.10	1.20	1.32	0.59	114

[a]1$ = 16.15 L.E. (Egyptian pound).

FCR: feed conversion ratio; ECR: economic conversion rate. (0, 30, 40 and 50%) levels of HP-DDG.

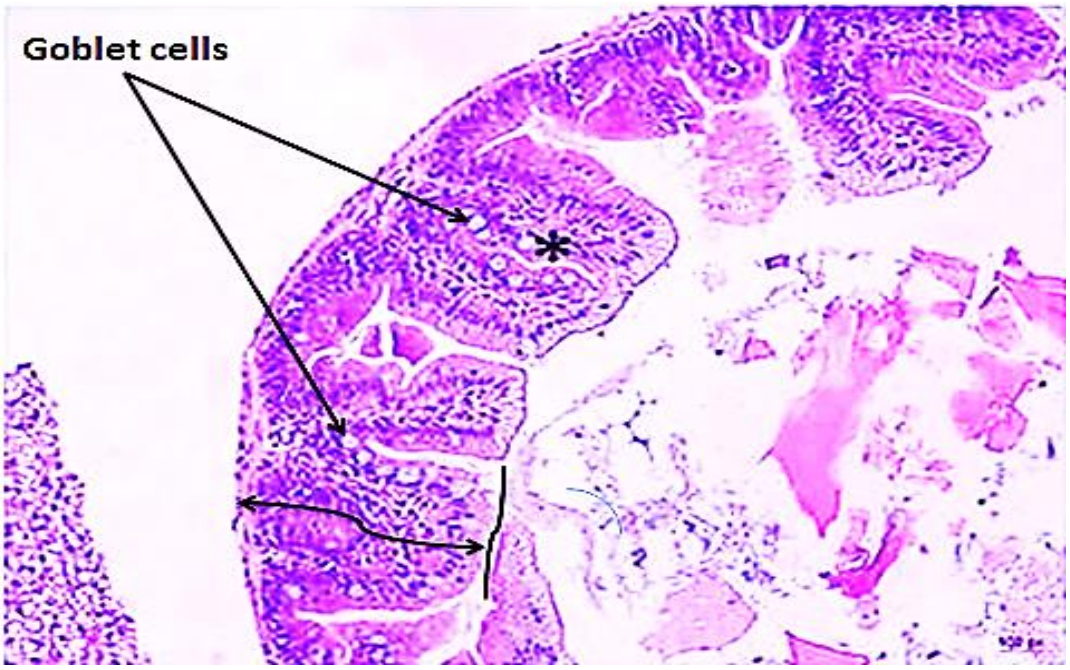

Figure (1a) Transverse section of Intestine of European sea bass, D.labrax, fingerlings fed the control diet (feed on basal diet + Phytase) for six weeks showing normal, intact intestinal wall intestinal villi as well as goblet cells.

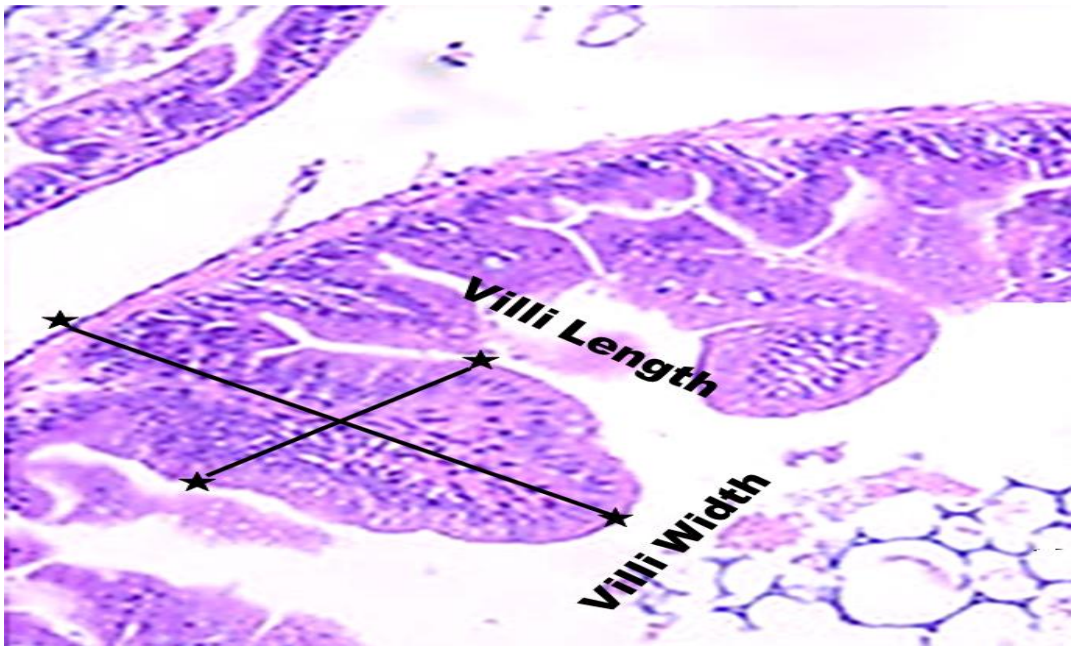

Figure (1b) TS of Intestine of European sea bass, *D.labrax*, fingerlings fed the (30% HP-DDG + phytase) for six weeks showing moderate improvement in length and width of intestinal (villi) as well as goblet cell density.

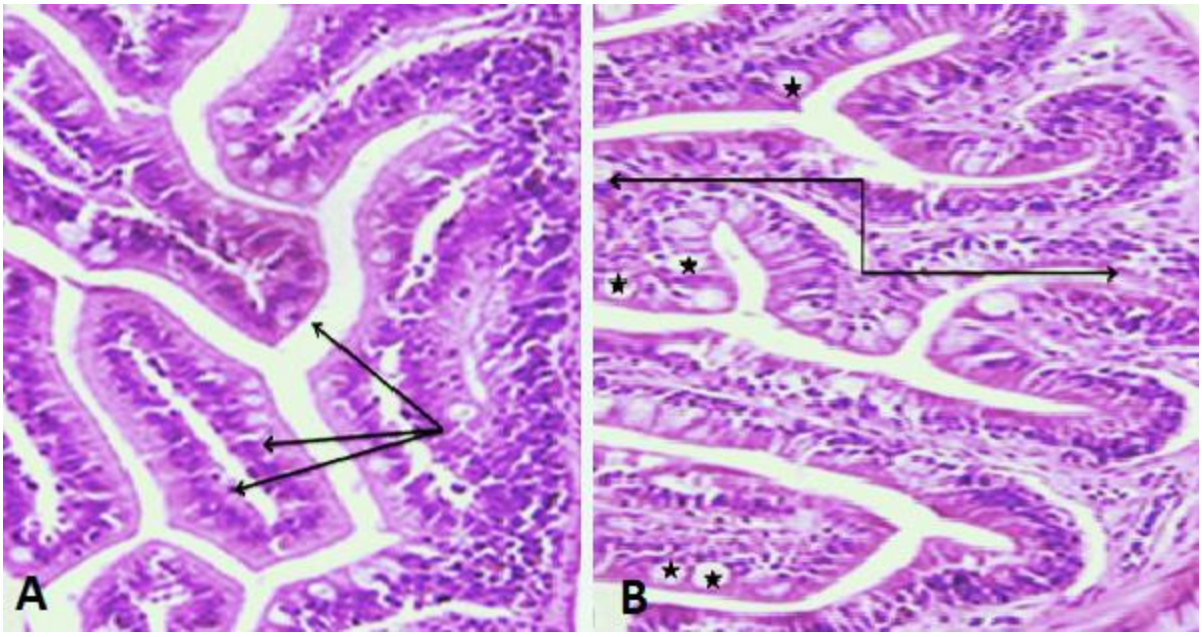

Figure (1c) TS of Intestine of European sea bass, D.labrax, fingerlings fed (40% HP-DDG + phytase) for six weeks showing highly increased length and width of the intestinal villi (arrows) and increase number of goblet cells. Stars show intracellular vacuolization indicative of lipid accumulation

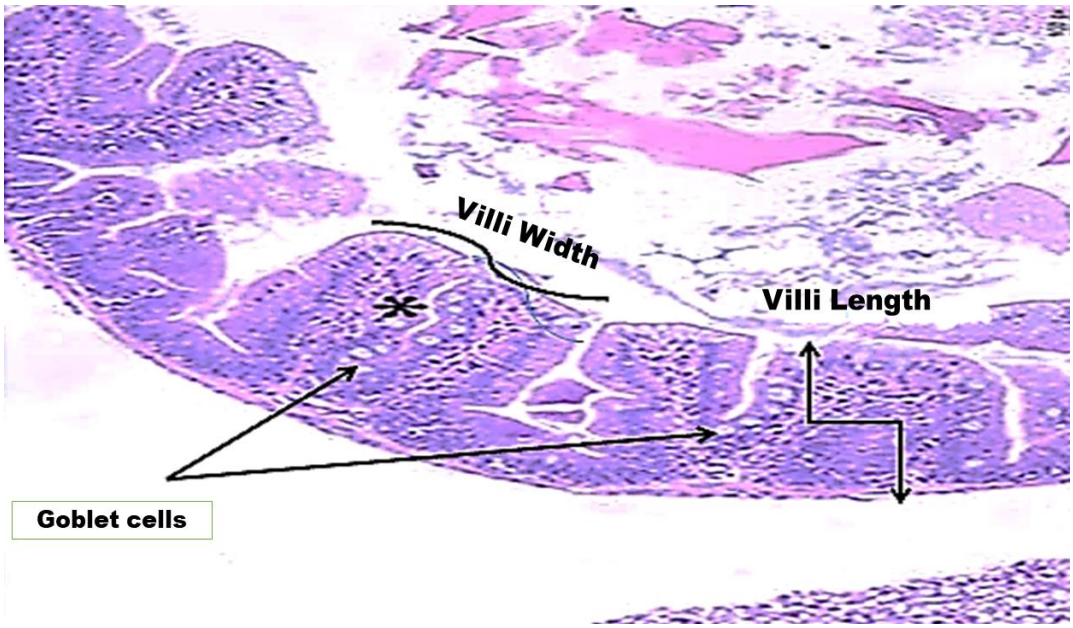

Figure (1d) TS Intestine of European sea bass, *D.labrax*, fingerlings fed (50% HP-DDG + phytase) for six weeks showing improvement in length and width of intestinal villus as well as increase number of goblet cells.

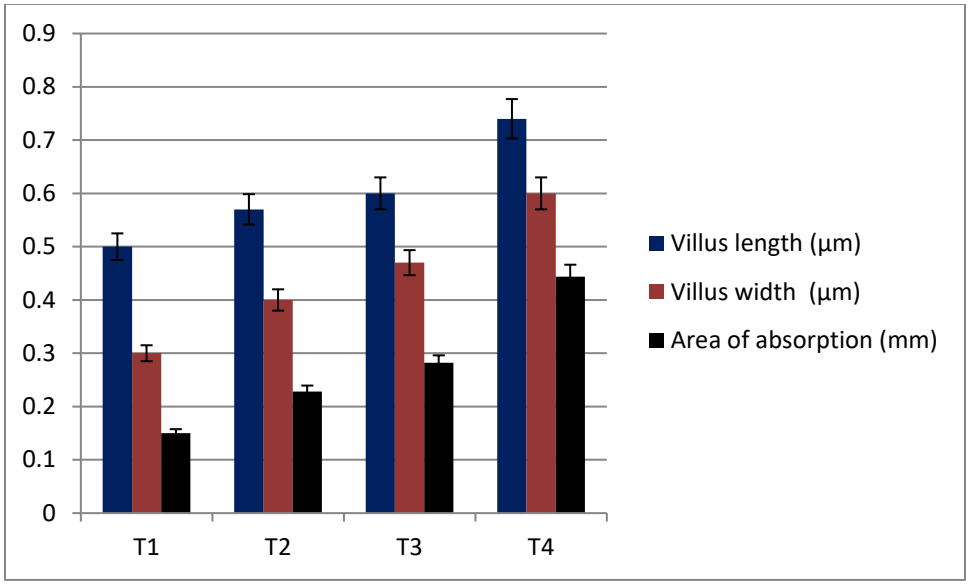

Figure (2) : Villi length and width and area of absorption in the (proximal? distal?) intestine of European sea bass fingerlings fed control (C), 30% HP-DDG (30), 40% HP-DDG (40), 50% HP-DDG (50) at the end of the 6-weeks feeding period.

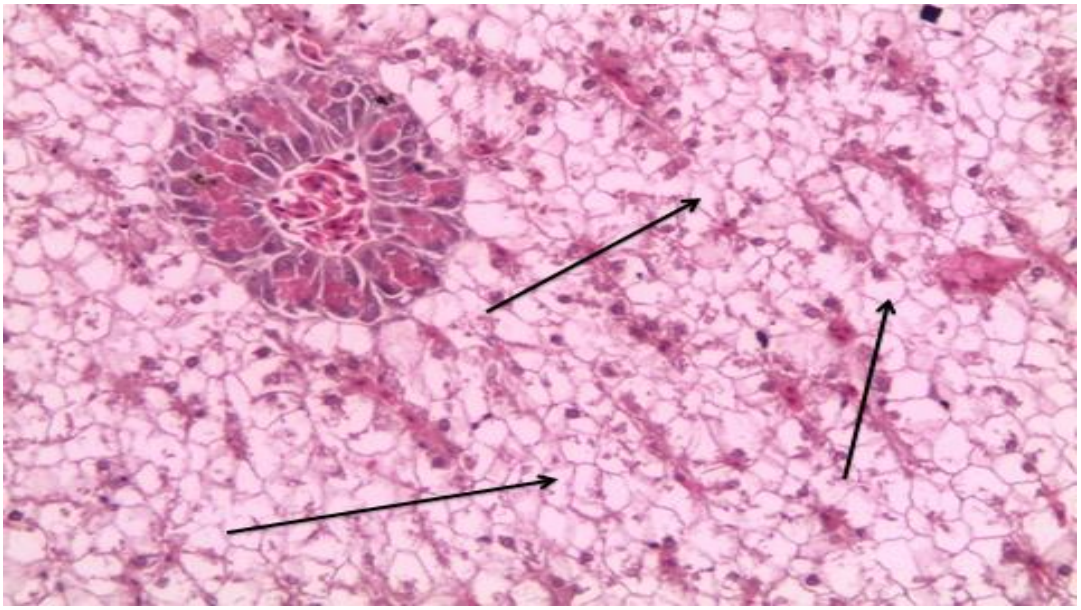

Figure (3a) Hepatopancreas of European sea bass, *D. labrax*, fingerlings (fed on the basal diet with phytase) for six weeks showing normal organization of the hepatic cell and blood capillaries (Arrows). (H&EX 400).

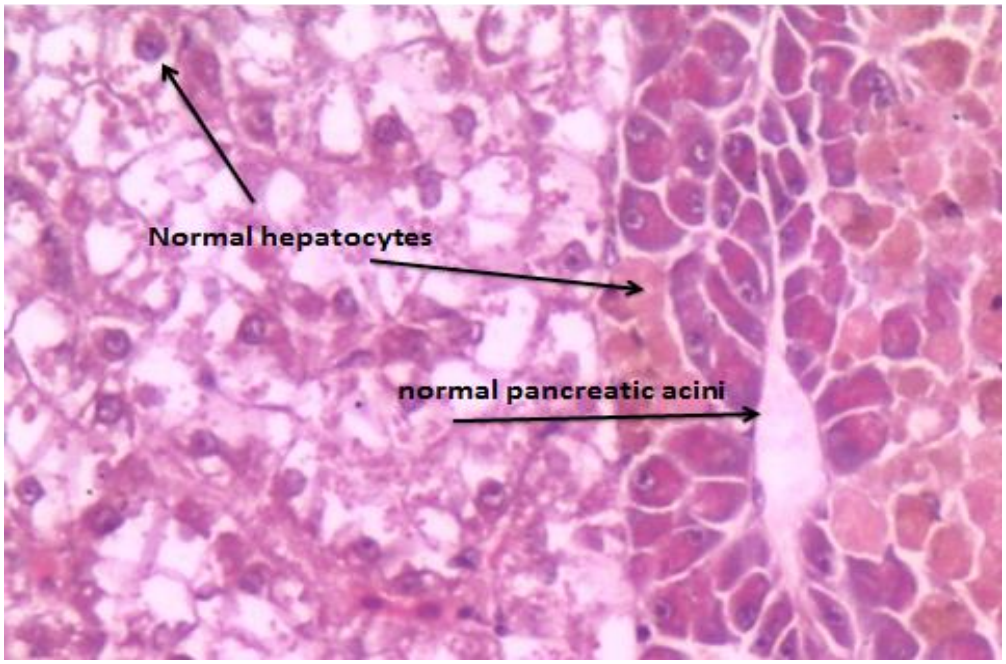

Figure (3b) Hepatopancreas of European sea bass, *D. labrax*, fingerlings fed (fed on 30% HP-DDG + Phytase) for six weeks showing activation of melano-macrophage centers and normal pancreatic acini and normal hepatocytes. (H&E X 400).

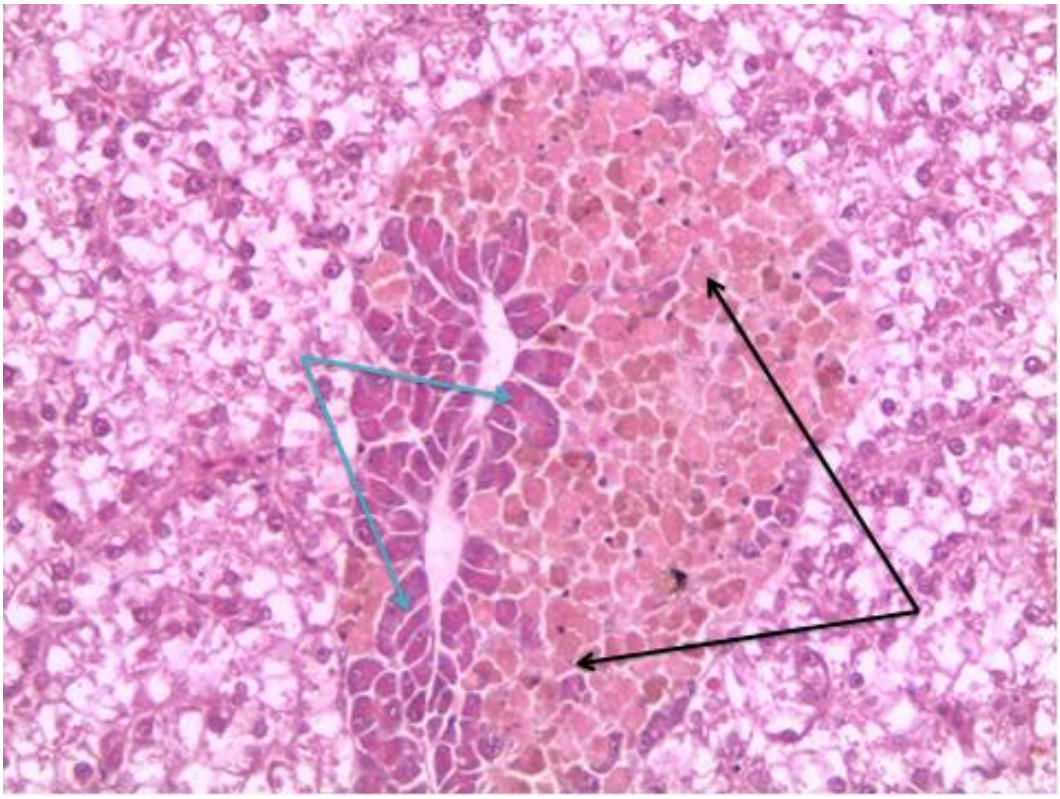

Figure (3c) Hepatopancreas of European sea bass, *D.labrax*, fingerlings (fed on 40% HP-DDG + Phytase) showing necrosis in pancreatic tissue (blue arrows) with activation of melano-macrophage centers (black arrows) (H&E X 20).

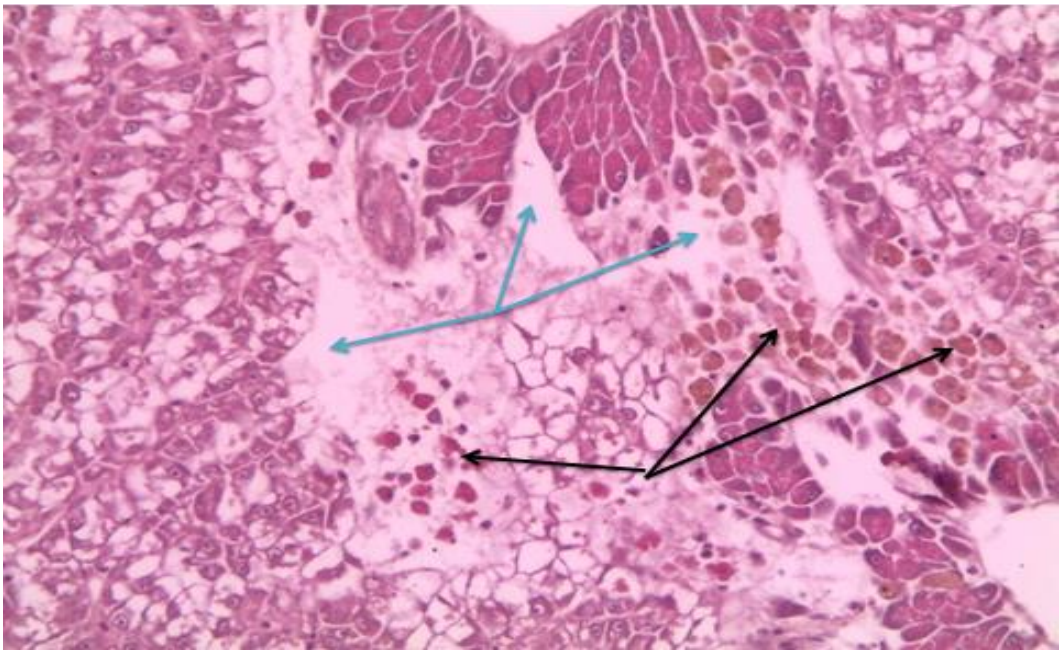

Figure (3d) Hepatopancreas of European sea bass, *D.labrax*, fingerlings (fed on 50% HP-DDG + Phytase) showing wide area of necrosis in hepatopancreas (blue arrows) with activation of melano-macrophage centers (black arrows) (H&E X 20).