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Partial replacement of dietary soybean meal by high protein distiller's dried grains (HPDDG) supplemented with protease enzyme for European seabass, *Dicentrarchus labrax* fingerlings

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Abstract

High protein distillers dried grains (HP-DDG) is a co-product of ethanol production that uses pre-fractionation technology. A 70-day's growth trial was conducted to investigate the effect of partial replacement of dietary soybean meal by high protein distiller's dried grains (HPDDG) with protease enzyme supplementation (PROXYM ULTRA®) on growth performance, physiological parameters and histological changes of the intestine of European sea bass, *Dicentrarchus labrax* fingerlings. The results indicated that increase dietary HP-DDG levels up to 50% of HP-DDG-supplemented with Protease significantly increases growth performance, feed utilization and improved FCR of sea bass. In addition, replacement of SBM by HP-DDG-supplemented with protease enhanced feed intake efficiency and the health status of fish. Hematology and serum biochemistry (hemoglobin (Hb), red blood cells (RBCs), white blood cells (WBCs) and humeral immune parameters including total protein, globulin, cholesterol, lysozyme activity and total antioxidant capacity significantly increased with increase HP-DDG-supplemented with protease in the diets. Results of this study indicated that HP-DDG-supplemented with protease is a good alternative protein source for aquaculture feed and can be included up to 50% as a replacement of SBM without compromising growth performance and physiological parameters of sea bass.

Keywords: *Dicentrarchus labrax,* HP-DDG-supplemented with protease, physiological parameters, growth performance, feed utilization, histology, humeral immune parameters

INTRODUCTION

Aquaculture production is expanding to fill the increasing demand of fish for human consumption globally. In 2016, aquaculture was responsible for the production of 171 million tons of fish products, most of which was for human consumption (FAO, 2018). With the world's population projected to reach 9.7 billion people by 2050 and global capture fisheries unstable and steadily declining, the spotlight turns to aquaculture production to contribute significantly to global food security and adequate global nutrition and human health (NASS, 2016). More than 70% of the total global aquaculture production is dependent upon the supply of external feed inputs. For the aquaculture sector to maintain its current growth rate, the supply of nutrient and feed inputs will have to grow at a similar rate, while aquatic ingredients production remains static and other sectors compete for same feed resources. There is an increasing need to seek alternatives, particularly underutilized commodities, such as by products obtained from food, fermentation and pharmaceutical industries, rather than being highly dependent of imported plant feedstuffs (Hassaan et al 2018 a,b et al., 2018; Hassaan et al., 2019), distillers dried grains with solubles (DDGS) is a by-product of cereal distillation for ethanol production. DDGS contains high protein, lipid and low fiber and Antinutritional factors (ANFs) levels (Liu, 2011). Studies on DDGS incorporation in aqua-feeds studies were mainly done in omnivorous fish species, such as channel catfish, *Ictalurus punctatus*; Li et al., 2011a). Nile tilapia, *Oreochromis niloticus* and hybrid tilapia, *Oreochromis niloticus* × *Oreochromis aureus* (Welker et al., 2014a). So far, studies performed on the potential use of DDGS in carnivorous species are limited to a few studies with rainbow trout, *Oncorhynchus mykiss* (Overland et al., 2013; Welker et al., 2014b), olive flounder, *Paralichthy solivaceus* (Rahman et al., 2015; Bae et al., 2015), meagre (Argyrosomus regius) and European sea bass (Magalhães et al., 2015). In addition, the use of various enzymes in aquatic feed has been on the rise to improve the overall quality of diets containing these economical protein sources. Exogenous enzymes have been shown to affect the digestibility of nutrients, including protein, carbohydrates and minerals (Forster et al. 1999). Earlier studies indicated that exogenous enzymes improve the growth performance by enhancing nutrient digestibility (Farhangi

and Carter, 2007) and improving the histological structure (Mathlouthi et al., 2002) and the health of intestine (reviewed by Castillo and Gatlin, 2015). Furthermore, digestive enzyme supplementation help to eliminate the effects of anti-nutritional factors and improve the utilization of dietary energy and amino acids, resulting in improved growth performance (Soltan 2009). The present study was undertaken to determine the effect of various dietary levels of high protein distiller's dried grains (HPDDG) supplemented with enzyme protease on growth performance, feed utilization, histology and haematological indices of European sea bass *Dicentrarchus labrax* fingerlings.

MATERIALS AND METHODS

Experimental Fish and Culture Technique

European seabass, with an average initial body weight of 7.47±0.8 g/fish were obtained from El-Shiref farm, Wady Marriott, Alexandria Governorate, Egypt; fish acclimated for one week to the water and were fed a control diet (5% body weight/day). During fish acclimation, the fingerlings were stocked in indoors circular fiberglass tanks (1 cubic meter) for one week and were fed with a control diet at a ratio of 5 % of body weight d^{-1}. The daily ration was divided into three equal amounts and offered three times a day (09.00, 12.00 and 15.00 h). Fish were randomly distributed into 12 glass aquaria (70×40×30 cm each) at El-Shiref farm, Alexandria, in a design of three replicate tanks for each of four dietary treatments. Fish were stocked at a density of 10 fish per aquaria. Water temperature, dissolved oxygen, pH, and ammonia were monitored during the trial, to maintain water quality at optimum range for *D. labrax*. Water temperature was maintain at 18.5 ± 0.9°C, dissolved oxygen (DO) at 6.1 mg L-1 and pH at 7.6 ± 0.7, under natural light (12:12 h light: dark schedule). Daily, 20% of water was exchange using underground filtered water (36 ppt).

Experimental diets

Four isonitrogenous (~448.27 g/kg CP) and isolipidic (~128.83 g/kg CL) experimental diets were formulated (Table 1). The control diet ($C_{0\%}$) had no high protein distiller's dried grains **(**HP-DDG) or Protease added.

Tested diets were formulated to contain 30% (P30%), 40% (P40%), and 50% (P50%) of HP-DDG added on the expanses of the soybean meal content and equally supplemented with 1.0 g/kg of Protease (PROXYM 102 ULTRA®), HPDDG and protease 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. HPDDG supplied from United States USA, by MIRASCO EGYPT Company. The chemical composition of HP-DDG used in the rations was crude protein 47%; Crude fat 4%; Crude fiber 4%; Ash 7% and Moisture 7%. During the 70-days experimental period, all fish were fed with their respective diets at 5% of body weight d^{-1} for 6 days/week. 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 protease enzyme (PROXYM ULTRA5®, Gloray Vet COMPANY, USA) contain the enzymatic activity of 2000000 Unit was dissolved into the 200 mL water at 37 °C (Yoo et al., 2005). The solution was incubated for 24 hours at room temperature according to the method of 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**.** Commercial protease enzyme product (PROXYM ULTRA®) was purchased from Gloray Vet COMPANY, USA. One gram of commercial protease enzyme (PROXYM ULTRA5®, Gloray Vet COMPANY, USA) contain the enzymatic activity of 2000000 Unit was dissolved into 100 mL water at 37 °C (Yoo et al., 2005. The solution was added to the experimental diets and incubated for 24 hours at room temperature according to the method of Danwitz et al. (2016).

126 **Growth Indices**

127 The mean final body weight (FBW) in experimental treatment was determined by dividing the
128 total fish weight in each aquarium by the number of fish. Weight gain (WG), specific growth rate (SGR),
129 feed conversion ratio (FCR), protein efficiency ratio (PER), protein productive value (PPV), fat retention
130 (FR), energy retention (ER), economical conversion rate (ECR) and survival (%) were calculated using
131 the following equations, according to Tiews (1980):

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

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

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

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

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

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

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

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

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

141 **Blood Samples and Haematological Analysis**

142 Blood samples were collected at the end of the experiment. From each of the dietary treatments,
143 five fish were used for hematological indices analysis and five for plasma content analysis. The fish were
144 anesthetized with t-amyl alcohol and the blood samples were taken by puncturing the caudal vessels.
145 Blood samples were collected into two tubes, one containing heparin as anticoagulant agent for
146 haematological assessment and the other was anticoagulant free for biochemical estimation. The
147 haematological parameters are expressed in international units (SI). The total red and white blood cell
148 counts (RBC; 10^6 mm^{-3} and WBC; 10^3 mm^{-3}, respectively) were obtained by using a standard Neubauer-
149 hemocytometer chamber using Shaw's solution as diluting fluid (Stoskopf, 1993). Hemoglobin (Hb; g
150 dL^{-1}) was determined colorimetrically using commercial kits (Diamond, Egypt) according to the cyan-

151 methemoglobin procedure (Drabkin, 1945). Hematocrit (Hct) were determined by using
152 microhematocrit-heparinized capillary tubes and a microhematocrite centrifuge (10000 g for 5 min)
153 Levels of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline
154 phosphatase (ALP) were estimated according to the method described by Reitman & Frankel (1957).

155 **Biochemical and immune parameters**

156 The total protein (g dL^{-1}) was determined in plasma samples of fish from the different
157 experimental groups by the Biuret method according to (Doumas et al. 1981). Albumin (g dL^{-1}) was
158 determined by the bromocresol green method (Reinhold, 1953) and globulin (g dL^{-1}) was calculated as
159 the difference between total protein and albumin, and cholesterol was measured by a commercial kit
160 (Pasteur, Lab, France, Egypt) (Yousefi et al. 2011). Lysozyme activity (U mg^{-1} protein) in serum was
161 determined according to the method of Ellis (1990) based on the lysis of the lysozyme sensitive gram-
162 positive bacterium *Micrococcus lysodeikticus* (Sigma, St. Louis, MO). Lysozyme acts upon susceptible
163 bacteria by combining with and breaking down a mucopolysaccharide. This mucopolysaccharidehas has
164 been shown to be situated in the bacterial cell wall *M. lysodeikticus*, is normally highly sensitive to
165 lysozyme dilutions of hen egg white lysozyme (Sigma) ranging from 0 to 25 µg mL^{-1} (in 0.1 M phosphate-
166 citrate buffer, pH 6) (Sigma, USA) were used as the standard.

167 Total antioxidant capacity (TAC) level was estimated spectrophotometrically at 532 nm following
168 the method with Tween 80 oxidation (Galaktionova et al. 1998). Briefly, 0.2 ml of tissue homogenate
169 was added to 2 ml of 1% Tween 80. Instead of the sample, the blank assay included 0.1 ml of distilled
170 water. The mixture was incubated for 48 hours at 37 ºC. After cooling, 1 ml of 40 % TCA was added.
171 The mixture was centrifuged at 3,000 g for 10 min. After centrifugation, 2 ml of supernatant and 2 ml of
172 0.25% TBA reagent were mixed in. The mixture was heated in a boiling water bath at 100 ºC for 15
173 minutes. The absorbance of the solution obtained was measured at 532 nm and was compared with the
174 blank. The TAC level was expressed in (%).

175 **Histological examination**

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

Economic evaluation

The economic evaluation was calculated according to the following equations (Salama et al., 2010).

Feed cost per Kg fresh fish (LE) = Cost / kg diet (LE) * consumed feed to produce 1 kg fish .

Relative feed cost/Kg fresh fish = Values of feed cost/Kg fresh fish / the minimum value of the same parameter

Feed cost / 1 Kg gain (LE) = Feed intake per kg gain (FCR) * cost/kg diet (LE).

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

Statistical analysis

One-way ANOVA and Duncan's, (1955) multiple range tests were calculated effects with a probability of $p<0.05$ were considered significant. The data from the experiments were statistically analyzed using GLM (general linear model) procedure according to Statistical Analysis System (SAS 2004). However, data is presented untransformed to facilitate comparisons.

RESULTS

Growth performance and feed utilization:

Fish growth performance presented in Table 2, shows that all fish fed HP-DDG supplemented with Protease had higher growth than the control diet, suggesting that the using of the tested ingredient supplemented with protease resulted in higher growth than the control diet, suggesting that the using of HPDDG-supplemented with Protease enhanced the growth performance of seabass fingerlings. The

FBW, BWG and SGR of *D. labrax* increased with the increasing of dietary HP-DDG supplemented with protease and higher in diet (50 % HPDDG with protease) compared to other treatments. The fish recorded high survival rate 100% in all tested diets and there were no mortality observed in the experiment (Table 3). Moreover, feed intake, FCR, PER and PPV were significantly enhanced with the increasing HP-DDG levels compared to fish fed the control diet (Table 3). The highest values of FCR, PER and PPV were recorded for fish fed 50% HP-DDG-supplemented with Protease, while the lowest values were observed for fish fed the control diet. The same trend was recorded for the highest values of FR and ER for fish fed 40% HP-DDG-supplemented with Protease (Table 3).

Hematological parameters:

The white blood cells (10^4), red blood cells (10^6), hemoglobin (g dl^{-1}) and hematocrit (Hct, %) values differed significantly ($P<0.05$) between the control fish and fish fed various HP-DDG-supplemented with Protease diets (Table 4). The WBCs, RBCs, Hb and Hct were tended to increase with increasing dietary HP-DDG levels (Table 4).

Biochemical parameters:

Total plasma protein and globulin (α Globulin, β Globulin and γ Globulin) were significantly higher in fish fed with the diets containing different levels of HP-DDG-supplemented with Protease compared to the control fish (Table 5) and the peak value recorded in 50% HP-DDG-supplemented with Protease diet compared to all treatments, but an opposite trend was observed for the total plasma Albumin. A similar increasing trend was observed for the Cholesterol, Triglyceride, Lysozyme and total antioxidant capacity (TAC) (Table 6). Levels of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) parameters are presented in Table 7. The highest values of ALT, AST and ALP were estimated in Seabass fed the control diet compared to HP-DDG-supplemented with Protease. The results observed that with increasing dietary HP-DDG levels the values of ALT, AST and ALP decreased compared to fish fed the control experimental diet group.

Histology of intestinal

Histology of the intestine of sea bass fed control diet and different levels of HP-DDG are showed in Figures (1–4). The histological analysis revealed that *D. labrax* fed on the control basal diet showing decreasing in the intestinal villi length and width and some evidence of damage and enteritis in some neighboring mucosal folds (Figure 1).

Fish fed 30% HP-DDG supplemented diet with Protease showed moderate improvement in the length of intestinal villi with few goblet cells in the lining epithelial (Figure 2). While, fish fed 40% HP-DDG supplemented diet with Protease showed improvement in length and width of intestinal villi with more regular uniformity as well as active goblet cells and filled with abundant acidic mucin secretion, due to increase absorption surface by supplementing protease and thus influence the intestinal health (Figure 3). Seabass fed 50% HP-DDG-supplemented diet with Protease showed moderate improvement in length and width of intestinal villi as well as goblet cells and filled with abundant acidic mucin secretion (Figure 4).

Economic evaluation:

Calculations of economic efficiency of the tested diets based on the cost of feed cost for one kg weight gain are shown in Table (8). The 50% HPDDG diet with protease exhibited significantly lower feed intake, best FCR and ECR values compared with the other diets, while fish fed the control diet recorded the highest in total feed cost / kg fish gain.

DISCUSSION

High protein content of HP-DDG products make them even more attractive for inclusion in fish diets because protein is the most expensive nutrient component in aqua-feeds. In the present study, fish fed up to 50% of HP-DDG-supplemented with Protease exhibited and improved growth indicating that enzyme is beneficial for the growth of sea bass (Table 2), these results could be attributed to: i) the role of protease to improve nutrient bioavailability and consequently enhance growth performance and feed efficiency, suggesting that the negative effects of plant ingredients were compensated to some extent by

addition of the enzymes, ii) HPDDG supplemented with exogenous enzymes improve the available energy of HPDDG by degrading the fiber content and increasing the digestibility of nonstarch polysaccharides (Barletta, 2012), iii) exogenous enzymes in HPDDG based diets by disrupting the cell wall matrix and enhancing the contact between digestive enzyme and cell content, which resulted in improved energy and nutrient digestibility (Wu et al., 2004). These results are consistent with Barletta (2012) who found that protease supplementation could degrade complex proteins in the diet into usable amino acids and peptides thereby resulting in improved protein digestibility and growth performance. In addition protease is capable of degrading grain storage proteins and liberating higher levels of available amino acids, energy and eliminate the effects of anti-nutritional factors in carnivorous fish diets containing high levels of plant-based feedstuffs resulting in improve performance (Dias et al., 2014; Gitoee et al., 2015). Growth responses of seabass fed enzyme protease supplemented diets were strongly related to enhance feed efficiency (Table 3). This indicates that the enzymes promoted feed intake in this group of fish, suggesting that they were effective in resisting the anti-nutritional effect of plant protein and increased growth. However, the mechanism of improving the feed intake in fish fed diets supplemented with enzymes is still unclear, but two possible explanations can be debated. One is the enhancement of palatability of the diets by free amino acids generated by protease activity enzyme (Carr et al. 1996) which resulted in higher feed intake in fish fed the diets supplemented with enzyme complex. Another explanation is the higher digestibility of nutrients in the diet. Debnath et al. (2005) reported that higher digestibility of dietary nutrients could lead faster gut passage of ingested feed. It was suggested that return of appetite is strongly correlated with rate of gastric evacuation of ingested food in fish (Lee et al. 2000). Faster digestion of ingested food could potentially promote feed intake of fish. The addition of exogenous enzymes to aqua-feed has been reported to enhance the digestion of indigestible ingredients in some species of fish and shrimp and it improve the growth performance such as Atlantic salmon (Carter et al. 1994), black tiger shrimp *Penaeus monodon* (Buchanan et al. 1997) and silver perch *Bidyanus bidyanus* (Stone et al. 2003; Yu et al., 2007). On contrary, Nalan et al. (2016) reported that dietary

supplementation of protease for rainbow trout, *Oncorhynchus mykiss* did not improve growth performance and FCR. Similarly, Dalsgaard et al. (2012) found that there were no differences in growth parameters and FCR with the addition of protease to soybean meal in rainbow trout diet. The discrepancy in their results may be associated with many factors affect the action of dietary enzyme, such as diet composition and feed processing. If the basal diet had a high nutrition level which had satisfied the nutrition requirement of aquatic animals, the supplementation of protease would not show positive effects on growth, while the basal diet had a low nutrition level or consisted of ingredients with relatively low quality, and the improvement of growth and nutrients utilization by dietary protease could be observed (Li et al., 2016).

Total serum protein is often used as an indicator of physiological condition in fish, as it is one of the most stable components of blood, and so an increase or decrease of total blood proteins, globulins and albumin has clinical relevance in fish (Peres et al., 2015). High plasma albumin and/or globulin has been related to stress, inflammatory, innate immune responses and feeding immunostimulants (Peres et al., 2015). Also higher levels of plasma non-specific humoral immune parameter, such as lysozyme and complement activity, have been used as indicative of immuno-enhancing properties to certain dietary compounds. In the present study, the hematological, biochemical and immune parameters WBC, RBC, Hb, PCV, total protein and globulin increased in fish diet containing HPDDG supplemented with protease enzymes compared with the control group (Table 4&5). These results are consistent with Peatman and Beck, (2016) found that commercial catfish fed diets with high levels of phytase enzymes could significantly improve the concentration of RBC's, WBCs, PCV% and Hb concentration as a result of phytase super dose fortification. The increase in hemoglobin concentration could be attributed to the higher oxygen consumption associated with more hemoglobin saturation and dissociation rates (Yahav et al., 1998). The serum albumin levels were significantly higher on the control diet without DDGS or enzyme supplementation (Table 5). These findings are in agreement with El-Katcha et al. (2014) who

found that enzyme supplementation had no significant effect on blood serum albumin concentrations compared with broiler fed on the same diet without enzyme supplementation.

Normally, AST exists in hepatocyte mitochondria, while ALT is spread around the hepatic cells and bile duct. The increasing activities of serum AST and ALT in fish may reveal the leakage of enzymes across damaged *p*lasma membranes and/or rising synthesis of enzymes by the liver tissue (Yang and Chen, 2003).Thus, the activity of serum AST and ALT are used as im*p*ortant indicators to reflect the health of liver and their functions of the fish (Zhai et al., 2014) as well as it can be used to assess the health status and as stress indicators in fish (Satheeshkumar et al., 2010). In the present study increasing dietary HP-DDG levels the values of ALT, AST and ALP decreased compared to fish fed the control experimental diet group (Table 6), while the levels of Cholesterol, Triglyceride, lysozyme, TAC increased (Table 7), su*pp*ress the release of liver damage enzymes such as AST and ALT into *p*lasma as stated *p*reviously by (Akrami et al. 2015). Also, the present results are consistent with (Shelby et al 2007) who found that Nile tilapia fed distillers dried grains with soluble improved immune, liver function and disease resistance. Similarly, Niamat (2017) found that Japanese quail fed diets partially or totally replaced yellow corn with DDGS recorded lower activities of AST and ALT compare to the control group. On Contrary, El-Saidy and Gaber (2003) showed that AST and ALT were not significantly affected by dietary contained 10, 15 and 20% DDGs. The present results are consistently with previous research conducted by (El-Saidy and Gaber., 2003) using DDGS in Nile tilapia and concluded that DDGS improve liver function of seabass.

Light and scanning electron microscopy revealed a normal and healthy morphology of the intestines of seabass fed HPDDG supplemented with protease diets compared to the control diet without DDGS or protease (Figure 1). The intestines of the fish showed intact epithelial barrier with well organized villi-like mucosal folds, abundant IELs and goblet cells. The fish intestines displayed healthy brush border with well organised and tightly packed microvilli revealing no signs of damage (Figure 2, 3 & 4). However, the microvilli of the brush border of seabass fed control without protease

supplementation appeared to be less tightly packed (Figure 1). Consequently, the microvilli density of the fish intestines was significantly different amongst seabass fed the experimental diets; the microvilli density of seabass fed the DDGS diets supplemented with protease were significantly higher ($P < 0.05$) than that of seabass fed the control without phytase. In terms of gastrointestinal morphology, there was no significant difference in mid-intestine with respect to perimeter ratios, goblet cells levels and IELs levels, but significantly higher microvilli density (a measure of absorptive intestinal surface area) was observed in seabass fed diets supplemented with protease (Figure 1 - 4). This is in line with improved growth performance and may have been a contributory factor to the observed growth parameters (Adeoye et al., 2016). In the study on rainbow trout by Zhang et al. (2012), activities of intestinal protease and gastric protease and structure of foregut tissue were improved by adding protease in to diet. In contrast to the aquacultural sector the use of enzymes seems to be evaluated adequately in terrestrial animal production, as it is used there since decades (Bedford, 2000). This is why the economic viability of enzymes products seems to be even more likely in the aquaculture sector, since most feed ingredients have high values of plant proteins compared to those of terrestrial animal farming. However, potentially adapted production and extrusion processes might be necessary and seem to be a major issue regarding the incorporation of enzymes in fish feeds.

In term of economic evaluation fish fed 50%HPDDG with protease achieved best FCR and ECR values (Table 8) compared with the other diets, while fish fed the control diet without DDGS and protease supplementation recorded the highest in total feed cost/kg fish gain. These results are consistent with Alam et al. (2003) who observed that feed cost per kg live weight was reduced by addition of exogenous enzymes in broiler diet. In addition, Khan et al. (2006) concluded that enzymes supplementation is more feasible and economical to obtain maximum profitability from broiler production. They also reported that enzyme supplementation decreased the relative cost of broiler feeds by 4 to 18% compared to other diets without enzyme supplement. Moreover, Peric et al. (2008) found that in regard to positive economical

effect, it is obvious that increase of cost of feed occurring because of the addition of enzyme, was annulled through increase of body weight of chickens and improved feed conversion.

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Table (1): Formulation and chemical composition of the experimental diets (g/kg)

Ingredients	Control	$P_{30\%}$	$P_{40\%}$	$P_{50\%}$
Fish meal 68 % CP	300	300	300	300
Soy bean meal 47% CP	375	262.5	225	187.5
Corn gluten 60% CP	90	90	90	90
Rice bran 12% CP	65	50	50	50
Wheat midllings13% CP	70	83.8	84.8	85.8
HP-DDG (BP50)	0	112.5	150	187.5
Soy bean oil	41	42	41	41
Fish oil	47.8	48	48	47
Di-calcium phosphate	8	8	8	8
Vit. and Min. premix[1]	2	2	2	2
Vitamin. C*	0.2	0.2	0.2	0.2
Protease (PROXYM ULTRA®)	1	1	1	1
Total	1000	1000	1000	1000
	Chemical composition (g/kg)			
Crude protein (CP)	448.6	448.8	449.7	446
Ether extract (EE)	125.6	131	129.8	128.9
Nitrogen free extract (NFE)[2]	283.3	257	266.5	278.6
Ash	110	120	120	114.5
Gross energy (GE; MJ/kg)[3]	20.98	20.93	20.91	20.96

[1]Vitamins and minerals mixture each 1 Kg of mixture contains: 12 m.IU vit. A, 12 mIU vit. D_3, 10g vit. E, 2g vit. K, 1g vit. B_1, 5g vit. B_2, 1.5g vit. B_6, 10mg vit. B_{12}, 30g niacin, 1000 mg folic acid, 50mg biotin, 10g banathonic acid, 50g zinc, 30g iron, 60g manganese, 10g copper, 100 mg cobalt, 100mg selenium, 1000mg iodine.

[2] NFE: nitrogen-free extract calculated using the following equation: NFE = 100 (crude protein + ether extract + crude fiber + ash).

[3] Gross energy (GE) = Calculated using gross calorific values of 23.63, 39.52 and 17.15 KJ g^{-1} for protein, fat and carbohydrate, respectively according to (NRC, 1993).

*Ascorbyl Phosphate

Table (2): Growth performances of European sea bass fed the experimental diets (means ± SE).

Diets	IBW (g fish^{-1})	FBW (g fish^{-1})	WG (g fish^{-1})	SGR (%/day)
Control	7.47±0.06	15.57±0.81^{b}	8.10±0.79^{b}	1.31±0.09^{b}
$P_{30\%}$	7.53±0.12	16.80±0.70^{ab}	9.27±0.60^{ab}	1.43±0.05^{ab}
$P_{40\%}$	7.43±0.06	17.07±0.45^{ab}	9.63±0.40^{ab}	1.48±0.04^{ab}
$P_{50\%}$	7.43±0.06	19.28±0.63^{a}	11.85±0.57^{a}	1.70±0.05^{a}

Values are mean ± SD of triplicate analyses. Means in the same column bearing different superscript differ significantly ($P \leq 0.05$).

IBW, initial body weight; FBW, Final body weight; WG, weight gain ; FCR, feed conversion ratio and SGR, specific growth rate.

Table (3): Feed efficiency of European sea bass fed the experimental diets (means ± SE).

Diets	Feed intake (g fish^{-1})	FCR (Feed : gain)	PER	PPV (%)	FR (%)	ER (%)	Survival rate
Control	16.93±0.40^{a}	2.09±0.24^{a}	1.07±0.13^{c}	11.08±0.17^{c}	22.42±2.16^{c}	6.72±0.09^{d}	100
$P_{30\%}$	15.57±0.31^{b}	1.68±0.12^{ab}	1.33±0.15 ^{b}	17.33±3.04^{b}	26.94±1.12^{b}	7.80±0.05 ^{cd}	100
$P_{40\%}$	14.17±0.29^{b}	1.47±0.04^{ab}	1.48±0.22 ^{b}	19.78±3.89^{b}	35.69±2.47^{a}	10.20±0.04^{a}	100
$P_{50\%}$	13.07±0.51^{c}	1.10±0.07^{b}	1.94±0.10^{a}	26.15±1.32^{a}	27.82±1.87^{b}	8.28±0.05^{b}	100

Values are mean ± SD of triplicate analyses. Means in the same column bearing different superscript differ significantly ($P \leq 0.05$).

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Table (4): Hematological parameters of European sea bass fed the experimental diets (means ± SE).

Diets	WBCs (10^4)	RBCs (10^6)	Hb (g dL^{-1})	Hct (%)
Control	23.95 ± 0.45^{d}	1.51 ± 0.03^{c}	8.10 ± 0.16^{c}	20.21 ± 0.18^{c}
$P_{30\%}$	26.98 ± 0.29^{c}	1.89 ± 0.06^{b}	9.27 ± 0.11^{b}	23.66 ± 0.14^{b}
$P_{40\%}$	31.42 ± 1.50^{b}	2.24 ± 0.13^{ab}	10.64 ± 0.36^{ab}	26.08 ± 0.18^{ab}
$P_{50\%}$	38.99 ± 1.03^{a}	2.81 ± 0.03^{a}	11.32 ± 0.08^{a}	27.62 ± 0.10^{a}

Values are mean ± SD of triplicate analyses. Means in the same column bearing different superscript differ significantly ($P \leq 0.05$).

WBCs, white blood cells; RBCs, red blood cells; Hb, Hemoglobin; Hct %, Hematocrit.

Table (5): Biochemical parameters of European sea bass fed the experimental diets (means ± SE).

Diets	Total protein ($g\ dL^{-1}$)	Albumin ($g\ dL^{-1}$)	Globulin ($g\ dL^{-1}$)	α Globulin (mg/dl)	β Globulin (mg/dl)	γ Globulin (mg/dl)
Control	3.50 ± 0.04^{c}	2.02 ± 0.13^{a}	1.48 ± 0.10^{d}	0.36 ± 0.01^{c}	0.34 ± 0.01^{c}	0.74 ± 0.02^{c}
$P_{30\%}$	3.60 ± 0.01^{c}	1.80 ± 0.04^{b}	1.80 ± 0.03^{bc}	0.44 ± 0.03^{b}	0.40 ± 0.01^{b}	0.78 ± 0.01^{b}
$P_{40\%}$	3.90 ± 0.04^{b}	1.62 ± 0.04^{c}	2.29 ± 0.01^{b}	0.53 ± 0.02^{a}	0.47 ± 0.05^{a}	0.80 ± 0.06^{b}
$P_{50\%}$	4.15 ± 0.02^{a}	1.55 ± 0.05^{d}	2.60 ± 0.03^{a}	0.56 ± 0.06^{a}	0.48 ± 0.01^{a}	1.03 ± 0.02^{a}

Values are mean ±SD of triplicate analyses. Means in the same column bearing different superscript differ significantly ($P \leq 0.05$).

Table (6): Biochemical parameters of European sea bass fed the experimental diets (means ± SE).

Diets	Cholesterol (mg dL^{-1})	Triglyceride (mg dL^{-1})	Lysozyme (U mg^{-1} protein)	TAC (%)
Control	147.04± 1.91^{c}	142.40±6.75^{d}	1.91± 0.04^{d}	8.23 ± 0.13^{b}
$P_{30\%}$	175.84± 4.04^{b}	162.71±6.26^{c}	2.90 ±0.04^{c}	8.89 ± 0.40^{b}
$P_{40\%}$	191.80 ± 5.43^{b}	215.77±2.26^{b}	3.88± 0.08^{bc}	9.92 ± 0.49^{ab}
$P_{50\%}$	239.53± 2.19^{a}	261.34±6.75^{a}	4.77 ± 0.01^{a}	13.78 ± 0.70^{a}

Values are mean ± SD of triplicate analyses. Means in the same column bearing different superscript differ significantly ($P \leq 0.05$).

TAC= Total antioxidant capacity.

Table (7): Liver function of European sea bass fed the experimental diets (means ± SE).

Diets	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Control	35.28± 1.51[a]	30.84±0.86[a]	17.50± 0.47[a]
$P_{30\%}$	32.77± 0.59[b]	28.19±1.38[b]	17.07±0.42[a]
$P_{40\%}$	26.23± 1.54[c]	23.87±0.76[c]	17.32± 0.04[a]
$P_{50\%}$	20.90± 0.34[d]	20.22±1.42[d]	15.86± 0.86[b]

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 distiller's dried grains; AST= serum aspartate aminotransferase, ALT= alanine aminotransferase and ALP= alkaline phosphatase

225 **Table (8):** Economical evaluation of European sea bass fed the experimental diets (means ± SE).

Diets	Feed cost per kg ($*)	FCR	ECR** ($*)	Cost/kg fresh fish($*)	Relative Feed cost/kg
Control	0.75	2.09	1.57	0.75	100
$P_{30\%}$	0.71	1.68	1.19	0.71	76.10
$P_{40\%}$	0.67	1.47	0.98	0.67	62.83
$P_{50\%}$	0.63	1.10	0.69	0.63	44.21

226 *1$=17.8 L.E. (Egyptian pound).
227 ** ECR = cost of diet ($ kg^{-1}) x Feed Conversion Ratio (FCR)
228 Price of SBM=8650 L.E
229 Price of HP-DDG= 7600 LE
230
231
232

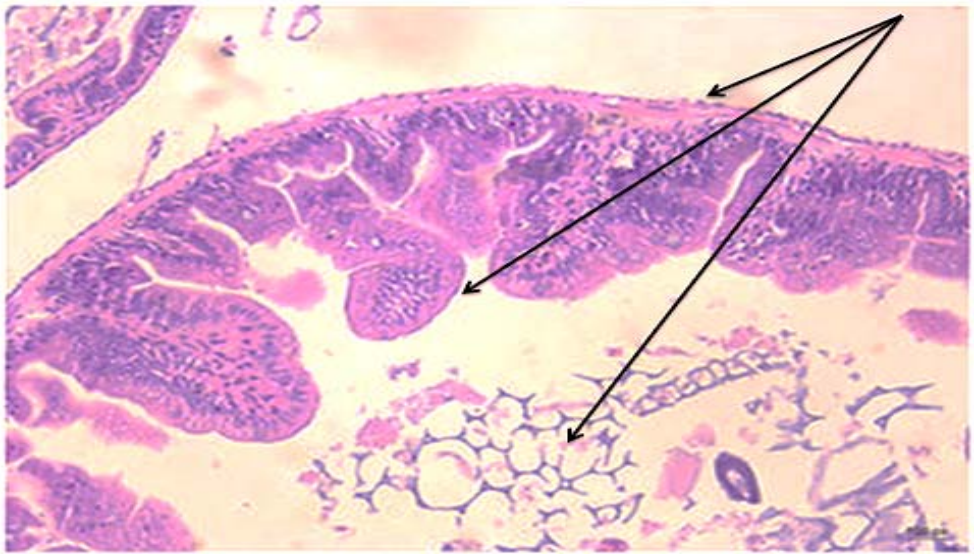

Figure (1): histological section of Intestinal tract of sea bass (**feed on control basal diet**) showing normal, intact intestinal wall and intestinal villi (Arrows) (H&E X10).

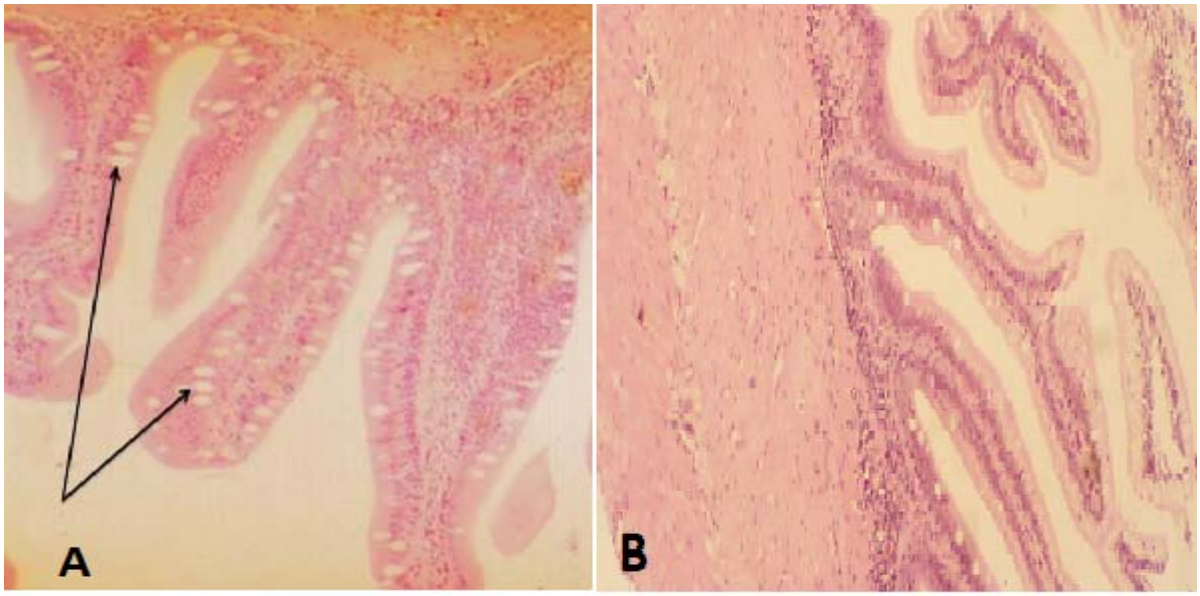

Figure (2): Photomicrograph of sea bass intestine stained with H&E (AX40 and B X20), group (**30%HPDDG +Protease**) showing increase in length and width of intestinal villi and mucosal folds (Arrow) as well as goblet cells (Stars).

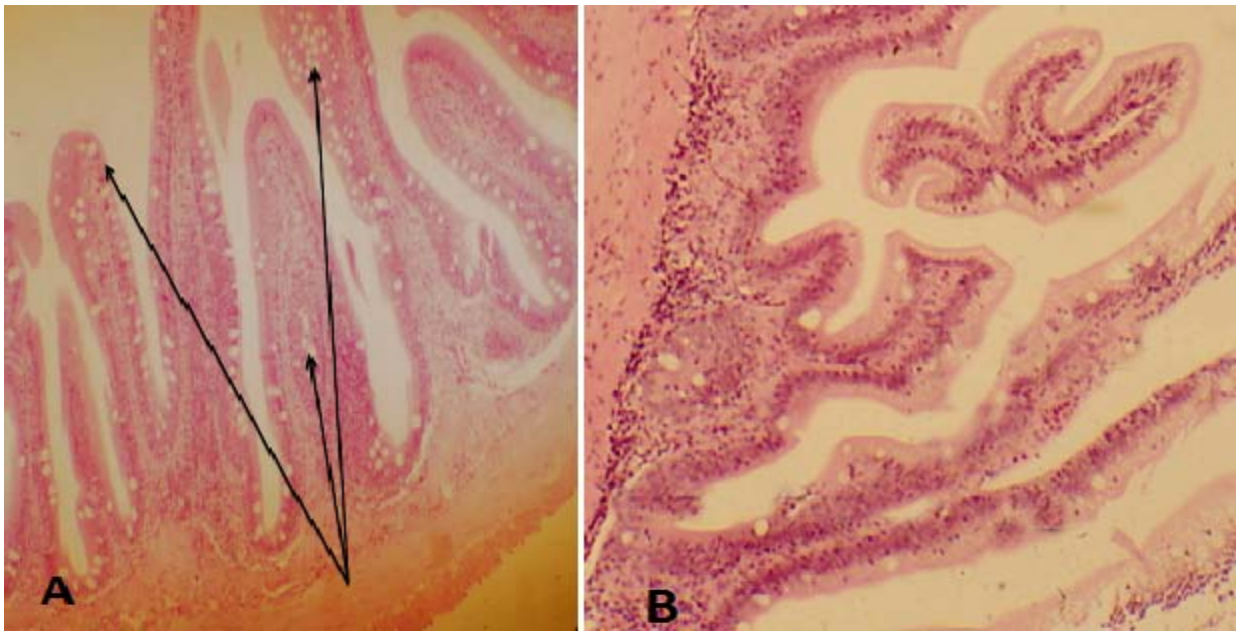

Figure (3). Photomicrograph of sea bass intestine stained with H&E (AX40 and B X20), group (**feed on 40% HPDDG + Protease**) showing increase in length and width of intestinal villi and mucosal folds (Arrow) as well as goblet cells (Stars).

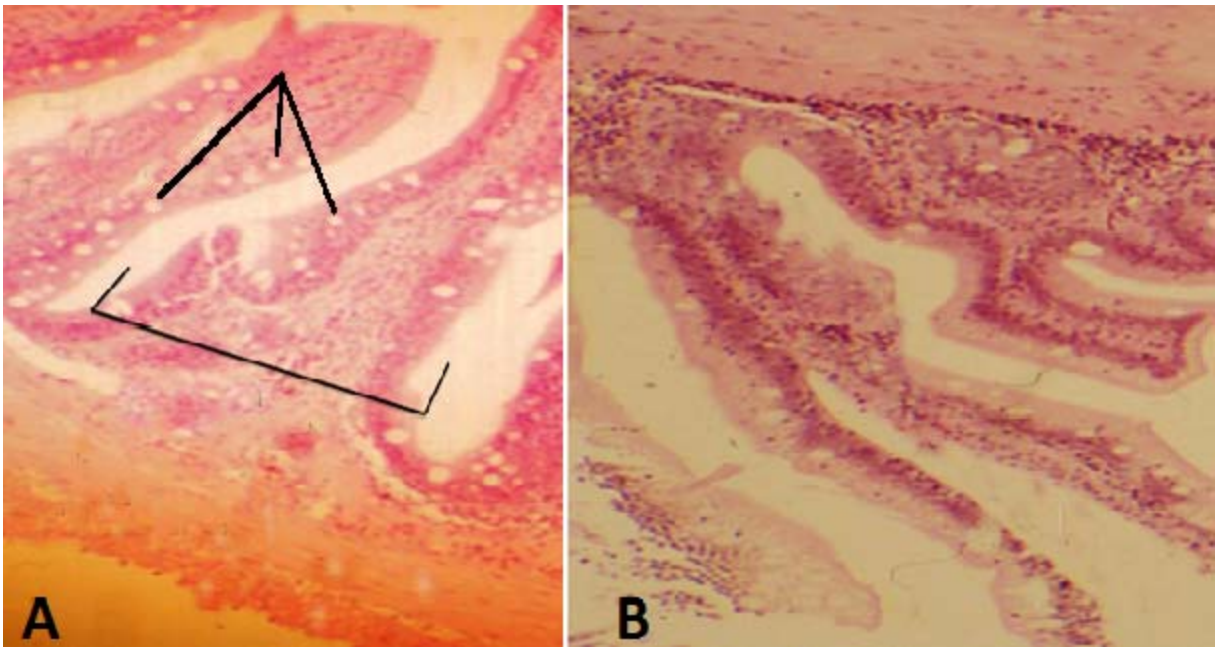

Figure (4). Photomicrograph of sea bass intestine stained with H&E (AX40 and B X20), group (**feed on 50% HPDDG + Protease**) showing increase in length and width of intestinal villi and thickness of intestinal mucosal (Arrow) as well as goblet cells (Star)