Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 23(4): 77- 91 (2019)
www.ejabf.journals.ekb.eg


# European seabass (*Dicentrarchus labrax*) performance, health status, immune response and intestinal morphology after feeding a mixture of plant proteins-containing diets

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## ARTICLE INFO

**Article History:**
Received: Aug. 24, 2019
Accepted: Sept. 30, 2019
Online: Oct. 6, 2019

**Keywords**:
*Dicentrarchus labrax*
European seabass
Plant proteins
Performance
Health status
Immunity
histomorphology

## ABSTRACT

The feasibility of reducing the fish meal (FM) content, as much as possible, in juvenile seabass diets by using a combination of four plant nutrients (plant mix, PX): soybean meal, SBM; soyprotein concentrate, SPC; corn gluten**,** CG and maize flour, MF was investigated . Seabass (mean initial body weight, 4.74±0.04 g) were fed with one of four isonitrogenous and isolipidic diets formulated to progressively reduce FM concentration of control diet (CTRL), by incorporation the PX, to be 30% (FM30 diet), 25% (FM25 diet), and 20% (FM20 diet) over a 13-week growth period. Fish were fed the experimental diets in triplicate and the resulting effects on growth performance, nutrient utilization, biochemical and amino acid composition, major blood constituents, immunity indicators and intestinal histology were evaluated. At the end of the trial, the FM25-fed fish presented growth, feed utilization, and survival rates comparable ($P>0.05$) to the CTRL fish. In addition, this diet did not negatively influenced major blood constituents, immunity indicators or intestinal histomorphology. Further dietary FM reduction to 20% (FM20 diet) and elevation of PX inclusion level (~ 59%) have caused a significant ($P<0.05$) decline in final fish weight, haemoglobin, erythrocyte, leukocyte and lymphocyte counts, elevated leukocytes phagocytic-activity and caused some abnormalities in the proximal intestine (PI) morphological features, but did not negatively impacted haematocrit value, mean erythrocyte cell volume (MCV), mean cellular haemoglobin content (MCH), mean cell haemoglobin concentration (MCHC), monocytes, eosinophils, serum total protein and lysozyme activity. Based on these results, the FM25 formulation (containing approximately 54% PX) can be considered the appropriate for reducing dietary FM down to 25% without compromising the performance, health, immunity or PI morphology of juveniles' seabass.

## INTRODUCTION

Currently, aquaculture production produces almost half of the fish consumed worldwide (FAO 2017), and the aquaculture sector is rapidly expanding to produce a greater supply of essential animal protein to the increasing world population. Generally, fish meal (FM) is the major protein source in aquafeeds, particularly for marine fish species; however, the increased demand for FM, coupled with a

significant shortage in its global production, has created an essential need to search for alternative protein sources for commercial aquafeeds. A wide variety of plant nutrients and economical feedstuffs that can be combined, with essential amino acid supplementation, to form feed products nutritionally similar to marine FM ingredient are being investigated. Based on many previous studies, soy products, such as standard soybean meals (SBM) and soyprotein concentrate (SPC), are reasonably priced high-protein ingredients that are available in large quantities worldwide, thus serving as excellent partial FM substitutes in aquafeeds (Gatlin *et al.* 2007). In the meantime, corn gluten (CG) is considered an appropriate alternative protein source for aqua/fish feeds owing to its high content of available protein (60%–70% DM), lower fiber and anti-nutritional factors, steady supply and competitive price as compared with other plant protein sources (Glencross, 2016). However, there are still apparent and unidentified biologically active compounds in plant products that inhibit the complete replacement of FM in carnivorous fish feeds (Gatlin *et al.*, 2007; Glencross, 2016). Although these plant protein ingredients are among the most commonly used for FM replacement in fish feeds, their use in salmonid, European seabass, gilthead seabream and turbot feeds is limited due to the development of induced inflammation in the distal intestine (enteritis) of these fish (Kokou *et al*., 2015; Rimoldi *et al*., 2016; Bai *et al*., 2019).

European seabass (*Dicentrarchus labrax* L.), (herein seabass) is a strictly carnivorous fish, mainly farmed in the Mediterranean region. Seabass has been regarded as a highly valuable table fish and is considered a delicacy in restaurants. Previous trials have shown that partial replacement of FM by either SBM or SPC individually in seabass diets is feasible (Vásquaz and Cueto 2014; Kousoulaki *et al*. 2015). However, the formulation of fish feeds is more complex with time. As no single plant protein will, by itself, be able to replace FM, the composition must be multi-ingredients to optimize the nutritional value of feed components. Kaushik *et al*. (2004) noted that almost total replacement of FM (95%) by a mixture of plant protein sources (a mixture of SBM, corn and wheat gluten meals and rapeseed meal) in seabass feeds was feasible with the supplementation of specific amino acids. Histological characteristics of the fish intestine have been used to analyse functional mechanisms that are helpful in diagnosing intestinal diseases and formulating suitable feeds (Khojasteh 2012). Nevertheless, there have been few studies examining the influence of combined dietary SBM, SPC and/or CG on seabass health status, immune-competence and intestinal histomorphology (Bonaldo *et al*. 2008; Rimoldi *et al*. 2016; Wassef *et al*. 2016, 2019; Bai *et al*., 2019), and the results have not been consistent.

Given the current trends in fish feed formulation, it is important to optimize nutritional inputs without reducing nutrient absorption or utilization to produce FM alternatives at a relatively lower cost than FM. With the expectation that a mixture of primarily SBM, SPC, CG and maize flour (MF) with specific AA supplementation is a promising FM replacement, the main objective of this research is to study the feasibility of reducing FM as much as possible in juvenile seabass diets. This will lower feed costs and future reliance on FM while optimizing growth performance as well as fish quality. The present research describes the resulting effects of a mixture of four plant nutrients (plant mixture, hereafter PX) on seabass growth performance, nutrient utilization, biochemical and AA composition, basal health status, immune response and intestinal histomorphology.

## MATERIALS AND METHODS

### Experimental Conditions

A total of 420 hatchery-bred, healthy juvenile seabass were obtained from the Marine Fish Hatchery of National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt. After transportation to the El-Max Experimental Fish Farm (15 km west of Alexandria), fish were housed in a 40 $m^3$ outdoor concrete raceway filled with filtered well-seawater (originating from the adjacent seashore line) that was continuously aerated via an air blower. Fish were acclimatized to the experimental conditions for two weeks and fed the control diet before the initiation of the feeding trial. Subsequently, 12 homogenous groups of 35 fish, with an initial mean body weight (IBW) of 4.74±0.04 g, were randomly selected, and each group was placed into a 1 $m^3$ blue-coloured and partly shaded concrete tank for each treatment and all treatment experiments were performed in triplicate. Each dietary treatment group was assigned one of the four experimental diets for a 13-week period. The experimental tanks were supplied with continuous flow of well water (32 ppt, 120 L/h) and air (8 L/min) using a flow-through system. Water quality parameters were monitored throughout the whole trial. The temperature and dissolved oxygen were recorded daily, and the pH, total ammonia and nitrite were measured weekly. The average temperature and oxygen values were 21.9±2.4°C and 7.0±0.5 mg/L (Oximeter: YSI, Pro 20, USA) respectively; pH was 7.3±0.1 (pH metre, YSI, Professional Plus), total ammonia was 0.03±0.02, nitrite was 1.80±0.01 mg/L and the natural photoperiod was 14 h light and 10 h dark throughout the feeding trial.

### Diets and Feeding Protocol

Four diets were formulated with the FM ingredient of the control diet (CTRL) to be progressively substituted by PX (SBM, SPC, CG, MF) and reduced to be 30%, 25% and 20% in diets named FM30, FM25 and FM20 respectively, and were fed to the seabass for 13 weeks. To compensate for low levels of some limiting essential amino acids (AA), due to the relatively low fishmeal formulations, diets were supplemented with DL-methionine, L-lysine and additionally with dicalcium phosphate (DCP). Shrimp meal was also added to enhance diet palatability since the inclusion of plant proteins in carnivorous fish feed has been associated with depressed voluntary feed intake levels. The pelleted diets were produced in Fish Nutrition Lab, NIOF, Alexandria, as previously described by Abdel-Mohsen *et al*. (2018).

Fish were hand fed to apparent visual satiety three times a day for 4 consecutive days and fish were fasted the fifth day according to the protocol suggested by Türkumen *et al*., (2012). Feed residues per tank were siphoned out, re-dried and weighed, and the amount of feed consumed was then quantified for each tank. The total number of feeding days throughout the whole trial (91 days) was 72 days. Detailed formulations and proximate diet analyses are presented in Table (1), and diet AA profiles are presented in Table (2).

### Haematology and Immunity Assays

At the end of the experiment, 24 h after the last meal, 5 fish were randomly sampled from each tank (15 fish per treatment) for analysis of the main blood composition constituents and serum immunity indicators. Blood sampling from fish fed the 4 dietary treatments was conducted simultaneously, immediately upon removal from the water. Fish were bled from the caudal vein with a heparinized syringe after light anaesthesia with clove oil (20 mg/L). Blood smears were prepared immediately after

sampling, and red blood cells (RBCs)/erythrocytes were counted using the standard haematological technique (Dacie and Lewis 2006).

Table 1: Formulation and proximate analyses (% DM) of experimental diets, as fed, to European seabass (*D.labrax*).

| Ingredient | Diets (g/Kg) | | | |
|---|---|---|---|---|
| | CTRL | FM30 | FM25 | FM20 |
| Fish meal [1] (FM) | 460 | 300 | 253 | 204 |
| Soya protein concentrate [2] (SPC) | 0 | 55 | 70 | 85 |
| Soybean meal [3] (SBM) | 0 | 110 | 138 | 167 |
| Corn gluten (CG)[4] | 130 | 161 | 180 | 200 |
| Maize flour (MF) [5] | 125 | 80 | 70 | 50 |
| Plant proteins mixture (PX) | 255 | 406 | 458 | 502 |
| Wheat flour [6] | 80 | 85 | 80 | 85 |
| %Total plant protein ingredients | 33.5 | 49.1 | 53.8 | 58.7 |
| Shrimp meal[7] | 75 | 75 | 75 | 75 |
| Fish oil [8] (FO) | 100 | 100 | 100 | 100 |
| Vitamins and Minerals Mix [9] | 30 | 30 | 30 | 30 |
| L-Lysine | 0 | 1 | 1 | 1 |
| DL-Methionine | 0 | 1 | 1 | 1 |
| Dicalcium phosphate | 0 | 2 | 2 | 2 |
| ***Proximate composition (%DM)*** | | | | |
| Crude protein (CP) | 43.95 | 44.24 | 44.16 | 44.08 |
| Total lipids (L) | 16.8 | 15.7 | 15.6 | 16.9 |
| Ash | 16.0 | 15.7 | 14.8 | 14.3 |
| Fiber | 1.30 | 2.1 | 2.6 | 2.9 |
| Nitrogen Free Extract (NFE)* | 14.05 | 13.86 | 15.44 | 13.82 |
| Moisture | 7.9 | 8.4 | 7.4 | 8.0 |
| Gross energy (GE, MJ/Kg) | 194.3 | 190.3 | 192.4 | 194.6 |
| CP/GE ratio | 22.63 | 23.25 | 22.95 | 22.66 |

1, FM Danish 999 LT (67% CP, 9% L)
2, SPC, USA (66% CP, 5.4% L)
3, Local SBM solvent extracted (45.5% CP, 3.1% L)
4, Local product from Maiz (*Zea mays*).
5, 6, Local products
7, Laboratory-made from local fishery undersize shrimps (50% CP, 4% L)
8, FO, Iceland SR, produced from fresh capelin (*Mallotus villosus*), herring (*Clupea harengus*) + and/or blue whiting (*Micromesistius poutassou*)
9, NRC (2011),
* NFE, calculated by difference

Table 2: Amino acid composition (means, % total, n=2) of experimental diets.

| Amino acid (AA) | Diets | | | |
|---|---|---|---|---|
| | CTRL | FM30 | FM25 | FM20 |
| ***Essential amino acids (EAA)*** | | | | |
| Arginine | 7.11 | 6.61 | 5.78 | 5.92 |
| Histidine | 2.17 | 2.73 | 2.65 | 2.38 |
| Isoleucine | 4.23 | 4.21 | 4.21 | 4.09 |
| Leucine | 9.07 | 7.26 | 7.3 | 7.33 |
| Lysine | 6.65 | 8.54 | 8.49 | 8.50 |
| Methionine | 1.59 | 2.71 | 2.51 | 2.61 |
| Phenylalanine | 4.74 | 4.02 | 4.19 | 4.13 |
| Threonine | 3.93 | 4.11 | 4.20 | 4.23 |
| Tryptophan | 1.03 | 0.51 | 0.53 | 0.59 |
| Valine | 4.54 | 4.65 | 4.85 | 4.9 |
| **Total EAA** | **45.06** | **45.35** | **44.71** | **44.68** |
| ***Non essential amino acids (NEAA)*** | | | | |
| Alanine | 6.18 | 6.12 | 6.36 | 5.8 |
| Aspartic acid | 8.43 | 9.34 | 9.59 | 8.96 |
| Cysteine | 1.0 | 0.8 | 0.62 | 0.55 |
| Glycine | 6.36 | 6.99 | 7.11 | 7.32 |
| Glutamic acid | 17.14 | 15.08 | 14.68 | 14.77 |
| Proline | 4.13 | 4.39 | 4.54 | 4.82 |
| Serine | 6.05 | 4.56 | 4.59 | 4.94 |
| Tyrosine | 3.68 | 3.41 | 3.15 | 3.18 |
| Total NEAA | **52.97** | **50.69** | **50.64** | **50.34** |
| Total Free AA | 98.03 | 96.04 | 95.35 | 95.02 |

Standard deviation (SD) values were omitted for table simplification

The mean erythrocyte cell volume (MCV), mean cellular haemoglobin content (MCH) and mean cell haemoglobin concentration (MCHC) were further calculated using the Dacie and Lewis (2006) formulae. The other major blood constituents, namely, haemoglobin (Hb); haematocrit (Hct); white blood cell (WBC)/leukocyte counts; and lymphocyte, granulocyte and monocyte percentages were measured (Svobodova *et al*., 1991).

The differential leucocyte count was carried out using blood smears stained with Wright-Giemsa, and the relative percentage of each leucocyte type was calculated separately. The rest of the blood specimens were left to clot at 4°C, and the coagulated blood samples were centrifuged at 4000 g for 10 minutes; obtainable sera were stored at –80°C for additional immunological assays.

The following immunity indicators were measured: (i) leukocyte phagocytosis activity (Kawahara *et al*., 1991), (phagocytic activity = percentage of phagocytic cells containing yeast cells); (ii) serum lysozyme activity, spectro-photometrically (Stat Lab, Germany) using the turbidimetric assay (Ellis 1990), where a unit of lysozyme activity was defined as the amount of serum causing a reduction in absorbance of 0.001/min; and (iii) total serum protein, by the biuret reaction (Doumas *et al*., 1981) with commercial kits (Pasteur labs, France). All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) and all samples were analysed in triplicate.

**Analytical Procedures**

The biochemical composition of diets and fish were determined according to the standard methodology of the AOAC (2005). Dry matter content was estimated by oven drying at 105°C, crude protein (total N x 6.25) was estimated by using a semi–automatic Kjeldahl method (VELP Scientifica, UDK 126, Italy) after acid digestion, and crude lipid was estimated gravimetrically after extraction with a mixture of chloroform and methanol (2:1 v/v). Ash content was measured by burning samples in a muffle furnace at 550°C for 6 h. The crude fibre was analysed by the acid hydrolysis method, and the nitrogen–free extract (NFE) was calculated by the difference. Gross energy content was estimated based on 23.6, 39.5 and 17.2 KJ/g for protein, lipid, and carbohydrates, respectively.

**Amino Acids**

Individual AA compositions of the experimental diets and fish at the end of the experiment were determined. Samples were hydrolysed using the methane disulphonic acid method (Simpson *et al*., 1976), and high–performance liquid chromatography (HPLC, Shimadzu Model LC–10AT, Japan) analysis was then carried out using the amino acid analyser manual.

**Intestinal Histology**

At the end of the feeding trial, thorough examinations of sections of seabass proximal intestine (PI) were carried out on 9 samples for each of CTRL, FM25 and FM20 dietary treatments (because sections of CTRL and/or FM30 fish were identical). PI samples were fixed in 2.5% glutaraldehyde with 0.1M sodium cacodylate buffer (1:1, v/v; pH 7.2, 3% $Na_2Cl$), then post-fixed in 1% osmium tetraoxide (1% cacodylate buffer, pH 7.2, 0.1M) for one h. The tissues were dehydrated through a graded alcohol series which was then replaced with agar low viscosity resin in increasing concentrations (30 resin: 70 alcohol, 50:50, 70:30) with 12h between each step until samples were in 100% resin. Samples were then placed in beam capsules and embedded overnight at 60°C. Semi-thin sections 1µm were cut on a Leica Ultra-microtome with a diamond knife, stained with toluidine blue and examined by light microscopy (Nikon Phase Contrast Dry, Tokyo, Japan). The

intestinal histomorphology was evaluated according to observations on the following criteria: (1) the shape of enterocytes (E) and their nuclei and cilia (2) goblet cells (GC) and (3) leucocyte infiltration in the lamina propria and submucosa (Khojasteh 2012). Photomicrographs of sections were taken using the Multi-Scan Base version 8.08 (Computer Scanning System Ltd., Warsow, Poland) and NIS-Elements F2.30 version 2.21 (Nikon, Tokyo, Japan) computer programs.

**Data Collection**

**Fish Performance**

At termination of the trial, growth and feed utilization criteria were calculated using the following indicators: weight gain (WG, g) = final body weight (FBW)– IBW; specific growth rate (SGR, %/d) = 100 (ln FBW–ln IBW) /t; daily growth index (DGI, g /fish/d) = 100 $[(FBW)^{1/3} - (IBW)^{1/3}]/t$; where FBW and IBW are the final and initial fish weights (g), respectively, and "t" is the time of the experiment (days); feed conversion ratio (FCR) = dry feed consumed (g)/fish weight gain (g); protein efficiency ratio (PER) = 100 (fish weight gain, g)/(protein intake, g); protein productive value (PPV) = 100 (protein gain, g)/protein fed (g); and survival (S, %) = the number of fish that survived at end of the experiment/the total number of fish present at the beginning of the experiment.

**Somatic Indices**

At the end of the trial, 5 fish were randomly removed from each tank (15 per treatment) for measurement of biometric indices. The total fish weight (TW, g) and length (L, cm) of each fish were recorded, and then the liver, viscera and intestine were carefully excised and weighed separately. The hepatosomatic index (HSI), viscerosomatic index (VSI), relative gut length (RGL), and condition factor (K) were all calculated. HSI and VSI were calculated as the proportion of either relative liver or viscera weight (g) to total body weight (g), and RGL was the proportion of gut length (cm) to total fish length (cm); Fulton's condition factor K equalled 100 x (TW, g /$L^3$ cm).

**Statistical Analyses**

All data are presented as the mean ± standard deviation (SD). Data were subjected to the Shapiro–Wilk test and univariate procedure, and the results indicated that the data were distributed normally (Shapiro–Wilk test, WC 0.90). All data expressed as percentages were subjected to arcsine square root transformation. One–way ANOVA was then used to determine differences among treatment mean values (n=3, unless otherwise stated) at the 5% significance level. Means were compared by Duncan's multiple range tests. All statistical analyses were performed using the SPSS 21 software package for Windows (ver16.0; SPSS, Chicago, IL, USA).

## RESULTS

**Growth, Feed Utilization and Somatic Indices**

In absolute terms, the level of individual essential amino acid (EAAs) in the low FM/PX diets was comparable and apparently similar to the corresponding levels in the CTRL diet, except for lysine and methionine which are slightly higher and for leucine which is lower than the corresponding's in CTRL diet (Table 2). Nevertheless, the total EAA contents of the PX diets (44.68–45.35%) were similar to that of the CTRL diet (45.06%). Besides, the total non-essential amino acid (NEAA) contents of the PX diets (50.34-50.69%) were slightly lower than that of the CTRL diet (52.97%).

After 13 weeks of feeding seabass PX diets, almost all measured growth performance criteria were unaffected (P>0.05) by reducing FM level, except for FBW, which was significantly (P<0.05) decreased by 2.5% at the lowest FM level (in FM20 diet) compared to the CTRL fish (Table 3). Under the prevailing conditions, all diets were equally consumed by fish during the whole growth trial, and average daily feed intake (DFI, % BW) ranged from 4.31–4.58%, with no significant difference among dietary treatments. Based on the results of growth and feed utilization only, the FM20 diet, which contained 25% FM (approximately 54% PX) and was supplemented with lysine and methionine, was efficiently utilized by juvenile's seabass without affecting growth performance, except for final weight, or feed utilization efficiency.

Table 3: Growth and feed utilization indices (mean ±SD) of seabass (*D. labrax*) fed Low-fish meal (FM)-diets for 13 weeks

| Parameter | Dietary treatments | | | |
|---|---|---|---|---|
| | **CTRL** | **FM30** | **FM25** | **FM20** |
| Initial body Weight (IBW, g) | 4.79±0.12 | 4.72±0.06 | 4.72±0.04 | 4.71±0.04 |
| Final body Weight (FBW, g) | 22.82a±0.20 | 22.7ab±0.1 | 22.32ab±0.33 | 22.25b±0.13 |
| Weight gain (WG, g) | 17.92±0.34 | 17.97±0.04 | 17.60±0.30 | 17.54±0.15 |
| Daily growth index (DGI, g/fish/d) | 1.28±0.01 | 1.28±0.01 | 1.26±0.01 | 1.26±0.01 |
| Specific growth rate (SGR, %/d) | 1.73±0.03 | 1.74±0.01 | 1.72±0.01 | 1.72±0.02 |
| Percentage weight gain (PWG, %) | 374.1±11.6 | 380.6±4.0 | 372.6±4.7 | 372.2±6.1 |
| Feed intake (%BW/d) | 4.58±0.04 | 4.35±0.35 | 4.34±0.27 | 4.31±0.32 |
| Feed conversion ratio (FCR) | 1.59±0.02 | 1.59±0.09 | 1.54± 0.07 | 1.49±0.08 |
| Protein Efficiency Ratio (PER, %) | 1.00±0.00 | 1.00±0.01 | 1.33±0.58 | 1.33±0.57 |
| Protein Productive Value (PPV, %) | 29.88±0.61 | 30.31±1.77 | 31.27±1.35 | 32.45±1.44 |
| Survival (S, %) | 95.0±5.00 | 95.0±5.00 | 96.7±2.90 | 95.0±8.10 |
| Condition Factor (K) | 1.09±0.13 | 1.08±0.04 | 1.08±0.03 | 1.08±0.07 |
| Hepato-somatic index (HSI) | 2.35±0.66 | 1.81±0.58 | 1.90±0.60 | 2.00±0.67 |
| Viscero-somatic index (VSI) | 8.18±2.30 | 7.24±2.06 | 7.44±1.70 | 6.90±1.84 |
| Relative gut length (RGL) | 0.91ab±0.17 | 0.85b±0.11 | 0.97ab±0.11 | 1.00a±0.15 |
| % feed cost-reduction | 0 | 11.52 | 17.50 | 23.48 |

Means in the same row with different letters are significantly different (P <0.05).

## Biochemical and Amino Acid Composition of Fish

At the end of the feeding trial, the final body composition of the fish was significantly altered by diet composition. Fish fed either the FM25 or FM20 diet showed a significant reduction (P<0.05) in protein content compared to CTRL- or FM30-fed fish (by approximately 4.5% or 3.8%, respectively, vs CTRL fish) (Table 4). A concomitant significant increase in lipid content was also noted in the FM20- and FM25-fed fish relative to the corresponding CTRL- or FM30-fed fish, which presented the lowest value (6.9% lower than CTRL) among all treatments. Ash content presented significantly (P<0.05) the highest value in FM25 fish among all treatments, whilst the other values were comparable to that of CTRL fish. Moisture content was significantly higher in all fish fed the PX diets than in CTRL fish, with no detectable variation trend (Table 4).

The AA composition of seabass fed the PX diets for 13 weeks is also presented in Table (4). For FM30-fed fish, only the isoleucine level was significantly (P<0.05) decreased compared to that of CTRL fish. In FM25- or FM20-fed fish, the levels of six EAAs (arginine, histidine, isoleucine, leucine, lysine and tryptophan) were reduced relative to the CTRL fish. Further changes in the concentrations of individual NEAAs were observed in seabass fed the PX diets relative to the corresponding fish fed the CTRL diet.

Table 4: Biochemical (mean ±SD, % wet weight) and amino acid composition (% total) of seabass (*D. labrax*) fed low-fish meal (FM)-diets for 13 weeks

| Parameter | Initial | Final | | | |
|---|---|---|---|---|---|
| | | CTRL | FM30 | FM25 | FM20 |
| Protein | 17.32±0.25 | 19.06a±0.35 | 18.40a±0.35 | 18.34b±0.32 | 18.21b±0.36 |
| Lipids | 7.54±0.38 | 8.38b±0.15 | 7.80c±0.06 | 8.40ab±0.42 | 8.41a±0.15 |
| Ash | 5.28±0.16 | 4.99b±0.31 | 4.94b±0.17 | 5.28a±0.10 | 4.88b±0.25 |
| Moisture | 68.23±0.52 | 67.10b±0.46 | 68.33a±0.32 | 67.40ab±0.26 | 67.93ab±0.06 |
| ***Essential amino acids (EAA)*** | | | | | |
| Arginine | | 6.48a±0.09 | 6.39a±0.08 | 6.27b±0.03 | 6.13c±0.02 |
| Histidine | | 2.94a±0.05 | 2.75ab±0.05 | 2.68b±0.13 | 2.55c±0.09 |
| Isoleucine | | 4.38a±0.06 | 4.29b±0.02 | 4.24b±0.02 | 4.11c±0.03 |
| Leucine | | 7.41a±0.03 | 7.35ab±0.02 | 7.32b±0.07 | 7.36ab±0.03 |
| Lysine | | 8.74a±0.13 | 8.68a±0.03 | 8.53b±0.04 | 8.52b±0.02 |
| Methionine | | 2.60±0.26 | 2.59±0.02 | 2.54±0.03 | 2.63±0.05 |
| Phenylalanine | | 4.20±0.10 | 4.13±0.03 | 4.20±0.10 | 4.15±0.03 |
| Threonine | | 4.28±0.04 | 4.30±0.03 | 4.22±0.06 | 4.26±0.05 |
| Tryptophan | | 0.61a±0.03 | 0.58a±0.03 | 0.53b±0.02 | 0.62a±0.01 |
| Valine | | 4.90±0.17 | 4.83±0.03 | 4.81±0.06 | 4.92±0.04 |
| **Total EAA** | | **46.54±0.09** | **45.89±0.04** | **45.34±0.05** | **45.25±0.05** |
| ***Non essential amino acids (NEAA)*** | | | | | |
| Alanine | | 6.23b±0.04 | 6.80a±0.26 | 6.38b±0.02 | 6.25b±0.04 |
| Aspartic acid | | 9.28a±0.03 | 9.15b±0.03 | 9.12b±0.01 | 8.47c±0.02 |
| Cysteine | | 0.73a±0.03 | 0.67b±0.02 | 0.54c±0.03 | 0.56c±0.03 |
| Glycine | | 7.32a±0.09 | 7.29ab±0.05 | 7.18b±0.03 | 7.34a±0.05 |
| Glutamic acid | | 14.26ab±0.04 | 14.18b±0.05 | 14.15b±0.03 | 14.60a±0.36 |
| Proline | | 4.12±0.03 | 4.11±0.10 | 4.16±0.03 | 4.18±0.04 |
| Serine | | 4.50±0.26 | 4.53±0.53 | 4.49±0.03 | 4.47±0.10 |
| Tyrosine | | 3.12b±0.02 | 3.11b±0.04 | 3.18a±0.10 | 3.21a±0.03 |
| **Total NEAA** | | 49.56±0.06 | 49.84±0.14 | 49.2±0.03 | 49.08±0.08 |
| **Total Free AA** | | 96.1±0.08 | 95.73±0.09 | 94.54±0.08 | 94.33±0.07 |

Means in the same row with different letters are significantly different ($P < 0.05$)

## Haemato-immunological Parameters

At the termination of the feeding period, the blood biochemistry of the juveniles' seabass was affected by the composition of the provided diets (Table 5). Feeding seabass PX-incorporated diets for 13 weeks significantly ($P<0.05$) altered their main blood composition constituents (count, level or ratio) in comparison with those of the CTRL fish (Table 5).

No significant differences ($P>0.05$) were found in the haemoglobin (Hb) content among fish fed the PX-diets up to 53.8% (CTRL, FM30, FM25); however, with the highest PX level of 58.4% (in FM20), the Hb level was 23.8% lower than that in the CTRL fish. Similarly, both RBC and WBC counts were significantly lower in FM20 fish than those in the CTRL, FM30 and FM25 fish.

With respect to erythrocyte indices, such as Hct values, the MCV, MCH and MCHC varied nonsignificantly ($P>0.05$) among treatments. The inclusion of PX in seabass diets at the highest FM-replacement level further resulted in a significantly lower lymphocyte percentage than that of fish fed the CTRL diet, whereas monocyte and eosinophil percentages remained unchanged with the different diets.

As for serum analysis, total protein and lysozyme activity remained unaltered in fish receiving the PX diets, whereas leukocyte phagocytosis was remarkably higher (by 3.4-fold) in the FM20-fed fish than in the CTRL fish (Fig. 1).

Table 5: Major blood constituents (mean±SD) of seabass (*D. labrax*) fed low-fish meal (FM)-diets for 13 weeks.

| Parameter | Dietary groups | | | |
|---|---|---|---|---|
| | CTRL | FM30 | FM25 | FM20 |
| Hemoglobin concentration. (Hb, g/dL) | 12.77ab±0.25 | 11.43ab±1.08 | 13.00a±0.40 | 9.73b±2.15 |
| Hematocrit value (Hct, %) | 41.67ab±3.79 | 36.00b±5.00 | 46.33a±5.51 | 32.67b±6.03 |
| Erythrocytes count ($x10^6$/L) | 4.69a±0.18 | 3.80b±0.53 | 4.60a±0.20 | 3.20b±0.26 |
| MCV (FL)* | 88.87±5.20 | 94.83±4.20 | 100.57±9.00 | 101.60±11.51 |
| MCH (pg)* | 27.27±0.50 | 30.23±1.50 | 28.27±1.04 | 30.17±4.38 |
| MCHC (mg/dl)* | 30.80±2.17 | 31.93±1.85 | 28.27±2.80 | 29.67±2.93 |
| Leucocytes count ($S×10^3$/L) | 31.33b±6.03 | 41.33ab±7.09 | 31.67b±5.69 | 47.67a±3.51 |
| Lymphocytes (%) | 32.33c±3.06 | 44.67a±2.52 | 36.00b±2.65 | 42.33ab±2.08 |
| Monocytes (%) | 3.67±1.53 | 4.67±2.52 | 5.33±3.21 | 5.67±2.08 |
| Eosinophils (%) | 0.67±0.58 | 1.00±1.00 | 1.00±1.00 | 1.33±0.58 |

Means in the same row with different letters are significantly different (P <0 .05).
*MCV, mean cell volume; MCH, mean cellular haemoglobin; MCHC, mean cellular haemoglobin concentration.

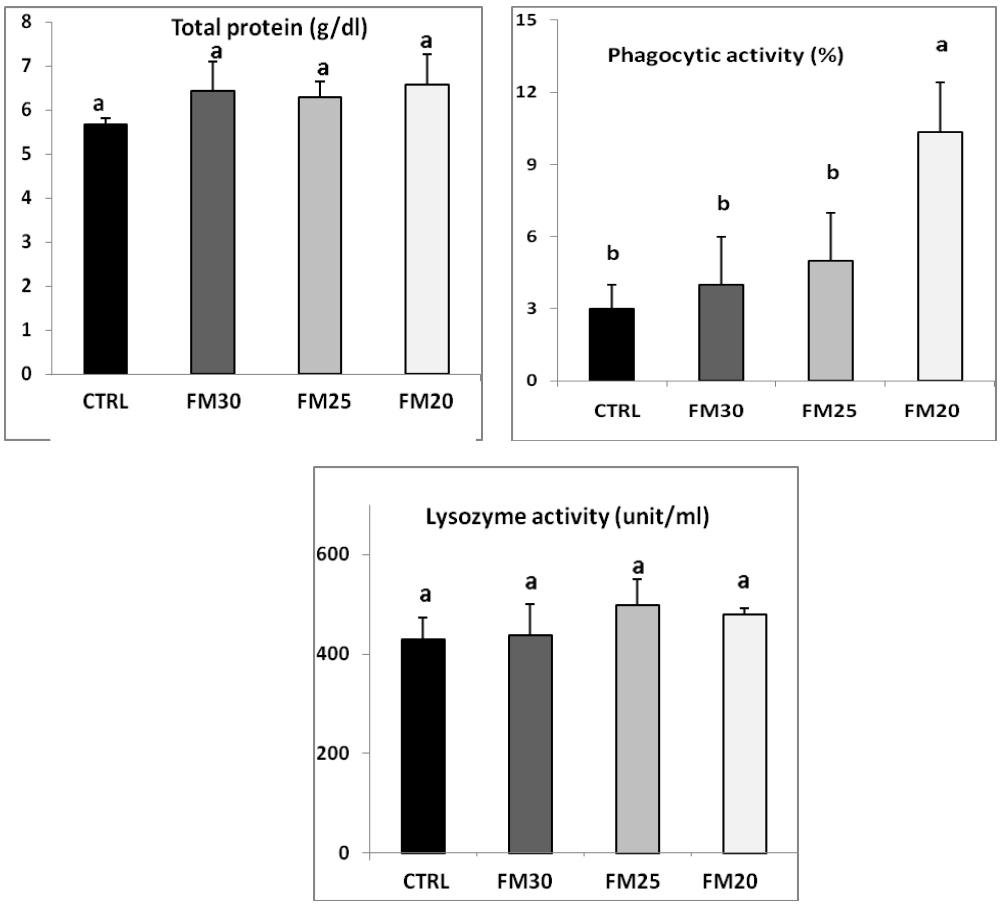


Fig. 1: Serum immunity indicators of seabass (*D.labrax*) fed low fishmeal (FM)- diets for 13 weeks.

## Proximal Intestine (PI) Histomorphology

Photomicrographs of PI semi-thin histological sections from juveniles' seabass following 13 weeks of feeding PX diets for CTRL, FM25 and FM20 fish are presented in Fig. 2 (A-C). Both fish fed the CTRL or FM25 diet showed normal overall morphology of the intestinal wall with normal long cilia at the top of the columnar-shape enterocytes (E) and the amongst very large goblet cells (GC) (Fig. 2A & 2B). No histological abnormalities were observed in the enterocytes structure or shapes, which aligned on basal lamina (BL) and appeared with one elongated nuclei (N) located below the middle of the cell and having one centric nucleolus.

However, further inclusion the dietary PX increase to 58.4% in the FM20 diet had a negative impact on some PI morphological characteristics. Deformed enterocytes with hyperplasia (HP) appearing, have many nucleoli (necrosis), sloughed intestinal cilia on the brush border of lumen, relative reduction in GCs size concomitant with many intra-epithelial leucocytes infiltration were apparent (Fig. 2C) in comparison to CTRL fish section. Consequently, these overall criteria may indicate tissue inflammation in seabass PI at the lowest FM dietary level (20%).

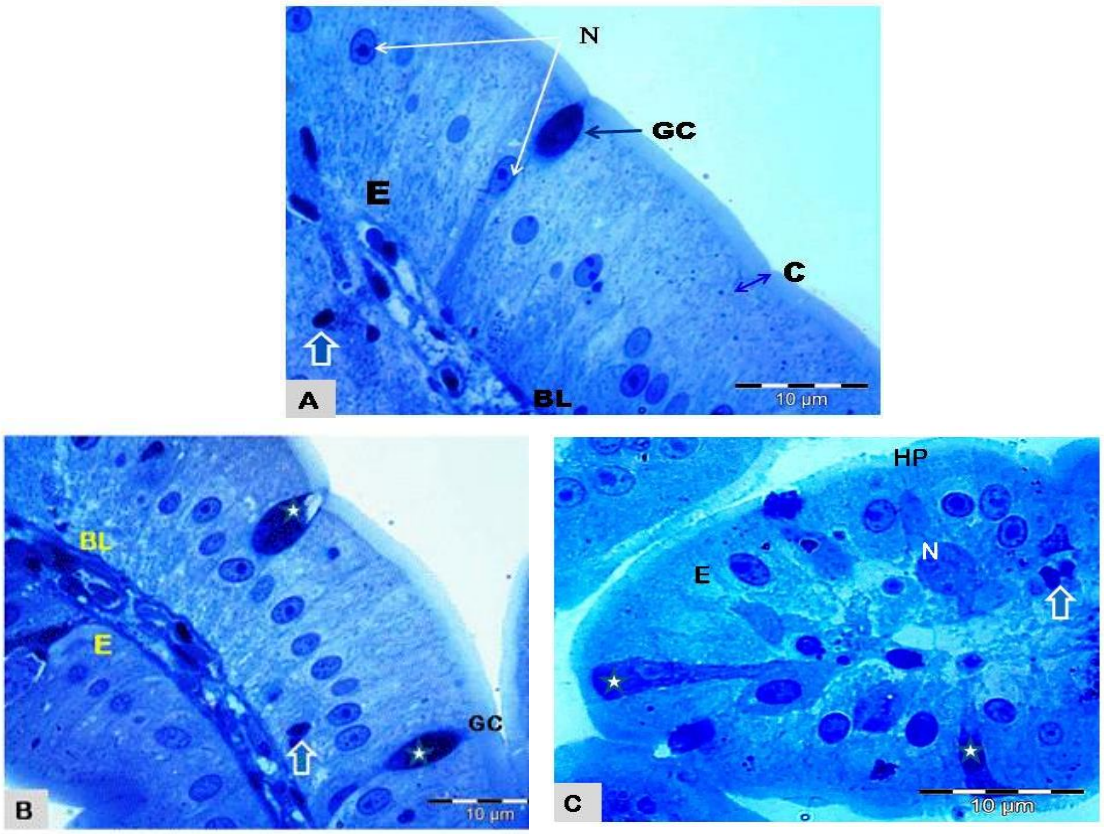


**Fig 2 A-C.** Semi-thin sections in proximal intestine of the seabass:

(**A**) fish fed the CTRL diet, showing normal columnar-shape enterocytes (E) aligned on basal-lamina (BL) with one elongated nuclei (N), normal long cilia at the top (C) and a secretion goblet cell (GC) amongst enterocytes, also a mucosa housing numerous baso-lateral lymphocytes (thick arrows);

**(B)** fish fed the FM25 diet showing normal appearance of enterocytes (E) aligned on basal lamina (BL), normal cilia (C), and very large goblet cells (GC) "stars" and intraepithelial leucocytes infiltration (thick arrows);

**(C)** fish fed the FM20 diet, illustrating that epithelia cells aligned on basal lamina (BL), abnormal enterocytes of columnar shape (E) with hyperplasia appearing (HP), have many nucleoli (N) arranged randomly in many levels, necrosis and sloughed for intestinal cilia (C), small goblet cells (GC) "stars" and many intra-epithelial leucocytes infiltration (thick arrows).

[Specimen were fixed with (F4 G1 stained with *Toluidine blue*) *Scale bar* = 10 µm] .

## DISCUSSION

Seabass is strictly a carnivorous fish species, and a large amount of studies on FM replacement with plant-based products or low FM diets have been conducted (e.g., Kaushik *et al*. 2004; Tibaldi *et al*. 2006; Bonaldo *et al*. 2008; Rimoldi *et al*. 2016, Ludec *et al*., 2018 among others). The results of these investigations showed that up to a 40% substitution rate of soybean product for dietary FM protein with the addition of supplemental AAs or other additives, such as soy lecithin, taurine and sodium butyrate was well tolerated by seabass. In carnivorous fish, feeds with a high vegetal content may lead to a reduction in survival and growth as well as low feed use efficiency. Poor growth results of seabass fed plant-protein diets have been linked to a reduction in voluntary consumption of the diet (Kaushik 2002). Additionally, the reduction in growth may be caused by a limiting supply of sulphur AAs and the scarcity of certain minerals (Tibaldi *et al*., 2006). In the present study, all PX diets were well consumed, even at the highest FM substitution level (FM20 diet), by the seabass, and voluntary feed intake was similar for all dietary treatments. This may be due to the use of shrimp meal as an appetite enhancer and diet enrichment with methionine and lysine. Therefore, juveniles' seabass showed good growth performance and feed efficiency when fed the PX diets, even at the highest inclusion level (approximately 59%). It was previously demonstrated that a 95% FM replacement by plant feedstuffs in the diets of seabass did not affect fish growth, diet digestibility or voluntary feed intake (Kaushik *et al*., 2004). However, in that study, the authors did not perform intestinal histomorphology examination. In the present study, the high survival (95~97%) and good overall health condition of the fish during the entire trial suggest the absence of any nutrient deficiency. The observed growth and survival rates of the juveniles' seabass were consistent with prior investigations on the species (reviewed by Vázquez and Cueto 2014; Kousoulaki *et al*., 2015). However, to gain a better picture of the effects of partial FM substitution by PX and determine its appropriate inclusion level, further analyses on fish health and intestinal histomorphology were conducted.

The unaltered organo-somatic indices, which are not influenced by diet composition of fish, indicated that the physiology of seabass was not altered when fed diets with 58.4% plant feedstuffs (Table 1). The obtained values of the seabass condition factor were similar to those previously reported by other researchers and reviewed by Kousoulaki *et al*., (2015) for the species. The RGL indicated that the utilization of experimental PX diets did not induce significant gut length alterations in the juveniles' seabass. These findings are contrary to those of Tibaldi *et al*., (2006) who observed that all biological indices were significantly impacted when seabass were fed soya protein diets. The difference between the results of the studies is probably due to the size of the seabass studied since Tibaldi *et al*., (2006) used 300 g fish while our study fish were smaller (IBW) of 4.74±0.04 g, indicating that larger-sized seabass of are more sensitive to diets with high levels of soy-based products. Additionally, the present study's findings regarding fish proximate composition contradict those of Bonaldo *et al*., (2008) who observed that seabass carcass composition was not influenced by the inclusion of 30% dietary soybean meal. Furthermore, the variations in some seabass NEAA levels at the end of the trial are less important than variations in EAA levels. Fish require specific concentrations of each individual EAA but not NEAAs, which can be synthesized by the fish (Kaushik 2002).

Haematological parameters are good indicators for fish health status (Faggio *et al*., 2014, Peres *et al*., 2014; Fazio *et al*., 2018). Normal ranges for blood parameters in seabass have been established in different studies carried out on wild and farmed fish subjected to different experimental conditions (Buscaino *et al*., 2010; Wassef *et al*., 2016, 2019, Abdel-Mohsen *et al*., 2018). In the present study, there were signs of impaired physiological condition caused by the highest dietary PX formulation, resulting in decreased Hb levels, RBC and WBC counts, which may indicate a stressful condition, in seabass fed the FM20 diet compared to the CTRL fish. Moreover, serum total protein (albumin and globulin) levels and lysozyme and phagocytic activities are also thought to be related to a stronger innate immune response in fish, and seabass are no exception (Scapigliati *et al*., 2002, Villegas and Mulero 2014). Our results showed that the measured serum immunity indicators were not significantly influenced by diet composition, except for the elevation of phagocytic activity in FM20 fish relative to the CTRL fish. These findings indicate that reducing dietary FM to 25% (diet FM25) has no negative effects on immunity competence of seabass, as the non-specific defence mechanisms play a key role in maintaining effective disease resistance to a variety of fish pathogens (Villegas and Mulero, 2014).

Intestinal morphology affects the physiology and metabolism of nutrient absorption (Khojasteh, 2012). Proximal portion of fish intestine (PI) is credited with higher nutrients digestibility and absorption and overall digestive capacity (Krogdahl *et al*., 2003). It is well documented that the anti-nutritional factors present in soybean products and/or CG can cause moderate or severe enteritis in European seabass (Cuoto *et al*., 2015, Rimoldi *et al*., 2016, Bai *et al*., 2019). In the present study, although no overt inflammation was observed, fish fed the lowest FM level (20%) presented some alterations in their PI morphological structure when compared with fish fed the CTRL or other PX diets (Fig 2A-C). Such an effect may be related to the high inclusion of plant nutrients in the seabass diet (~59% in the FM20 diet), which may induce morphometric changes in the PI, as was previously observed by Rimoldi *et al*., (2016). SPC contains a high level of protein in addition to a high level of non-starch polysaccharides, which are considered indigestible by fish (Koukou *et al*., 2016). Therefore, the inclusion of high levels of soyproducts plus CG and MF in the FM20 diet has caused some modifications in the seabass PI morphological characteristics (Fig 2C). Thus, the PI modifications observed in the present study were likely a consequence of the combination of at least 4 plant ingredients (Table 1) and thus different anti-nutrients in the diet. These results are consistent with the previous study of Leduc *et al*., (2018) that seabass fed a diet containing low FM levels had an altered intestinal mucosa. On the contrary, Couto *et al*., (2015) fed seabass with two purified soybean anti-nutrients, saponins and phytosterols, and observed no severe effects on fish gut histology, supporting the speculation that the deformed PI structure observed in the present study (Fig 2C) may have been due to the effects of different anti-nutrients, from the four plant nutrient sources in the FM20 diet. The presence of slight inflammation in the PI of seabass may be related to the increased sensitivity of this intestinal portion to plant feedstuff anti-nutrients. This criterion may reduce the capacity of the enterocytes lining the epithelium to absorb nutrients (Rimoldi *et al*., 2016). These changes have been referred to as "noninfectious enteritis" in seabass or just generally as "inflammation," characterized in certain seabass by the previously mentioned criteria for Fig (2C). Such intestinal changes likely occur very rapidly and then either continue to deteriorate or stabilize depending on the amount of fish meal/plant proteins in the diet (Rimoldi *et al*., 2016).

## CONCLUSION

Based on the current research findings, the highest but efficiently utilized PX level was approximately 54% (in FM25 diet) which resulted in good seabass rearing performance under the prevailing conditions. Reducing FM content to 25% of the dry diet for seabass in this size, presented similar basal-health status, immune-competence and PI-morphology to those of the CTRL fish and can lower the cost of feed by 17.5%. Further reduction in FM level (to about 20%) negatively affect Hb, RBC counts, WBC counts, lymphocytes and elevated leukocyte phagocytic activity in juveniles' seabass. Moreover, this level of plant proteins (~59%) further induced morphometric changes in the enterocytes of the PI of fish, which may alter nutrient use and absorption.

## ACKNOWLEDGMENTS

The authors are greatly indebted to Dr. N. E. Abdel-Meguid Professor of cell biology, Zoology Dept., Fac. Sciences, Alexandria University and H. H. Abdel-Mohsen for their help in the histology section.

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