Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 26(5): 707 – 727 (2022)
www.ejabf.journals.ekb.eg

Do marine synbiotics decrease estradiol impacts in the early-weaned European seabass (*Dicentrarchus labrax*) larvae?

Ahmed Md. Salem[1, *], M. S. Abokadah[2], N. M. El-Bermawi[2], Nader R. Abdelsalam[3]

1. National Institute of Oceanography & Fisheries (NIOF), Egypt.
2. Animal and Fish Production Department, Faculty of Agriculture,Alexandria University, Egypt.
3. Agricultural Botany Department, Faculty of Agriculture, Alexandria University, Alexandria, Egypt.

[*]**Corresponding Author: am.salem@niof.sci.eg, drsalemniofaqua@gmail.com**

ARTICLE INFO
Article History:
Received: Aug. 4, 2022
Accepted: Sep. 13, 2022
Online: Oct. 7, 2022

Keywords:
Synbiotic,
Estradiol,
Oxidative stress,
Sea bass,
Larvae,
Weaning

ABSTRACT

A European sea bass (*Dicentrarchus labrax*) weaning trial was conducted from the 35th to the 50th dph. Larvae were reared and fed on enriched rotifers and *Artemia* in the same 2 m^2 tank from 3 to 34 dph in NIOF hatchery larval rearing unit. In the present study, the total antioxidant capacity (TAC), superoxide dismutase activity (SOD), catalase (CAT) activity, and some hepatic enzymes in early life stages after exposure to four treatments in triplicates: green water control (G), marine synbiotic (MS), estradiol (E2), MS and E2 mixture (ES) treated micro-diets were investigated. The larval length, growth weight, and survival considerably decreased in the E2 *vs*. the other treatments, mixing MS with E2 in ES treatment increased significantly larvae growth and survival compared to E2 and G control. The total protein significantly decreased in larvae exposed to the E2 treatment compared to the other treatments. Furthermore, albumin was significantly reduced after E2 treatment, while globulin significantly increased after MS treatment. Larvae fed ES showed the highest body glucose in g/l. The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities decreased significantly after E2 treatment compared to all other treatments, while ES treatment increased them significantly compared to E2 treatment. The alkaline phosphatase (ALP) activity increased significantly after MS treatment compared to all other treatments. Triglycerides (TAG) increased significantly after E2 treatment *vs*. the other treatments. The SOD and CAT activities increased considerably, while TAC showed a significant decrease after E2 treatment *vs*. all other treatments. SOD and CAT activities were significantly reduced, while TAC showed a significant increase after ES treatments compared to E2 and G treatments. In conclusion, mixing MS with E2 in ES treatment decreased the negative impacts of E2 in the European sea bass larval growth, survival and antioxidant capacity.

INTRODUCTION

The European seabass (*Dicentrarchus labrax*) is considered one of the aquaculture and fisheries most valuable marine fish species in the Mediterranean Sea (FAO, **2022)**. However, the lack in the fry is still the bottleneck for more aquaculture development **(Verhaegen, 2012)**. The dietary lipids are remarkably an essential source of energy that provides essential fatty acids, phospholipids, sterols, and fat-soluble vitamins needed for

optimum functioning of physiological processes, maintenance of biological structure, and function of cell membranes **(Desouky *et al*., 2020)**. Though sparing protein using high dietary lipid levels improves different fish species growth and feed efficiency **(Abasubong *et al*., 2018)**, yet fatty liver, health and yield reduction resulted in high fat deposition in fish **(Abasubong *et al*., 2018)**. Fish larvae oxidation risks are due to high larval water, long-chain PUFA contents, metabolic rate, and oxygen demand (Betancor *et al*., 2012). Therefore, to avoid lipid peroxidation, adequate larval dietary antioxidant elements are important **(Mourente *et al*., 1999; Betancor *et al*., 2011)**.

Baroiller and D'Cotta (2016) reported that, one sex grows faster than the other, males (tilapia) or females (sea bass). **Wang *et al*. (2008)** stated that finfish monosex female production has increased using estrogens recently. Estrogens controlled 56 different fish species sex differentiation **(Piferrer, 2001)**. Notably, females grow faster than males in the European seabass, largemouth bass (*Micropterus salmoides*), Atlantic halibut (*Hippoglossus hippoglossus*) and black crappie (*Pomoxis nigromaculatus*) females **(Hendry *et al*., 2003; Arslan, 2004; Gorshkov *et al*., 2004)**. **Flynn and Benfey (2007)** postulated that, caviar is the main production advantage of the short nose sturgeon (*Acipenser brevirostrum*). For successfully controlling the juveniles sex, critical factors are determined; namely, hormone dosage, gonad development stage, and hormone treatment. **Piferrer (2001)** observed that, estradiol-17β (E2) hormone may change the sex ratio, and negatively affect the survival and growth. **El-Gerisy and El-Gamal (2012)** revealed in a study using tilapia (*Oreochromis niloticus*) fry that when the sex reversing hormone increased, growth and survival decreased. noted that, endocrine disrupting chemicals were detected in aquatic environments **(Sumpter & Johnson, 2005; Thilagam *et al*., 2010)**. **Thomas-Jones *et al*. (2003)** revealed that, oestrone, E2 and 17a ethynylestradiol (EE2) are considerable estrogenic compounds in aquatic environments, and are efficient for exerting an impact on organisms even at very low concentrations (1 ng L^{-1}). **Maciuszek *et al*. (2020)** demonstrated that, oestrogens modulated the carp (*Cyprinus carpio*) innate immune response, and estradiol induces a direct and indirect immunomodulatory effects. **Livingstone (2001)** detected an oxidative damage in the aquatic organisms caused by xenobiotics ROS production. Remarkably, ROS might act as a biomarker to designate estrogen pollution since the E2 exposed fish liver significantly increase the ROS level **(Thilagam *et al*., 2010)**. **Betancor *et al*. (2012)** reported that enzymes and vitamins E and C, as well as carotenoids are considered the most effective antioxidants used in systems to prevent oxidative damage in fish. **Sayed and Abou Khalil (2016)** results linked hormonal sex reversal with oxidative stress induction. The fish interactive antioxidant system is performed through SOD, CAT, glutathione S-transferase (GST), and GPx **(Hamed *et al*., 2020)**.

Synbiotics, the combination of probiotics and prebiotics, have revealed ameliorated growth, immune and antioxidative influences on fish and shellfish **(Lamari**

***et al*., 2016; Ringø & Seong, 2016; Huynh *et al*., 2017)**. Many additives have been developed to replace or decrease the use of antibiotics in aquaculture, including prebiotics **(Ringø *et al*., 2010; Akrami *et al*., 2013;; Bindels *et al*., 2015; Zaki *et al*., 2015; Akter *et al*., 2016; Widanarni *et al*., 2018)**, , probiotics **(Abu-Elala *et al*., 2013; Salem *et al*., 2015; Lamari *et al*., 2016; Banerjee & Ray, 2017; Tarnecki *et al*., 2019; Ringø *et al*., 2020; Serradell *et al*., 2020)** and synbiotics **(Salem *et al*., 2015; Ringø & Seong, 2016; Azimirad & Meshkini, 2017; Okay *et al*., 2018; Villumsen *et al*., 2020)**.

The current experiment addressed the effects of E2 and MS or their mixture on the European sea bass larvae, and the subsequent effects on larval growth, survival, and antioxidant enzymes.

MATERIALS AND METHODS

Larval rearing and experimental design

Newly hatched larvae of the European seabass were obtained from farmed broodstock induced spawning at the Fish Reproduction and Spawning lab. (N: 31°12'46.2" E: 29°53'06.1"), Marine hatchery, Aquaculture Division, NIOF and stocked in green water flowing through a larval rearing unit. Samples were reared and enriched rotifers and *Artemia* were fed in the same 2 m^2 tank from 3 to 34 dph in the hatchery larval rearing unit. On the 35^{th} dph, triplicate groups of larvae were stocked randomly, and equally as 100 larvae/30 L^3 aquarium in 12 glass aquaria. Weaning experiment was conducted from the 35^{th} to the 50^{th} dph on different dietary treatments. The experimental tanks were subjected to a partial daily water exchange rate of 30% /tank of water. This step was conducted using hose to plankton net covered basket. Aeration extended for 24 hours/day using electric air power. Light was 50-100 lux at water surface on 16 hours light: 8 hours darkness in a light cycle.

Larvae were fed on enriched rotifers, *Brachionus B. plicatilis* and *B. routindiformis* from 2 to 12 dph at 20 rotifers/ml and enriched *Artemia franciscana* (GSL) nauplii for 14 dph starting with 1 nauplii/ml and increasing the density up to 2 nauplii/ml until the 34^{th}dph. *Artemia metanauplii* and treated 100-200 micron Orange® microdiets co-feeding started at 35 dph 6 times/day. From the 40 dph, larvae were fed 4 metanauplii/ml and treated O. Range® with 15g/m^3 until the 43^{th} dph. Then, larvae were fed on 2 metanauplii/ml and treated O. Range® with 30 g/m^3 until the 46^{th} dph. Larvae fed only on O. range® with 45 g/m^3 at 50^{th} dph and the addition of *Artemia* metanauplii stopped. Rotifers and *Artemia* were enriched for 4 and 6 hours at 28ºC, respectively, using DHA SELCO® (INVE, Belgium). Every day, the tanks' bottom was siphoned.

Four weaning microdiets treatments were performed: control green water (Inve O.Range® microdiet without treatment) (G), marine synbiotic (*Bacillus (B.) subtilis* HS1 probiotic bacteria 1x 10^7 CFU + 1 mg chitosan g^{-1} microdiet) treated Inve O.Range®

microdiet (MS), estradiol sex reversing hormone treated Inve O. Range® microdiet (150 mg kg^{-1} microdiet) (E2) and marine synbiotic and estradiol sex reversing hormone treated Inve O.Range® microdiet (ES) treated Inve O.Range® microdiet (E2). The marine synbiotic used in this experiment was developed to contain a mixture of Suez Gulf locally isolated marine bacterial probiotic (*B. subtilis* HS1) **(Salem *et al*., 2015**) and locally extracted marine chitosan prebiotic **(Zaki *et al*., 2015)**, which showed effective results on the European seabass larvae and fry, respectively. Estradiol dose used in this experiment was 150 mg E2 kg^{-1} feed following the method of **Wang *et al*. (2008)**.

Growth performance and feed utilization

Larvae growth and feed utilization were analyzed in terms of length gain (LG, mm), percentage length gain (LG, %), length average daily gain (LADG, mm d^{-1}), specific length growth rate (LSGR, % day^{-1}), weight gain (WG, mg), percentage weight gain (WG, %), weight average daily gain (WADG, mg day^{-1}), specific growth rate (SGR, % day^{-1}), survival (S, %) and condition factor (K).

To measure the parameters, the following formulae were used:
LG (mm) = final length (FL) (mm) – initial length (IL) (mm),
LG (%) = 100 × [(FL – IL)/IL]
LADG (mm day-1) = = FL – IL/ experimental days
LSGR = 100 × [(Ln FL) – (Ln IL)]/experimental days
WG (mg) = final weight (FW) (mg) – initial fish weight (IW) (mg)
WG (%) = 100 x [(FW – IW)/IW]
WADG (mg day^{-1}) = FW – FI/experimental days
WSGR = 100 × [(Ln FW) – (Ln IW)]/experimental days
Survival (S %) = (final fish count/initial fish count) × 100
$K = 100 \times (FW/FL^3)$

Measurement of water quality

Water quality was measured using Hanna® HI9828 portable electric device weekly from the beginning to the end of the experiment at 2 pm. The measurements during the experiments ranged between 18.77 - 21.73 ºC for temperature, 97.13 - 103.03% for dissolved oxygen %, 6.88 - 7.36 for pH, 180.83 - 203.97 Ms/cm for conductivity, 115.20 - 129.90 ppm for total dissolved solids and 36.40 - 36.73 ppt for salinity.

Microbiological measurements

B. (BBC) and *Vibrio (V.)* (VBC) colony forming units (CFU) were done in the Microbiology Lab., Marine Environment Division, NIOF. Serial dilutions of $10^{-2} \pm 10^{-4}$ were made using filtered sterilized sea water. For each water sample, 100 µl was inoculated on sterile plates and incubated at 30°C for 24 - 72 h. Plates of the selective media of sea water agar for BBC and thiosulfate citrate bile salt sucrose agar (TCBS) for

VBC were inoculated with 0.1 ml of the diluted samples, and the different bacterial genera were counted as described in the study of **Salem *et al*. (2015)**.

Measurement of antioxidants biomarkers and enzymes

Reagents used for the different techniques were purchased at Biodiagnostic Company kits, Cairo, Egypt purchased, spectrophotometer model: 01102, LAXCO, Inc., USA for measuring antioxidants and enzymes. Whole larvae CAT and SOD activities and TAC were measured according to protocol given by **Aebi (1984)**, **Nishikimi *et al*. (1972)** and **Koracevic *et al*. (2001)**, respectively. Total protein, albumin, and globulin were assessed in g/l according to biuret method for protein **(Gornall *et al*., 1949)** using modified bromocresol green method **(Doumas *et al*., 1971)** for albumin, and the total globulin fraction was determined by subtracting the albumin from the total protein. Moreover, GOD-PAP enzymatic colorimetric method **(Weissman & Klien, 1958)** was used for glucose. ALP activity was determined using the modified method of **Befield and Goldberg (1971)**; while acid phosphatase (AP) activity was determined using the **Kind and King (1954)** modified method. On the other hand, AST and ALT activities were measured according to the methods of **Murray (1984)**. Furthermore, TAG was determined using the modified method of **Fassati and Prencipe (1982)**. Specific activities of enzymes were calculated by dividing the total enzyme activity on the total protein content.

Histological methodology, samples preparation and fixation

Five larvae from each tank were collected at the end of the weaning trial, fixed in 10% formalin saline for 24 – 48 hours, dehydrated through graded alcohols, then xylene and finally embedded in paraffin wax. Two paraffin blocks containing two larvae per tank were sectioned at 5 μm, and sections were stained with hematoxylin and eosin (H&E) for histopathological evaluations **(Martoja & Martoja-Pearson, 1970)**.

Statistical analysis

All data were subjected to a one-way analysis of variance (ANOVA) at a 95% confidence limit, and means compared by Duncan's test ($P < 0.05$), using SPSS software (SPSS for Windows 24; SPSS Inc., Chicago, IL, USA).

RESULTS

Larval growth and survival

The larvae weaned with MS achieved the best significant ($P < 0.05$) FL, LG, LADG, LSGR, LG% and S%. (Table 1). On the contrary, the larvae weaned in MS achieved non-significant ($P > 0.05$) FW, WG, WADG, WSGR and WG%. While, the

larvae weaned in G displayed inconsequential ($P > 0.05$) K, and the larvae weaned in E2 showed the best (not significant, $P > 0.05$) SK (Table 2).

Table 1. Effects of estradiol (E2) hormone and marine synbiotic treated microdiets on the European seabass (*D. labrax*) larval length growth performance

Treatment	G	MS	E2	SE2
Larval length growth performance between 5dph and 50dph				
5 dph IL mm	**3.35 ± 0.05**	**3.35 ± 0.05**	**3.35 ± 0.05**	**3.35 ± 0.05**
50 dph FTL mm	**10.47^{c} ± 0.15**	**12.03^{a} ± 0.35**	**10.23^{c} ± 0.06**	**11.57^{b} ± 0.15**
Gain mm	**7.07^{c} ± 0.15**	**8.63^{a} ± 0.35**	**6.83^{c} ± 0.06**	**8.17^{b} ± 0.15**
LADG mm/day	**0.16^{c} ± 0.00**	**0.19^{a} ± 0.01**	**0.15^{c} ± 0.00**	**0.18^{b} ± 0.00**
LSGR %/day	**1.89^{c} ± 0.02**	**2.08^{a} ± 0.04**	**1.85^{c} ± 0.01**	**2.03^{b} ± 0.02**
Gain %	**212.50^{c} ± 7.58**	**259.30^{a} ± 13.55**	**205.50^{c} ± 3.20**	**245.31^{b} ± 5.70**
Larval length growth performance between 35dph and 50dph				
35 dph IL mm	**6.07 ± 0.47**	**6.07 ± 0.47**	**6.07 ± 0.47**	**6.07 ± 0.47**
50 dph FTL mm	**10.47^{c} ± 0.15**	**12.03^{a} ± 0.35**	**10.23^{c} ± 0.06**	**11.57^{b} ± 0.15**
50 dph FSTL mm	**9.37^{c} ± 0.49**	**10.40^{a} ± 0.30**	**8.80^{d} ± 0.10**	**9.90^{b} ± 0.36**
Gain mm	**3.93^{b} ± 0.68**	**5.97^{a} ± 0.15**	**4.17^{b} ± 0.51**	**5.50^{a} ± 0.62**
LADG mm/day	**0.26^{b} ± 0.05**	**0.40^{a} ± 0.01**	**0.28^{b} ± 0.03**	**0.37^{a} ± 0.04**
LSGR %/day	**4.27^{b} ± 0.42**	**5.17^{a} ± 0.07**	**4.12^{b} ± 0.37**	**4.92^{a} ± 0.34**
Gain %	**65.12^{d} ± 12.72**	**98.84^{a} ± 9.54**	**69.38^{c} ± 13.43**	**91.53^{b} ± 16.85**
Survival %	**47.67^{c} ± 2.52**	**56.67^{a} ± 1.53**	**42.67^{d} ± 2.52**	**52.33^{b} ± 1.53**

Different letters in the same row are for treatments effects' significance ($P < 0.05$).
LG: length gain, LADG: length average daily gain, LSGR: length specific growth rate, LG: length gain%.
G: green-water control non treated, MS: marine synbiotic treated, E2: estradiol treated and SE2: marine synbiotic and estradiol treated microdiets.

Larval body protein, glucose, and enzymes activities

The larvae fed MS exhibited the highest considerable values ($P < 0.05$) of body total protein in g/l. The larvae fed ES revealed the highest substantial ($P < 0.05$) body glucose in g/l (Fig. 1). The larvae fed MS and ES presented higher consequentially ($P < 0.05$) body globulin in g/l, and larvae fed G recorded high significant value ($P < 0.05$) of body albumin. The larvae fed E2 exhibited the highest considerable ($P < 0.05$) body TAG in mmol/l. Larvae fed E2 exhibited the highest noteworthy ($P < 0.05$) body globulin in mg/mg protein, and larvae fed G revealed the highest considerable ($P < 0.05$) body albumin in mg/mg protein and the larvae fed E2 displayed the highest substantial ($P < 0.05$) body TAG in mmol/g protein (Fig. 2).

The larvae fed MS exposed the highest considerable ($P < 0.05$) body ALP in U/l and in mu/mg protein. The larvae fed ES exhibited the highest substantial ($P < 0.05$) body AP in U/l and in mu/mg protein. While, the larvae fed G exposed the highest considerable ($P < 0.05$) body AST and ALT in U/l and in mu/mg protein (Fig. 3).

Table 2. Effects of estradiol (E2) hormone and marine synbiotic enriched microdiets on the European seabass (*D. labrax*) larval weight growth performance

Treatment	G	MS	E2	SE2
Larval weight growth performance (5 - 50dph)				
5 dph IW mg	**3.25 ± 0.25**	**3.25 ± 0.25**	**3.25 ± 0.25**	**3.25 ± 0.25**
50 dph FW mg	**17.17 ± 4.90**	**21.50 ± 6.68**	**15.37 ± 1.44**	**20.43 ± 7.01**
Gain mg	**13.92 ± 4.65**	**18.25 ± 6.92**	**12.12 ± 1.64**	**17.18 ± 7.13**
WADG µg/day	**309.26 ± 103.34**	**405.56 ± 153.72**	**269.26 ± 36.35**	**381.85 ± 158.34**
WSGR %/day	**2.50 ± 0.354**	**2.75 ± 0.42**	**2.40 ± 0.13**	**2.68 ± 0.43**
Gain %	**422.53 ± 110.76**	**574.19 ± 251.23**	**376.51 ± 78.55**	**535.93 ± 232.21**
Larval weight growth performance (35 - 50dph)				
35 dph IW mg	**6.45 ± 0.05**	**6.45 ± 0.05**	**6.45 ± 0.05**	**6.45 ± 0.05**
50 dph FW mg	**17.17 ± 4.90**	**21.50 ± 6.68**	**15.37 ± 1.44**	**20.43 ± 7.01**
Gain mg	**10.72 ± 4.93**	**15.05 ± 6.67**	**8.92 ± 1.39**	**13.98 ± 7.03**
WADG mg/day	**0.71 ± 0.33**	**1.00 ± 0.44**	**0.59 ± 0.09**	**0.93 ± 0.47**
WSGR %/day	**2.21 ± 0.48**	**2.54 ± 0.51**	**2.10 ± 0.15**	**2.45 ± 0.53**
Gain %	**166.35 ± 76.71**	**233.22 ± 102.77**	**138.14 ± 20.48**	**217.11 ± 110.41**
KTL	**1.51 ± 0.48**	**1.26 ± 0.48**	**1.43 ± 0.11**	**1.31 ± 0.43**
KSTL	**2.17 ± 0.97**	**1.95 ± 0.74**	**2.25 ± 0.16**	**2.08 ± 0.61**

Different letters in the same row are for treatments effects significance ($P < 0.05$).
G: green-water control non treated, MS: marine synbiotic treated, E2: estradiol treated and SE2: marine synbiotic and estradiol treated microdiets.
FW: final weight in mg, WG: weight gain in mg, WADG: weight average daily gain in µg, WSGR: weight specific growth rate in %/day, WG%: total weight gain%, KTL: condition factor of total length and KSTL: condition factor of standard length.

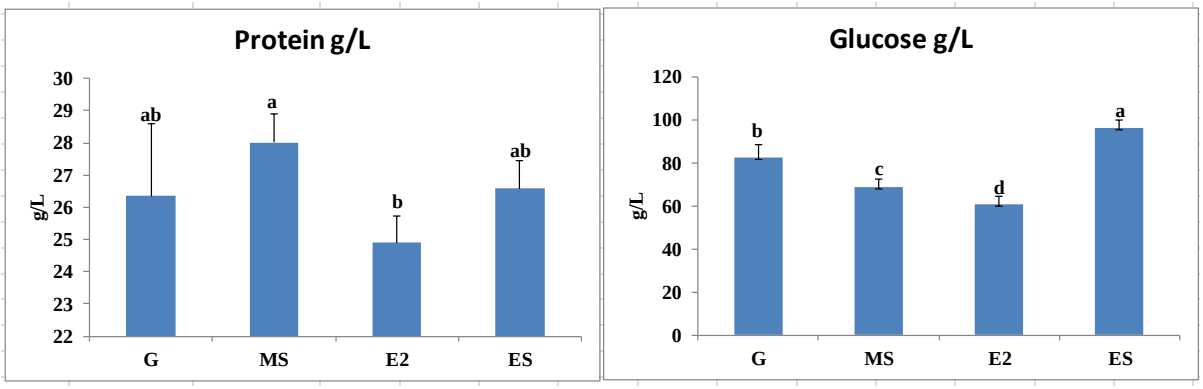

Fig. 1. Effect of estradiol (E2) sex reversing hormone and marine synbiotic treated microdiets on European seabass (*D. labrax*) larval protein and glucose body content
Different letters in the same figureare for treatments effects significance ($P < 0.05$).
G: green-water control non treated, MS: marine synbiotic treated, E2: estradiol treated and SE2: marine synbiotic and estradiol treated microdiets.

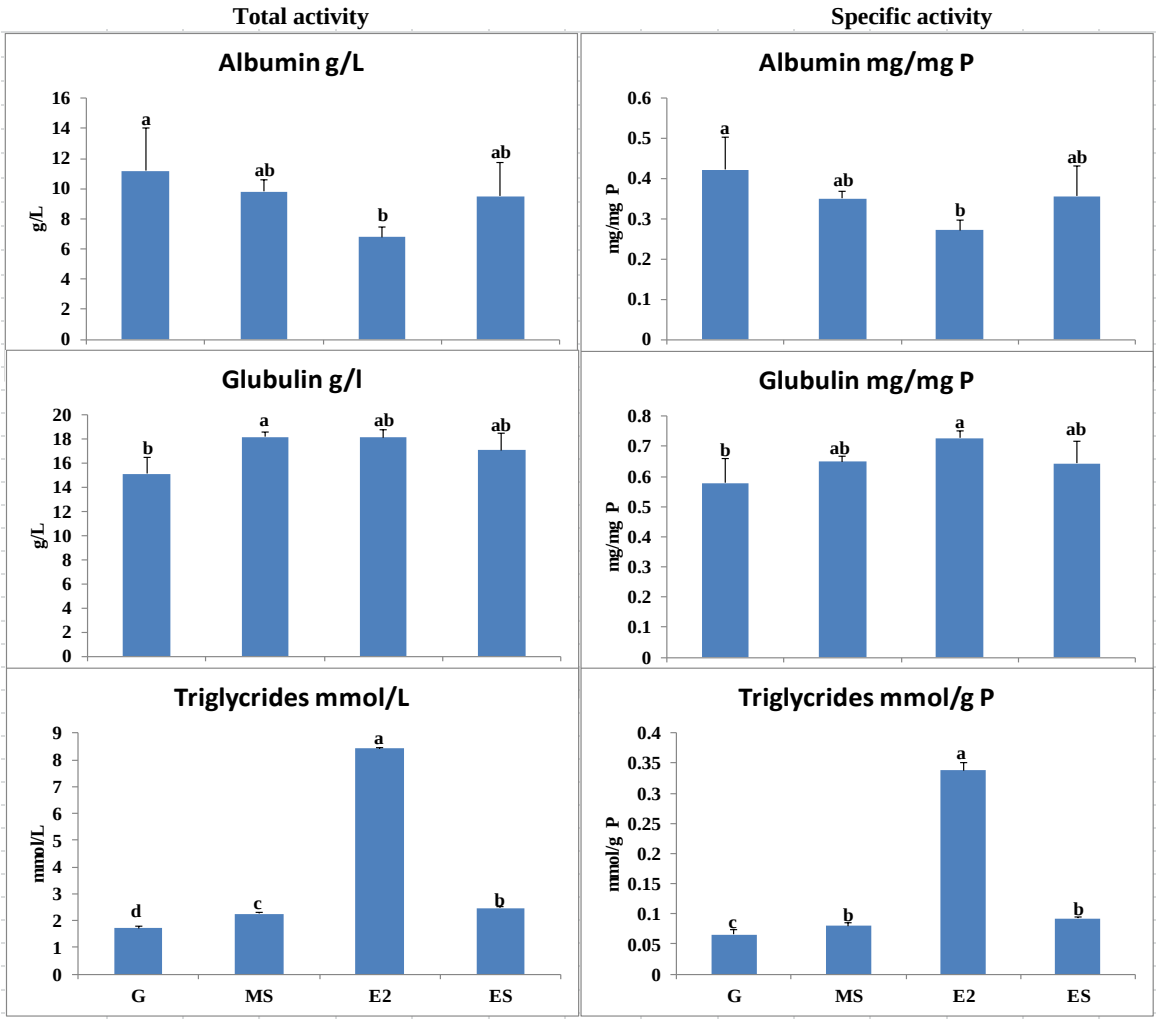

Fig. 2. Effect of estradiol (E2) sex reversing hormone and marine synbiotic treated microdiets on European seabass (*D. labrax*) larval albumin, globulin and triglycerides total and specific activities
Different letters in the same figure are for treatments effects significance ($P < 0.05$).
G: green-water control non treated, MS: marine synbiotic treated, E2: estradiol treated and SE2: marine synbiotic and estradiol treated microdiets.

Antioxidants biomarkers

The current results revealed that the larvae fed E2 displayed high substantial effect ($P < 0.05$) on body SOD and CAT activities in U/g larvae and in U/g protein. In addition, larvae fed MS and ES had the highest considerable ($P < 0.05$) amount of body TAC in in Mmol/g protein (Fig. 4).

Bacterial accounts

The *V.* sp. was not noticed in the rearing water samples or in larvae of the four treatments. MS administration revealed significant ($P < 0.05$) effect of *Bacilli* counts in larvae. The ES larval rearing water achieved the highest significant ($P < 0.05$) *B.* sp. bacterial counts results (Table 3).

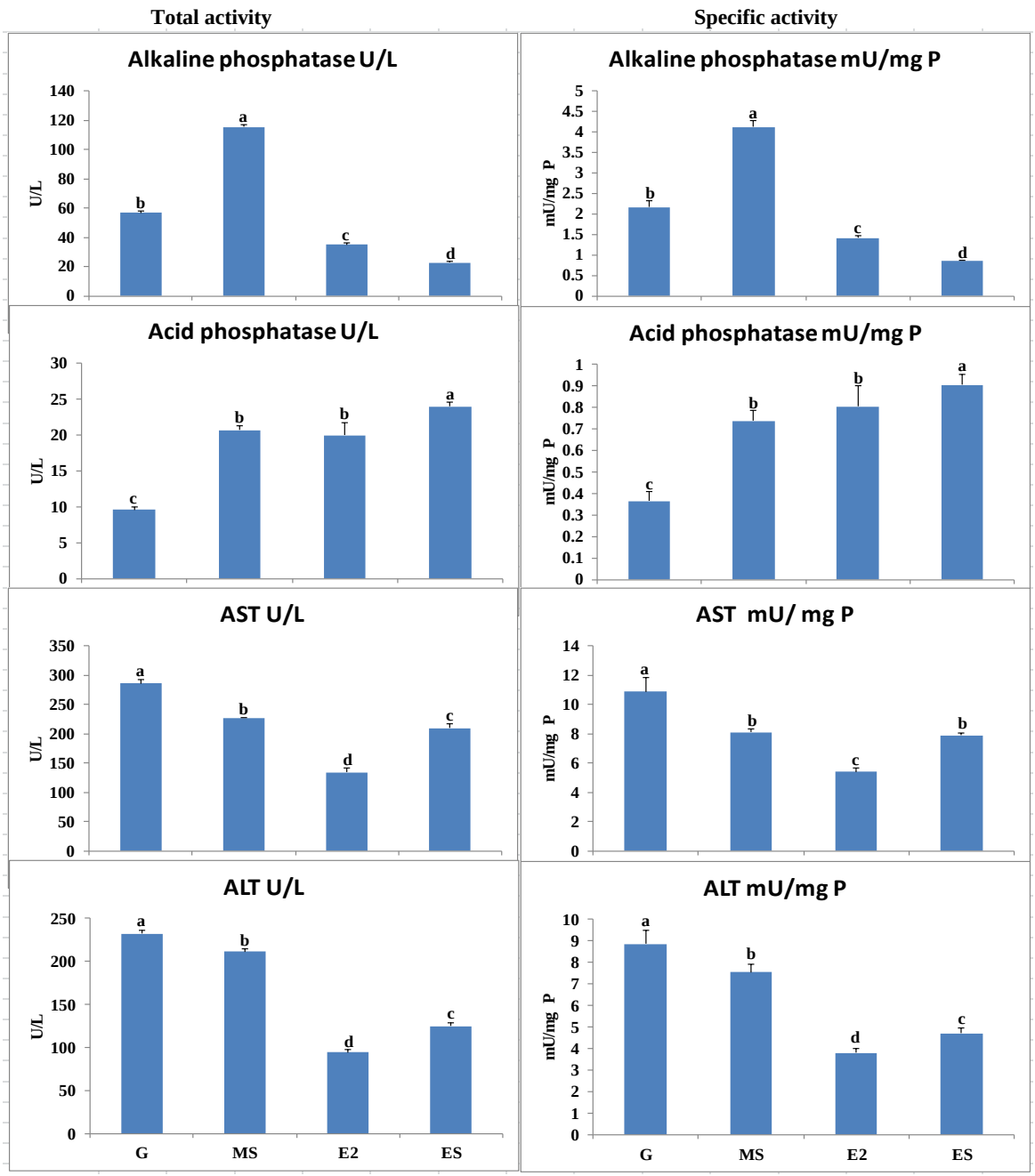

Fig. 3. Effect of estradiol (E2) sex reversing hormone and marine synbiotic treated microdiets on European seabass (*D. labrax*) larval liver enzymes total and specific activities
Different letters in the same row are for treatments effects significance ($P < 0.05$).
G: green-water control non treated, MS: marine synbiotic treated, E2: estradiol treated and SE2: marine synbiotic and estradiol treated microdiets.

Histological examinations

The liver of G control larvae showed homogenous hepatic parenchyma; the hepatocytes were polyhedral shaped cells with a cord-like arrangement of two or more hepatocytes thick. Hepatocytes have spherical centrally located nucleus, with densely staining chromatin margins and eosinophilia cytoplasm. Hepatocytes showed irregular

vacuolization. Sinusoids appearing throughout in the interstitial connective tissue between the hepatic plates. The sinusoidal capillaries were narrow and irregularly shaped. Abundant red blood cells were found in the liver sinus of G control larvae (Fig. 5A). In the liver of control fish as well as MS treated larvae, (Fig. 5B) and 35 dph larvae, (Fig. 5E), the tissue structure was clear and hepatic cells were regularly arranged. The hepatic cord and the hepatic sinusoid were connected to each other forming a net. Whereas in the liver of E2 treated larvae, edema of hepatocytes vacuolization, nuclear pyknosis were detected (Fig. 5C), and in ES mixture treated larvae focal area of hepatocytes undergoing necrosis. The liver sinus expanded and the red blood cells reduced and do not even exist (Fig. 5D).

Table 3. Effect of estradiol (E2) sex reversing hormone and marine synbiotic enriched microdiets on the 45 and 50 dph European seabass (*D. labrax*) larval water quality, water and larval bacterial counts quality

Treatment	dph	G	MS	E2	SE2
Water *Bacillus* bacterial count	**50**	**$2.00^{b} \pm 1.00$**	**$1.33^{b} \pm 0.58$**	**$1.33^{b} \pm 0.58$**	**$9.00^{a} \pm 2.00$**
Larvae *Bacillus* bacterial count	**50**	**$121.67^{c} \pm 7.64$**	**$230.67^{a} \pm 9.02$**	**$4.00^{d} \pm 1.00$**	**$200.00^{b} \pm 5.00$**

Different letters in the same row are for treatments effects significance ($P < 0.05$).
G: green-water control non treated, MS: marine synbiotic treated, E2: estradiol treated and SE2: marine synbiotic and estradiol treated microdiets.

The results in Fig. (6) show the effect of larval weaning using G, MS, E2 and ES treated microdiets on the histological sections in pancreas. The pancreatic exocrine tissue of G control larvae consisted of acini with centroacinar cells. Acinar cells were small cells with a euchromatic nucleus in base and a very dark basophilic cytoplasm; the apex of each cell contained bright, esinophilic zymogen secreting granules. Endocrine components of pancreas was observed among exocrine acinar cells consisting of a number of lightly capsulated, spherical cell in various sizes found scattered throughout the pancreas tissue (Fig. 6A). No clear differences were detected between control and treated larvae, except in MS treated larvae that has a well differentiated pancreatic exocrine cells structure (Fig. 6B), and in E2 treated ones, large vacuoles appeared in between acinar cells (Fig. 6C). Pancreatic tissue of ES and 35 dph larvae illustrated normal appearance (Figs. 6D, E).

Fig. (7) shows the effect of larval weaning using G, MS, E2 and ES treated microdiets on the histological sections in kidney. The kidney of G control larvae (Fig. 7A) showed urineferous tubules with distal and proximal tubular types. Each tubule consists of a layer of columnar epithelial cells resting on a basement membrane, with a wide lumen and hemopoietic tissue between tubules. No structural differences were detected in kidney structure of ES mixture treated larvae (Fig. 7D), 35 dph larvae (Fig. 7E) and G control (Fig. 7A). In MS treated larvae, some epithelial cells of proximal tubules showed vacuolization (Fig. 7B). Compared to the kidney of G control larvae (Fig. 7A), some of

the renal tubules in the kidneys of E2 treated larvae (Fig. 7C) displayed a lot of debris from necrotic epithelium and lost the normal shape. The interstitial areas between the tubules (Fig. 7C), were also enlarged, in which fewer red blood cells are identified.

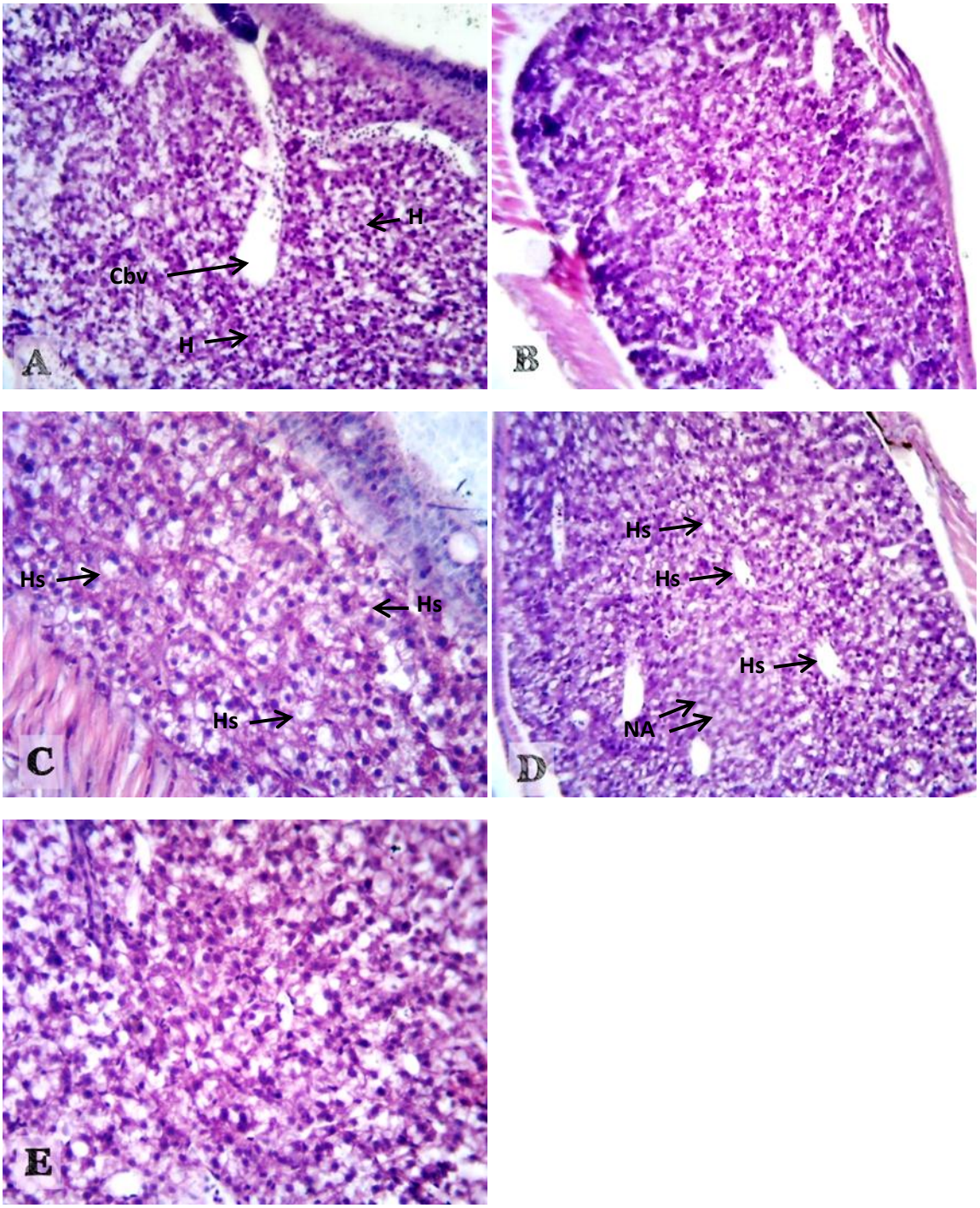

Fig. 5. Photomicrograph of liver (L) histological appearance of 50 dph *D. labrax* larvae G (A), MS (B), showing normal hepatocytes structure., E2 (C), illustrating extensive vacuolized hepatocytes and pyknotic nuclei and ES (D), showing necrotic area, hepatic sinus expansion and 35dph (E) larvae, showing normal liver structure. Hepatocyte (H), Central blood vessel (Cbv), Hepatic Sinusoid (HS), pycnotic nuclei (pn), (H&E X400).

DISCUSSION

The E2 hormone changes the sex ratio, gonads morphology, and negatively influences survival and growth **(Piferrer, 2001)**. Tilapia fry growth and survival decreased after the sex reversing hormone dose increased **(El-Gerisy & El-Gamal, 2012)**. In the current experiment, the larval length, weight growth, and survival decreased significantly after E2 treatment, compared to all other treatments, mixing MS with E2 in ES treatment and subsequently increased significantly the larvae growth and survival, compared to E2 and G control treated larvae. In support of the present study, **Salem *et al*. (2015)** recorded that, *B. subtilis* HS1 (the same dose and strain of included in the present study MS) and commercial synbiotic treating rotifers and *Artemia* enrichment and sea bass yolk sac larvae, and first fed larvae to weaning tanks until weaning showed significant improvement of larval growth and survival. **Zaki *et al*. (2015)** suggested that the dietary chitosan marine prebiotic (the same source and dose include in MS used in the present study) of sea bass fry increased the growth, survival and the non-specific responses. **Wang *et al*. (2008)** recorded that athough E2 changed the sex ratio, it reduced the survival and growth.

The Biomin IMBO synbiotic significantly improved common carp specific growth rate, protein efficiency rate, carcass protein, hematocrit, hemoglobin, leucocyte, serum albumin and glucose **(YarAhmadi *et al*., 2014)**. Synbiotic supplementation decreased feed conversion ratio, carcass lipid, serum cholesterol and globulin **(Ringø & Song, 2016). Hassan *et al*. (2014)** showed that synbiotic significantly increased total protein content and albumin. Rainbow trout serum protein, albumin and globulin level improved when synbiotic (Biomin IMBO) was applied **(Mehrabi *et al*., 2011)**. In the current experiment, total protein significantly increased after MS treatment. While, albumin was significantly reduced after E2 treatment. Globulin showed a significant increase after MS treatment. Hence, the larvae fed ES showed the highest significant body glucose in g/l.

Kumar *et al*. (2011) and **Hassan *et al*. (2014)** assessed that, ALT and AST enzymes are indicators for liver health and function through controlling the transferring amino group function of alpha-amino acids to alpha-keto acids. Large amounts of ALT and AST are released into animal blood, mostly during liver cell damage. In the current study, the E2 treatment significantly reduced ALT and AST enzyme activities, while mixing E2 with MS in ES treatment significantly increased them compared to E2 treatment. In the Nile tilapia fed diets supplemented with probiotics, the ALT and AST levels were considerably reduced **(Soltan & El-Laithy, 2008)**. Dietary fructooligosaccharide and *B. licheniformis* significantly enhanced leucocyte counts, ALP, plasma AP, lysozyme, plasma alternative complement (ACH50), phenoloxidase, total serum protein, globulin and IgM **(Zhang *et al*., 2013)**. Furthermore, improved liver and plasma antioxidant capacity was observed **(Ringo & song, 2016). Li *et al*. (2009)** revealed that inclusion *B.* and IMOS increased growth performance, phagocytosis of hemocyte, phenoloxidase, respiratory burst, AP and ALP activities. Intestinal total viable

bacterial counts and counts of presumptive *V.* were lower in synbiotic groups. **Salem *et al*. (2019)** deduced that, the dietary orange peal prebiotic decreased ALP in sea bream. Whereas, **Dehaghani *et al*. (2015)** noted that the ALP activity in the control was considerably higher compared to the synbiotic groups.

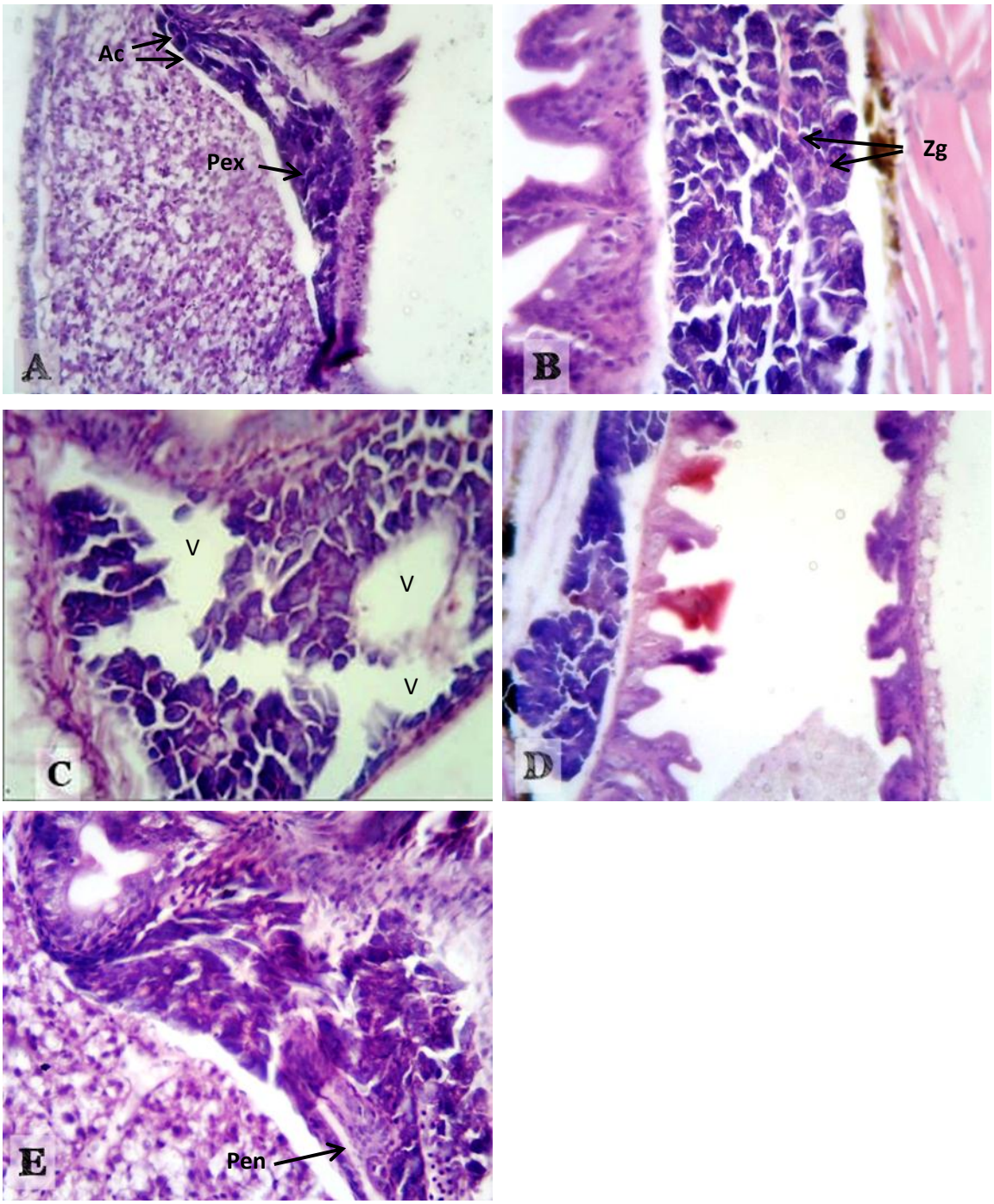

Fig. 6. Photomicrograph of pancreaic tissue histological appearance (SP) of 50dph *D. labrax* larvae G (A), showing normal pancreatic tissue, MS(B), showing well differentiated pancreatic tissue, E2(C), illustrating large vacuoles, and ES(D), showing normal acinar cells and 35 dph (E) larvae, showing normal pancreatic structure. Acinar cells (Ac), Pancreatic exocrine (Pex), Zymogen granules (Zg), Vacuole (V), Pancreatic endocrine (Pen), (H&E X400).

It is worthy to mention that, *Lactobacillus* spp. bacteria improved ALP activity in gilthead sea bream (*Sparus aurata* L.) larvae **(Suzer *et al*., 2008)**. In the current study, the ALP enzyme recorded a substantial reduction after ES (E2 and MS mixture) treatment compared to all other treatments, and the ALP enzyme showed a significant increase after MS treatment, compared to all other treatments in agreement with the findings of **Suzer *et al*. (2008)**. Coinciding with the current study, ES (E2 and MS mixture) treatment significantly increased AP activities compared to all other treatment; AP activity increased in *Miichtys miicy* fed with *C. butyricum* and recorded immune system increase **(Song *et al*., 2006)**.

The ROS induction could lead to lipid peroxidation and oxidative stress. The induction of lipid peroxidation correlated to ROS generated and scavenged by antioxidants (CAT or SOD) in Japanese sea bass liver under E2 stress **(Thilagam *et al*., 2010). Mensinger *et al*. (2005)** and **Desouky *et al*. (2020)** reported that, the levels of triglycerides and cholesterols are also considered essential indices of health status (). Consequently, increases in plasma/liver low density cholesterol, TAG, and cholesterol levels, the decreasing of high-density cholesterol, are considerable risk factors for steatosis in mammals **(Kim & Spiegelman, 1996). Desouky *et al*. (2020)** related this to the higher crude lipid and growth reduction in high fat diet treatment. However, the plasma/liver TAG, cholesterol and low density cholesterol values reduction were detected in high-fat diets supplemented with glycyrrhetinic acid treatment. In the current study, the TAG exhibited a considerable increase in E2 treatment *vs*. the other treatments, while mixing E2 with MS in ES treatment significantly reduced the TAG compared to E2 and G treatments. The hepatic tissue oxidative stress of sea bass was induced by E2 **(Thilagam *et al*., 2010)**. Orange peel prebiotic improved the oxidative stress (TAC, SOD, GSH-Px and CAT activities) resistance in sea bream **(Salem *et al*., 2019)**. CAT gene expression levels at 20 dph sea bass were significantly dissimilar among the three probiotics, *Lactobacillus casei* probiotic upregulated CAT **(Lamri *et al*., 2016)**. However, antioxidant enzymes (CAT, SOD and GPX) at 20 and 41 dph sea bass had no considerable differences. In the current study as well as in Lamri *et al*. (2016), Sea bass larvae were not infected by pathogenic *V.* In the current study, the SOD and CAT activities revealed a significant increase, while TAC exhibited a substantial reduction after E2 treatment confirming the negative impacts of E2 on larvae compared to all other treatments. The activities of SOD and CAT noticeably reduced after ES treatment, while TAC exposed a considerable increase after ES treatment compared to E2 and G.

The influences of dietary prebiotics, probiotics and synbiotics on the fish and shellfish rearing water and gut microbiota investigated **(Merrifield *et al*., 2011; Kühlwein *et al*., 2013; Salem *et al*., 2015)**. The current experiment larval rearing water samples or the larvae of the four treatments detected no *V.* sp. The MS and ES achieved the best significant *B.* sp. bacterial counts results of the larvae and water, respectively. In

accordance, **Salem *et al*. (2015)** recorded that *B. subtilis* HS1 marine probiotic and commercial synbiotic treating rotifers, *Artemia* enrichment, sea bass yolk sac larvae and first fed larvae to weaning tanks until weaning showed significant improvement of total bacterial count and *B.* sp., while significantly decreased the counts of *Aeromonas* sp., *Staphylococcus* sp. and *V.* sp., compared to control treatment.

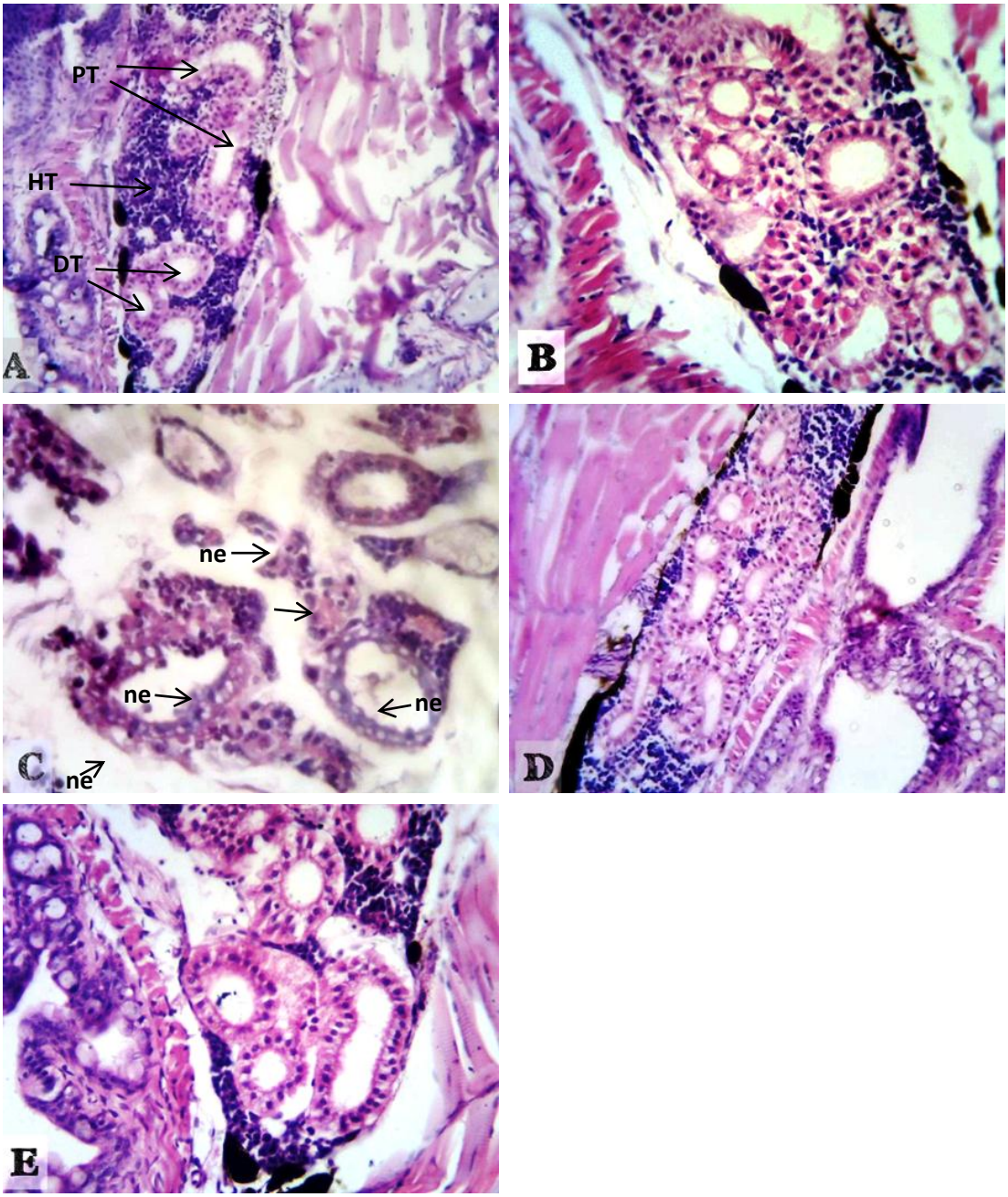

Fig. 7. Photomicrograph of kidney histological appearance (K) of 50 dph *D. labrax* larvae G (A), showing normal kidney structure., MS(B), showing vacuolized tubular tissue., E2(C), illustrating disintegrated tubular cells, cells debris and reduced hemopietic tissue., and ES(D), showing normal tubular cells structure., and 35dph(E) larvae, showing normal kidney shape. Proximal Tubules (PT), Distal Tubules (DT), Hemopoietic Tissue (HT), Epithelial vacuolization (Ev), necrotic epithelium (ne). (H&E X400).

Salem *et al*. (2018) determined that, the European sea bass fry sources (wild and two hatcheries including NIOF, the same source of the present study larvae and water) impacted on water and fry *V.* sp. and *B. sp.* bacterial counts. *V. sp*. was not detected in the water source sample of the three fry sources. The wild collected fry achieved the best significant water and fry results in *B.* bacterial counts, supporting the results of the present study because NIOF hatchery feeds or water are not supplemented with *B.* sp. or any other probiotics, prebiotics and synbiotics. **Maciuszek *et al*. (2020)** indicated that in the head kidney of *Aeromonas salmonicida* infected fish, E2-treated feeding induced an upregulation of gene expression. Moreover, in infected fish fed with E2-treated food, a higher gene expression of the estrogen receptors and of the aromatase CYP19 was detected. **Salem *et al*. (2019)** deduced that, the decline in the bacterial count of dietary orange peel prebiotic is due to active components with wide antimicrobial activities to resist the diseases.

CONCLUSION

The current findings revealed a significant improvement of larval length, weight growth, and survival with MS dietary supplementation, significantly decreased after E2 treatment. ES treatment increased significantly the larvae growth and survival compared to E2 and G control treated larvae. Total protein, albumin, glucose, ALT and AST enzymes significantly decreased after E2 treatment, while ES treatment significantly increased them compared to E2 treatment. The larvae fed ES showed the highest significant ($P < 0.05$) body AP. The ALP enzyme showed a significant increase after MS treatment. The TAG showed a significant increase after E2 treatment, while ES treatment noteworthy decreased the TAG. The SOD and CAT activities showed a significant increase after E2 treatment, and significantly reduced after ES treatments. While, TAC showed a significant decrease after E2 treatment, and reversely, TAC showed a significant increase after ES treatments. MS treated sea bass larvae improved growth rate and survival, decreased the negative impacts of E2 and increased and ameliorated morphological structure of liver and kidney of ES, compared to E2 treatments. In conclusion, mixing MS with E2 in ES decreased the negative impacts of E2 on the European sea bass larval weaning growth, survival, antioxidant capacity and oxidative stress.

REFERENCES

Abasubong, K. P.; Li, X. F; Zhang, D. D.; Jia, E. T.; Xiang-Yang, Y.; Xu, C. and Liu, W. B. (2018). Dietary supplementation of xylooligosaccharides benefits the growth performance and lipid metabolism of common carp (*Cyprinus carpio*) fed high-fat diets. Aquac Nutr 00:1–9.

Abu-Elala, N.; Marzouk, M. and Moustafa, M. (2013). Use of different *Saccharomyces cerevisiae* biotic forms as immune-modulator and growth

promoter for *Oreochromis niloticus* challenged with some fish pathogens. International Journal of Veterinary Science and Medicine, 1(1), 21–29.

Aebi, H. (1984). Catalase in vitro. Methods Enzymol 105:121–126

Akrami, R.; Iri, Y.; Rostami, H. K. and Mansour, R. M. (2013). Effect of dietary supplementation of fructooligosaccharide (FOS) on growth performance, survival, *Lactobacillus* bacterial population and hematoimmunological parameters of stellate sturgeon (*Acipenser stellatus*) juvenile. Fish & Shellfish Immunology, 35: 1235–1239. https ://doi. org/10.1016/j.fsi.2013.07.039

Akter, M. N.; Sutriana, A.; Talpur, A. D. and Hashim, R. (2016). Dietary supplementation with mannan oligosaccharide influences growth, digestive enzymes, gut morphology, and microbiota in juvenile striped catfish, *Pangasianodon hypophthalmus*. Aquaculture International, 24(01): 127–144.

Arslan, T. and Phelps, P. (2004). Directing gonadal differentiation in bluegill, *Lepomis marochirus* (Rafinesque), and black crappie, *Pomoxis nigromaculatus* (Lesueur), by periodic estradiol-17β immersions. Aquaculture Research 35, 397–402.

Azimirad, M. and Meshkini, S. (2017). Determination of the optimal enrichment *Artemia franciscana* with a synbiotic combination of probiotics *Pediococcus acidilactici* and prebiotic fructooligosaccharide. Journal of Veterinary Research Forum, 8 (1): 49–54.

Banerjee, G. and Ray, A. K. (2016). Bacterial symbiosis in the fish gut and its role in health and metabolism. Symbiosis 72: 1–11.

Baroiller, J.F. and D'Cotta, H. (2016). The reversible sex of gonochoristic fish: insights and consequences. Sex Dev.10:242–266

Belfield, A. and Goldberg, D. M. (1971). Colorimetric determination of alkaline phosphatase activity. Enzyme, 12, 5: 561-568.

Betancor M. B.; Atalah, E.; Caballero, M. J.; Benítez -Santana, T.; Roo, J. and Montero, D. (2011). Alpha-tocopherol in weaning diets for European sea bass (*Dicentrarchus labrax*) improves survival and reduces tissue damage caused by excess dietary DHA contents. Aquaculture Nutrition 17: E112–E122.

Betancor, M. B.; Caballero, M. J.; Terova, G.; Saleh, R.; Atalah, E.; Benítez-Santana, J. T.; Bell, G. and Izquierdo, M. (2012). Selenium inclusion decreases oxidative stress indicators and muscle injuries in sea bass larvae fed high DHA microdiets. Br. J. Nutr. 108/12: 2115–2128.

Bindels, L. B.; Delzenne, N. M.; Cani, P. D. and Walter, J. (2015). Towards a more comprehensive concept for prebiotics. Nature Reviews Gastroenterology & Hepatology, 12: 303–310. https ://doi. org/10.1038/nrgas tro.2015.47

Dehaghani, P. G.; Baboli, M. J.; Moghada, A. T.; Nejad, S. Z. and Pourfarhadi, M. (2015). Effect of synbiotic dietary supplementation on survival, growth performance, and digestive enzyme activities of common carp *Cyprinus carpio* fingerlings. Czech Journal of Animal Science 60: 224–232.

Desouky, H. E.; Jiang, G.; Zhang, D.; Abasubong, K. P.; Yuan, X.; Li, X. and Liu, W. (2020). Influences of glycyrrhetinic acid (GA) dietary supplementation on growth, feed utilization, and expression of lipid metabolism genes in channel catfish (*Ictalurus punctatus*) fed a high-fat diet. Fish Physiol Bioch. 46:653–663.

Doumas, B. T.; Watson, W. A. and Biggs, H. G. (1971). Albumin standards and the measurement of serum albumin with bromocresol green. Clinica Chimica Acta, 31: 87-93. doi:10.1016/0009-8981(71)90365-2

El-Greirsy, Z. A. and El-Gamal, A. E. (2012). Monosex production of tilapia, Oreochromis niloticus using different doses of 17a-methyltestosterone with respect to the degree of sex stability after one year of treatment. Egypt. J. Aquat. Res. 38: 59–66.

FAO. (2022). The State of World Fisheries and Aquaculture 2022. Towards blue transformation. Rome, FAO. https://doi.org/10.4060/cc0461en

Flynn, S. R. and Benfey, T. J. (2007). Effects of dietary estradiol-17β in juvenile shortnose sturgeon, Acipenser brevirostrum, Lesueur. Aquaculture 270:405–412.

Fossati, P. and Prencipe, L. (1982). Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. Clinical Chemistry, 28: 2077-2080.

Gornall, A. G.; Bardawill, C. J. and David, M. M. (1949). Determination of serum proteins by means of the Biuret reaction. J. Biol. Chem., 177, 751–766.

Gorshkov, S.; Gorshkova, G. and Colorni, B. (2004). Effects of natural estradiol-17β and synthetic 17α- ethynylestradiol on direct feminization of European sea bass *Dicentrarchus labrax*. Journal of the World Aquaculture Society, . 35/ 2: 167-177.

Hamed, M.; Soliman, H. A. M.; Osman, A. G. M. and Sayed, A. H. (2020). Antioxidants and molecular damage in Nile Tilapia (*Oreochromis niloticus*) after exposure to microplastics. Environmental Science and Pollution Research 27:14581–14588

Hassaan, M.; Soltan, M. and Ghonemy, M. (2014). Effect of synbiotics between *Bacillus licheniformis* and yeast extract on growth, hematological and biochemical indices of the Nile tilapia (*Oreochromis niloticus*). Egyptian Journal of Aquatic Research, 40: 199-208.

Hendry, C. I.; Deborah, J.; Martin-Robichaud, J. D. and Benfley, J. T. (2003). Hormonal sex reversal of Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture, 219:769–781

Huynh, T. G.; Shiu, Y. L.; Nguyen, T. P.; Truong, Q. P.; Chen, J. C. and Liu, C. H. (2017). Current applications, selection, and possible mechanisms of actions of synbiotics in improving the growth and health status in aquaculture: A review. Fish & Shellfish Immunology, 64, 367–382.

Kim, J. B. and Spiegelman, B. M. (1996). ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. Genes Dev 10:1096–1107

Kind, P. R. and King, E. J. (1954). J.Clin, Path. 7,322

Koracevic, D.; Koracevic, G.; Djordjevic, V.; Andrejevic, S. and Cosic, V. (2001). Method for the measurement of antioxidant activity in human fluids. J Clin Pathol 54:356–361

Kühlwein, H.; Emery, M.; Rawling, M.; Harper, G.; Merrifield, D. and Davies, S. (2013). Effects of a dietary β - (1, 3)(1, 326 6) - D - glucan supplementation on intestinal microbial communities and intestinal ultrastructure of mirror 327 carp (*Cyprinus carpio* L.). Journal of Applied Microbiology. 115: 1091-1106. 328

Kumar, R. M.; Fasalu, R.; Tamizahm, T.; kumar, B.; Phaneendra and Surendra, B. (2011). Evaluation of hepatoprotective activity of *Asparagus racemosus* root against paracetamol induced acute liver injury in rats Pharmacology on line Ind. J. of Novel Drug Dlivery 1:1059-1066.

Lamari, F.; Mahdhi, A.; Chakroun, I.; Esteban, M. A.; Mazurais, D. and Amina, B. (2016). Interactions between candidate probiotics and the immune and antioxidative responses of European sea bass (*Dicentrarchus labrax*) larvae. Journal of Fish Diseases39: 1421–1432.

Li, J.; Tan, B. and Mai, K. (2009). Dietary probiotic Bacillus OJ and isomaltooligosaccharides influence the intestine microbial populations, immune responses and resistance to white spot syndrome virus in shrimp (*Litopenaeus vannamei*). Aquaculture 291:35e40.

Livingstone, D. R. (2001). Contaminated-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42/ 8: 656-666.

Maciuszek, M.; Pijanowski, L.; Pekala-Safinska, A.; Kemenade, B. and Chadzinska, M. (2020). 17β-Estradiol affects the innate immune response in common carp. Fish Physiol Biochem. 46:1775–1794

Martoja, R. and Martoja-Pearson, M. (1970). Tecnicas de Histologia Animal. Toray-Masson S.A., Barcelona.

Mehrabi, Z.; Firouzbakhsh, F. and Jafarpour, A. (2011). Effects of dietary supplementation of synbiotic on growth performance, serum biochemical parameters and carcass composition in rainbow trout (*Oncorhynchus mykiss*) fingerlings. Journal of Animal Physiology and Animal Nutrition, 96/ 3:474–481.

Mensinger, A. F.; Walsh, P. J. and Hanlon, R. T. (2005). Blood biochemistry of the oyster toadfish. J Aquatic Animal Health. 17:170–6. doi: 10.1577/H04-021.1

Merrifield, D. L.; Harper, G.; Mustafa, S.; Carnevali, O.; Picchietti, S. and Davies, S. J. (2011). Effect of dietary alginic acid on juvenile tilapia (*Oreochromis niloticus*) intestinal microbial balance, intestinal histology and growth performance. Cell and Tissue Research, 344: 135–146.

Mourente, G.; Tocher, D. R.; Diaz-Salvago, E.; Grau, A. and Pastor, E. (1999). Study of the high n-3 highly unsaturated fatty acids requirement and antioxidant status of *Dentex dentex* larvae at the *Artemia* feeding state. Aquaculture 179: 291–307.

Murray, R. (1984). alanine aminotransferase clin Chem the C.V. Toronto Princeton 1088-1090.

Nishikimi, M.; Roa, N. A. and Yogi, K. (1972). The occurrence of supeoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochemical Biophysical Research Communications, 46: 849-854.

Okey, I. B.; Gabriel, U. U. and Deekae, S. N. (2018). The use of synbiotics (prebiotic and probiotic) in aquaculture development. Sumerianz J. Biotechnol., 1(2): 51-60.

Piffrrer, F. (2001). Endocrine sex control strategies for the feminization of teleost fish. Aquaculture, . 197/ 1-4: 229-281.

Ringø, E. and Song, S. K. (2016). Application of dietary supplements (synbiotics and probiotics in combination with plant products and β-glucans) in aquaculture: Aquaculture Nutrition 22: 4-24, doi: 10.1111/anu12349.

Ringø, E.; Olsen, R. E.; Gifstad, T. Ø.; Dalmo, R. A.; Amlund, H.; Hemre, G-I. and Bakke, A. M. (2010). Prebiotics in aquaculture: A review. Aquaculture Nutrition 16: 117-136.

Ringø, E.; van Doan, H.; Lee, S. H.; Soltani, M.; Hoseinifar, S. H.; Ramasamy, H. and Song, S. K. (2020). Probiotics, lactic acid bacteria and bacilli: interesting supplementation for aquaculture. Journal of Applied Microbiology 129: 116-136,

Salem, Ahmed Md.; El-Bermawi, N. M.; Abdelsalam, N. R. and Abokadah, M. S. (2018). Effect of European Seabass (*Dicentrarchus Labrax*) Fry Source (Wild, NIOF and GAFRD K21) on its Growth Performance and Physiology Egypt. J. Vet. Sci., 49/1: 13-23.

Salem, Ahmed Md.; Nour, A. M.; Srour, T. M.; Assem, S. S.; Ibrahim, H. A. and El-Sayed, H. S. (2015). Greenwater, marine *Bacillus subtilis* HS1 probiotic and synbiotic enriched artemia and rotifers improved European Seabass *Dicentrarchus labrax* larvae early weaning length growth, survival, water and bacteriology quality. American Journal of Life Sciences. Special Issue: New Horizons in Basic and Applied Zoological Research, 3 (6- 1): 45-52.

Salem, M. E- S.; Abdel- Ghany, H. M.; Sallam, A. E.; El-Feky, M. M. and Almisherfi, H. M. (2019). Effects of dietary orange peel on growth performance, antioxidant activity, intestinal microbiota and liver histology of Gilthead sea bream (*Sparus aurata*) larvae. Aquacult Nutr. 25:1087– 1097.

Sayed, A. H. and Abou Khalil, N. S. (2016). Estimation of the Modulatory roles of thieno [2,3-cpyrazole compounds versus the toxicity of 4-nonylphenol in African catfish (*Clarias gariepinus*). Int. J. Biochem. Res. Rev. 11: 1–8.

Serradell, A.; Torrecillas, S.; Makol, A.; Valdenegro, V.; Fernández-Montero, A.; Acosta, F.; Izquierdo, M. S. and Montero, D. (2020). Prebiotics and phytogenics functional additives in low fish meal and fish oil based diets for European sea bass (*Dicentrarchus labrax*): Effects on stress and immune responses. Fish & Shellfish Immunology, 100:219-229

Soltan, M. A. and El-Laithy, S. M. (2008). Effect of probiotics and some spices as feed additives on the performance and behaviour of the Nile tilapia, *Oreochromis niloticus*. Egypt. J. Aquat. Biol. Fish., 12/2: 63-80.

Song, Z.; Wu, T.; Cali, L.; Zhang, L. and Zheng, X. (2006). Effects of dietary supplementation with Clostridium butyricum on the growth performance and humoral immune response in *Miichthys miiuy*. J. Zhejiang Univ Science B, 7(7):596-602

Sumpter, J. P. and Johnson, A. C. (2005). Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment. Environ Sci Technol 39:4321–4332

Süzer, C.; Çoban, D.; Kamaci, H.; Saka, S.; Firat, K.; Otgucuoglu, Ö. and Küçüksari, H. (2008). *Lactobacillus* spp. bacteria as probiotics in gilthead sea bream (*Sparus aurata*, L.) larvae: effects on growth performance and digestive enzyme activities. Aquaculture, 280: 140–145.

Tarnecki, A. M.; Wafapoor, M.; Phillips, R. N. and Rhody, N. R. (2019). Benefits of a *Bacillus* probiotic to larval fish survival and transport stress resistance. S. R. 9:4892.

Thilagam, H.; Gopalakrishnan, S.; Qu, H.; Bo, J. and Wang, K. (2010). 17b estradiol induced ROS generation, DNA damage and enzymatic responses in the hepatic tissue of Japanese sea bass. Ecotoxicology 19:1258–1267

Thomas-Jones, E.; Thorpe, K.; Harrison, N.; Thomas, G.; Morris, C.; Hutchinson, T.; Woodhead, S. and Tyler, C. (2003). Dynamics of estrogen biomarker responses in rainbow trout exposed to 17beta-estradiol and 17alpha-ethinylestradiol. Environ Toxicol Chem 22:3001–3008

Verhaegen, B.; Rekecki A.; Decostere, A. and Broeck, W. (2012). Bottlenecks in aquaculture: Some risky aspects of early life stages of fish. Vlaams Diergeneeskundig Tijdschrift 81(2):63-69

Villumsen, K. R.; Ohtani, M.; Forberg, T.; Aasum, E.; Tinsley, J. and Bojesen, A. M. (2020). Synbiotic feed supplementation significantly improves lipid utilization and shows discrete effects on disease resistance in rainbow trout (*Oncorhynchus mykiss*). Sci. Rep. 10: 1–12. doi: 10.1038/s41598-020-73812-8

Wang, Y.; Gao, Z.; Beres, B.; Ottobre, J.; Wallat, G.; Tiu, L. and Yao, H. (2008). Effects of estradiol-17β on survival, growth performance, sex reversal and gonadal structure of bluegill sunfish *Lepomis macrochirus*. Aquaculture 285: 216–223

Weissman, M. and Klien, B. (1958). Evaluation, of glucose determination in untreated serum samples. Clin chem. 4:420-422.

Widanarni, W.; Taufik, A.; Yuhana, M. and Ekasari, J. (2018). Dietary mannan oligosaccharides positively affect the growth, digestive enzyme activity, immunity and resistance against *Vibrio harveyi* of Pacific White Shrimp (*Litopenaeus vannamei*) Larvae. Turk. J. Fish.& Aquat. Sci. 19(4): 271-278.

Yarahmadi, P.; Miandare, H. K.; Hoseinifar, S. H.; Gheysvandi, N. and Akbarzadeh, A. T. (2014). Effects of stocking density on hematoimmunological and serum biochemical parameters of rainbow trout (*Oncorhynchus mykiss*). Aquac. Int. 23: 55–63.

Zaki, M. A.; Salem, M. E. S.; Gaber, M. M. and Nour, A. M. (2015). Effect of chitosan supplemented diet on survival, growth, feed utilization, body composition and histology of Sea bass (*Dicentrarchus labrax*). World J. Eng. Technol.;3(4):38-47.

Zhang, C. N.; Li, X. F.; Xu, W. N.; Jiang, G. Z.; Lu, K. L.; Wang, L. N. and Liu, W. B. (2013). Combined effects of dietary fructooligosaccharide and *Bacillus licheniformis* on innate immunity, antioxidant capability and disease resistance of triangular bream (*Megalobrama terminalis*). Fish & Shellfish Immunology, 35: 1380–1386.