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Effects of polyphenol administration to European farmed sea bass (*Dicentrharcus labrax* L): Special focus on hepatopancreas morphology

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Abstract

Hepatopancreas is an accessory organ associated to the liver in some fish, even including sea bass (*Dicentrharcus labrax* L). Hepatopancreas contains an exocrine portion but until now its function has poorly been investigated. Here, European farmed sea bass have been treated with a feed enriched in polyphenols extracted from seeds of red grape (Nero di Troia cultivar) at two different doses (100 and 200 mg/kg, respectively) from day 273 to day 323. In fish samples, hepatopancreas area sizes have been measured to evaluate the effects of this dietary regimen on its morphology. Quite interestingly, in treated fish area sizes of hepatopancreas were higher than those detected in untreated fish. Two hundred dose polyphenols was more effective than that of 100 mg/kg polyphenols. Finally, hepatic polyphenol concentration was diminished in fish receiving 100 mg dose polyphenols and normalized with 200 mg dose in comparison to untreated fish. This evidence suggests the utilization of polyphenols for liver function, even including hepatopancreas development. Conclusively, these data suggest an expansion of hepatopancreas induced by polyphenol administration that is also associated to less mortality in farmed fish.

Keywords: Aquaculture, fish, hepatopancreas, liver, macrophages, melanomacrophage centers, polyphenols

Introduction

In fish, pancreas consists of two portions, the exocrine and the endocrine compartments, respectively, which exert distinct functions [1]. In fact, the former secretes digestive juice, while the latter produces insulin and glucagon. Of note, the exocrine pancreas is incorporated in the liver of some fish and known as hepatopancreas [2-5]. Histologically, it appears as an acinar structure with zymogen granules accumulated in the apical part of the exocrine cells [5]. According to fish species, hepatopancreas can remain extra-hepatic separated from hepatic compartment by a thin layer of connective tissue or penetrate into the liver parenchyma, even deeply [3, 6].

There is evidence that signals which induce hepatic and pancreatic genes seem to proceed in a parallel way, as observed in mammalians [7]. From a phylogenic point of view, this event may also occur in fish and, in some cases, the two organs may coexist, thus explaining the formation of the hepatopancreas.

To the best of our knowledge, hepatopancreas in farmed fish has poorly been investigated, especially, in terms of protective functions. In fact, in aquaculture systems fish have to cope with many microbial antigens and water contaminants. In this respect, the development of aquaculture with intensification of production systems necessitates of high quality feedstuff for the improvement of fish health in the absence of side effects for consumers [8, 9]. Fish meal is commonly used as fish feed, however, because of its shortage prices have recently increased and, therefore, alternative feeds are currently employed [10]. In addition, the use of antibiotics and chemotherapeutics in aquaculture has allowed the development of resistant pathogens, bioaccumulation and environmental pollution [10]. In this framework, in European sea bass (*Dicentrharcus labrax* L.), derived from a polluted place (Bizerte Lagoon, Tunisia), more elevated hepatocyte DNA damage was detected along with hepatic altered morphology in comparison to wild samples collected from the Mediterranean sea [11]. In farmed European sea bass, when compared to wild samples from the Mediterranean sea (Sicily, Italy), organochlorine compound concentrations in muscles and liver were higher in the former than those detected in the latter [12].

In farmed European sea bass the effects of acute treatment with mercury was evaluated [13]. Modifications of gills, liver and enterocytes were detected in terms of severe oedema, teleangeactasia, hydropic cell swelling and alteration of both endoplasmic reticulum and mitochondria. These results suggest the potential *in vivo* effects of water contamination with mercury in the aquatic environment.

Therefore, in view of the putative impairment of fish immune response depending on environmental factors, immune stimulants and vaccines are currently utilized to increase farmed fish resistance to microbes [14]. Furthermore, dietary attempts to improve fish growth performance and immune functions have been made *via* administration of dietary substances, such as pre- and probiotics [15]. In a recent paper, evidence has been provided that administration of prebiotics to fish increased intestinal macrophage and dendritic cell functions, as well as epithelial barrier function, with production of short chain fatty acids [16]. Also combined administration of kefir and low molecular weight sodium alginate (a synbiotic) increased resistance of Nile tilapia (*Oreochromis niloticus*) against *Streptococcus agalactiae* [17]. From the above data one can better appreciate the mechanisms of action exerted by pre- and probiotics [18, 19]. Other natural products such as xylan oligosaccharides from corncobs, when administered to European sea bass, could enhance growth performance, both serum immunoglobulin levels and lysozyme activity with reduction of hepatic reactive oxygen species production [20, 21]. Also, administration of arachidonic acid rich oil to European farmed sea bass juveniles positively affected growth performance, tissue fatty acid profile and lipid metabolism [22]. Mannan oligosaccharides supplemented to the diet of European sea bass lowered lipid vacuolization and regular shape of hepatocytes around the sinusoidal spaces [23]. Sukumaran and associates [24] have reported the effects of dietary ginger *Zingiber officinale* administration in *Labio rohita* fingerlings with a dramatic increase of anti-oxidants (zinc/copper superoxide dismutase and glutathione peroxidase) and anti-inflammatory [interleukin (IL)-10, transforming growth factor-β] genes and of signaling molecules (nuclear factor erythroid 2-related factor and inhibitor protein κβα) in hepatopancreas, as well as in kidney and intestine. Seeds of

Achyranthes aspera supplemented to feed in carp *Catla catla* up-regulated gene expression of tumor necrosis factor-α, lysozyme c and g in kidney and IL-10 in hepatopancreas [25]. In a few studies, administration of polyphenols, natural products, largely present in vegetables, fruit, cereals, cocoa, tea, wine and extra virgin olive oil, has been investigated in farmed fish for their anti-inflammatory and anti-oxidant activities. Sakuna and associates [26] demonstrated that quercetin, when incorporated into the diet, improved the original transport-stress mortality in farmed red claw cray fish (*Cherax quadricarinatus*) infected with Chequa iflavirus. In farmed turbot, administration of resveratrol reduced scuticociliatosis in virtue of its anti-protozoal activity [27]. In *Carassius auratus*, fed a hypercolesterolemic diet, administration of polyphenols, extracted from waste water from an olive mill, reduced steatotic damage of the liver [28]. In farmed sea bass, administered with polyphenols extracted from the seeds of *Canosina* Nero di Troia (*Vitis Vinifera)*, intestinal pro-inflammatory cytokine release was evaluated [29]. Such a dietary regimen led to a reduction of intestinal pro-inflammatory cytokines (IL-1β and IL-6) and an enhancement of splenic interferon (IFN)-γ release with an expansion of fish melanomacrophage centers (MMCs).

On these grounds, here we will describe the effects of polyphenol administration to sea bass on the hepatopancreas morphology, using the same dietary regimen above mentioned [29].

Data will show that in treated animals with 200 mg/kg dose of polyphenols area sizes of hepatopancreas significantly increased at T2 when compared to T1 values and controls (untreated fish). Moreover, polyphenol concentrations in liver increased at T2, in comparison to T1 values, thus falling within ranges comparable to those of controls. This may suggest the potential utilization of polyphenol, at T1, for liver function performance, even including development of hepatopancreas.

In conclusion, for the first time our data will show the effects of dietary polyphenols on hepatopancreas, thus, indicating a putative contribution of this organ to immune protection in farmed sea bass.

Materials and Methods

Polyphenol Extracts

Canosina red grape from Nero di Troia, a typical *Vitis vinifera* grape cultivar from Apulia (South Italy), was used for this study. Frozen seeds from berries were extracted by percolation using ethanol/water (70:30), and, then, extracts were analyzed by means of liquid chromatography with diode array detection to determine the polyphenol composition [30]. Thereafter, the extract was purified on a synthetic adsorbent brominated resin and percentage of polyphenol content (cathechins and epicatechins) was equal to 10.37.

Preparation of fish feed and experimental design

Diet consisted in conventional feed mixed with two different doses of grape extracts, administered 3-4 times a day to 273 days old sea bass (*Dicentrharcus labrax* L.) reared in captivity in a farm near Lesina lake (Foggia, Italy). Administration lasted from day 273 up to day 323. Accordingly, three groups of fish were selected:

1) fish (n=30) fed mix composed by 50 gr of grape extracts in 5 kg of cornstarch, namely 10 gr of mix (100 mg of extract) for 1 kg of conventional pellet (100 mg/kg);

2) fish (n=30) fed mix composed by 100 gr of grape extracts in 5 kg of cornstarch, namely 10 gr of mix (200 mg of extract) for 1 kg of conventional pellet (200 mg/kg);

3) Controls (n=30) were represented by fish fed conventional feed.

Of note, 100 mg/kg and 200 mg/kg doses of polyphenols represented optimal amounts according to preliminary experiments where lower doses or higher doses have been used.

Sample collection

Liver and muscles samples were collected from a total of 90 sea bass. Sample collection took place during winter at 273 days (T1) and at 323 days (T2) from the beginning of the experiment.

Fish, reared in experimental conditions, were treated according to the "Council Directive 86/609 EEC for the protection of animals used for experimental and other scientific purposes" [31] and the "Ethical justification for the use and treatment of fishes in research" [32].

Collection and preparation of liver and muscles homogenates

Sea bass livers, once collected, were immediately immersed in ice to eliminate any residual blood. Once weighed, each individual liver was gently pressed through a cell filter with a nylon membrane 70 µm (Becton Dickinson, Bedford, MA). Filtrates were resuspended into saline solution to obtain a final concentration of the suspension equal to [0.3-0.5 g/ml]. Samples were aliquoted and stored at -30°C, until use.

Total polyphenol concentrations in cell pellets from 30 samples of each group in triplicates were measured by the Folin-Ciocalteau method [33], modified by Serafini and associates [34] to remove protein interferences. For hydrolyzing the conjugated forms of polyphenols, HCl 1M (Sigma-Aldrich, Milan, Italy) was added to 100 µL of cell pellets, and incubated at 37 °C for 30 min. Then, a mixture of NaOH 2M and methanol was added and incubated at 37 °C for 30 min. Then, metaphosphoric acid 5M was added to cell pellets while they were centrifuged at 1500 × *g* for 10 min. Cell supernatants were kept in ice in the dark, while polyphenols were extracted again adding a solution of acetone 1:1 (v:v) and centrifuged at 2700 × *g* for 10 min. The two supernatants obtained were combined and, before adding Folin-Ciocalteau reagent (Carlo Erba, Milan, Italy), were centrifuged at 5000 × *g* for 5 min. The results were expressed as picogram gallic acid equivalent *per* 1×10^{6} cell pellets (GAE) using a calibration curve obtained with gallic acid standards. Similar procedure was applied to sea bass muscles (data not shown).

Basic Histology and Histochemistry

All fishes were anaesthetized with Tricaine 1:5000 (Fluka BioChemika, Buchs, Switzerland) according to the guidelines for Euthanasia of Nondomestic Animals American Association of Zoo Veterinarians [32]. Liver of each fish was removed, fixed in 10% buffered formalin, later washed in

running water, dehydrated in increasing ethanol concentrations and embedded in paraffin wax. Sections of tissue (5µm thick) were stained with Hematoxylin-Eosin (H&E) (Merck, Darmstadt, Germany) and Perls Van Gieson staining (Bio-Optica, Milan, Italy) to identify ferric iron deposits. Furthermore, the identification of macrophages was performed using the Peroxidase methods (Perox) (Sigma Diagnostics, St. Louis, MO, USA). Histology and histochemistry were run on 30 samples for each group and microscopic observation was randomly performed on 30 different fields.

Quantification of hepatopancreas

The surface occupied by hepatopancreas (μm^2 liver parenchyma) was measured randomly at selected digital fields. Each digital field was photographed with a 40X objective with a digital camera (XC-003P, Sony, Tokyo, Japan) connected to a light microscope (Laborlux 12, Leitz, Wetzlar, Germany). Measurements were performed using image analysis software (QWin, Leica, Cambridge, England).

The various steps of the experimental design are indicated in **Figure 1.**

Starting samples: Farmed sea bass treated with 2 different doses of polyphenol extracts (100 and 200 mg/Kg, respectively) from day 223 to day 273 (T1).
Step 1: Administration of the same dosage of polyphenols will follow from day 273 to day 323 (T2).
Step 2: Morphological analysis of the liver and of the hepatopancreas in treated and untreated fish will be performed at T1 *vs* T2.
Step 3: Measurement of hepatopancreas area sizes under the same experimental conditions will be conducted.
Step 4: Determination of total content of polyphenols in the liver and muscles of treated and untreated fish will be run at T1 *vs* T2.

Figure 1. Description of the experimental design related to polyphenol administration to farmed sea bass.

Statistical studies

Statistical analyses were performed using the GraphPad Prism statistical software release 5.0 for Windows Vista. To compare differences between surfaces occupied by hepatopancreas, at T1 and T2 age, in untreated and treated samples, and to compare the total polyphenolic contents at the same time points, a Student t test was carried out. Differences were considered statistically significant when $p<0.05$.

Results

Basic Histology and Histochemistry

At T1, the histological appearance of the liver, stained with H&E, is depicted in **Figure 2**. Liver parenchyma is arranged according to an heterogeneous architecture. The hepatocytes are polygonal in shape, their cytoplasm is homogenous and weakly basophilic, also showing a conspicuous fat content, represented by one to a few lipid droplets. The hepatocytes arranged as cords are surrounded by a network of sinusoids (**Figure 2, panel a**). These sinusoidal capillaries are narrow and irregularly shaped, appearing throughout the interstice between the hepatic plates. The hepatocyte cords are constituted by two or sometimes more cell layers (**Figure 2, panel b**). Furthermore, several layers of hepatocytes give rise to spherical lobules containing a vein in a more or less central position (central-lobular vein) (**Figure 2, panels b** and **c**). Hepatic artery branches are difficult to be distinguished from veins due to their very thin wall. Bile ducts (BDs) are either associated to a portal vein branch or isolated (**Figure 2**, **panels c** and **d**).

The hepatopancreas develops around the portal vein and consists of a large number of acini present into the liver parenchyma. It can be differentiated from hepatic tissue by its acinar arrangement and characteristic stain with H&E with a basophilic basal pole and a cytoplasm rich in eosinophilic zymogen granules. A thin connective septum separates the hepatocytes from exocrine pancreatic cells (EP) (**Figure 2**, **panels e** and **f**).

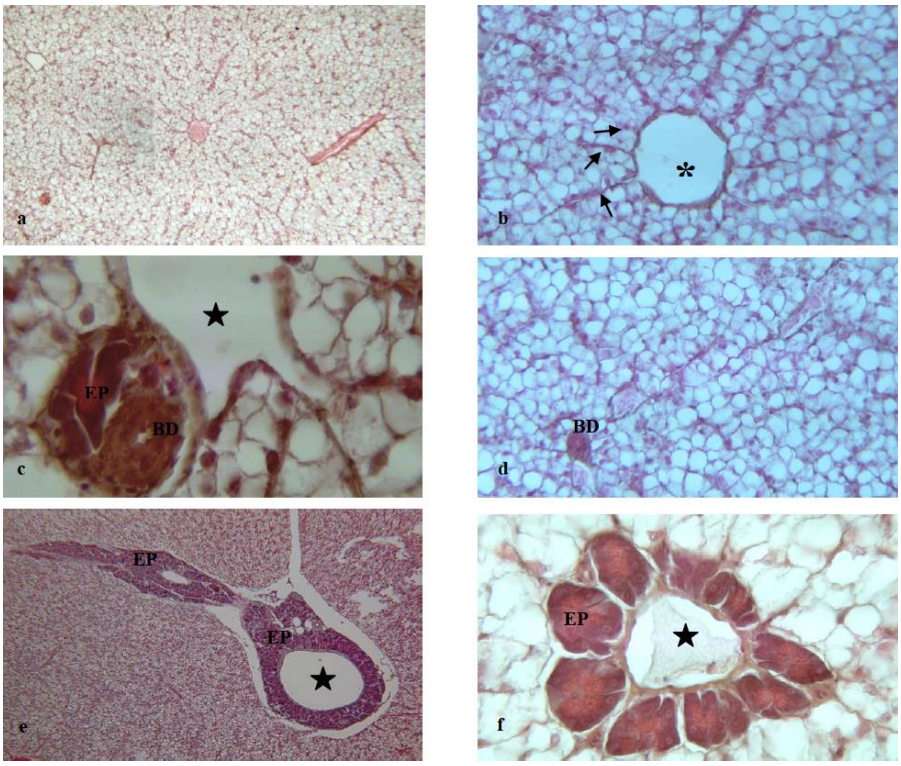

Figure 2. **Panel a:** Hepatic parenchyma of sea bass (*Dicentrharcus labrax* L.) constituted by hepatocytes with conspicuous fat content (H&E., 100X). **Panel b:** The hepatocyte-sinusoidal structures are defined in a tubular form. The hepatocyte lining is double-layered. The sinusoidal capillaries (arrow) are narrow and irregularly shaped with sinusoids appearing throughout the interstice between the hepatic plates. A central vein is indicated with an asterisk (H&E., 250X). **Panel c:** High magnification light micrographs of portal vein (star) accompanied by exocrine pancreatic cells (EP) and a bile duct (BD) (H&E., 400X). **Panel d**: An isolated BD is indicated (H&E., 250X). **Panels e** and **f:** Organization of the intrahepatic exocrine pancreatic tissue around a blood vessel (star), separated from the hepatocyte cords by means of a thin septum of connective tissue (H&E., 250X and 400X, respectively).

Free macrophages **as residual cells** and MMCs are observed in H&E sections, when identified with Perox staining (**Figure 3, panel a**). The presence of ferric iron was detected with Perls Van Gieson staining (**Figure 3, panel b**).

Of note, liver and hepatopancreas morphology, at T2, was quite overlapping that described at T1 (data not shown).

Images are representative of a series of histological observations conducted on all fish.

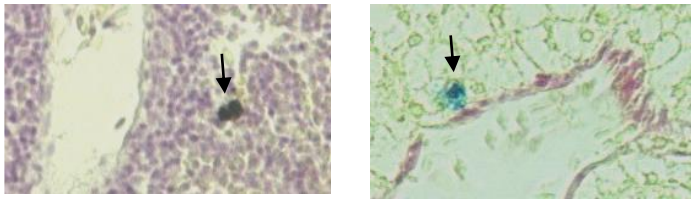

Figure 3. Free macrophages (arrows) in hepatic parenchyma of sea bass (*Dicentrharcus labrax* L.) identified with Perox staining (**Panel a**) and Perls Van Gieson (**Panel b**) (400X).

Quantitative analyses of hepatopancreas

In order to evaluate the effects of dietary polyphenols on sea bass hepatopancreas development, sizes of various areas were measured. The size of intrahepatic exocrine pancreatic tissue greatly varied within the same section around a blood vessel, ranging from 905,950 μm^2 to 3,169 x 10^6 μm^2 at T2, as observed in **Figure 4.** Hepatopancreas area sizes were always higher in treated animals than those in controls (untreated fish), and between samples at T1 versus T2 in the presence of 200 mg grape extract.

Differences were statistically significant.

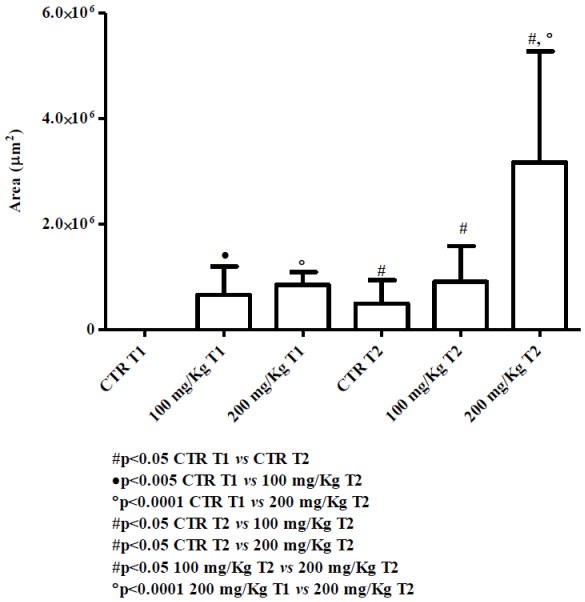

Figure 4. Effects of polyphenols on hepatopancreas area sizes in sea bass (*Dicentrharcus labrax* L.). The surface occupied by hepatopancreas was measured at selected digital fields and photographed with a 40X objective with a

digital camera. CTR=untreated fish hepatopancreas; T1=hepatopancreas at 273 days; T2=hepatopancreas at 323 days. Statistical analysis was performed using the GraphPad Prism statistical software release 5.0 for Windows Vista. To compare differences between different stimuli, a Student t test was carried out. Differences were considered statistically significant when $p<0.05$.

Total polyphenol content in liver and muscles

In liver samples at T1, as indicated in **Figure 5**, concentration of polyphenols decreased in treated fish with 100 and 200 mg polyphenols, respectively, in comparison to controls. At T2 (**Figure 6**) in treated samples polyphenol concentration was overlapping that observed in controls, using 200 mg polyphenols. Conversely, at the dose of 100 mg polyphenols, concentration was still lower than that detected in controls.

With respect to polyphenol concentrations in muscles, the two doses of polyphenols did not modify values in comparison to controls (data not shown).

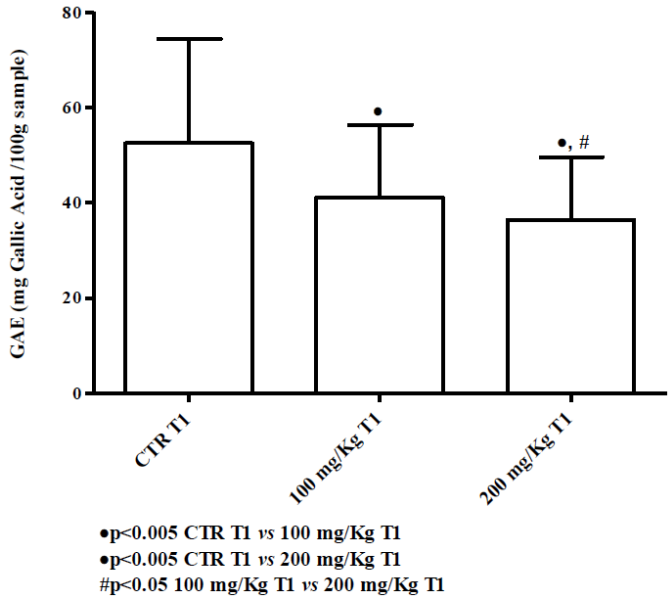

Figure 5. Total polyphenol content in liver sea bass (*Dicentrharcus labrax* L.) at 273 days (T1). Polyphenol contents were expressed as GAE as described in materials and methods. For abbreviations and statistical analyses see **Figure 4**.

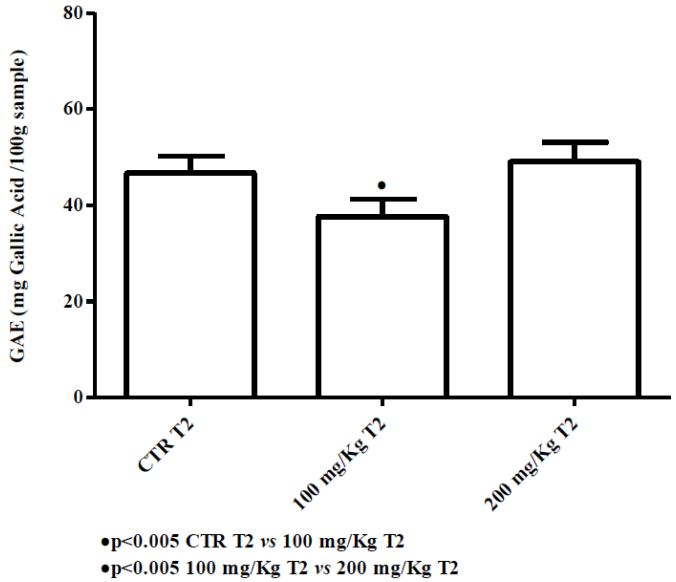

Figure 6. Total polyphenol content in liver sea bass (*Dicentrharcus labrax* L.) at 323 days (T2). Polyphenol contents were expressed as GAE, as described in materials and methods. For abbreviations and statistical analyses see **Figure 4**.

Discussion

Our data clearly demonstrate that administration of polyphenol-enriched feed to 273 day old sea bass leads to a significant increase in area sizes of hepatopancreas at day 323 in comparison to T1 and controls (untreated fish).

As observed in **Figure 3 panel a**, macrophages and MMCs are distinguishable in the context of hepatopancreas. MMCs represent precursors of germinal centers of high vertebrates, endowed with protective functions [35]. In this respect, they perform either destruction or recycling of exogenous and endogenous matter [36]. Moreover, this process leads to the genesis of lipofuscin, melanin and haemosiderin deposits detected in MMCs [37]. Finally, MMCs are able to store iron following erytrophagocytosis and engulf bacterium and parasite spores, also participating to antigen processing [38].

In a recent paper, we have demonstrated that 273 day old sea bass fed polyphenols extracted from seeds of Nero di Troia (*Vitis Vinifera*) underwent an increased release of splenic IFN-γ and a parallel augmentation of MMC area sizes in the spleen [29]. In a parallel study, evidence has been

reported that prolonged dietary treatment with polyphenols could increase kidney MMC activity in European farmed sea bass, thus, suggesting that all MMC system in the fish body is able to respond to this dietary treatment [39]. The fish samples employed in this study have been treated with polyphenols for longer time up to 323 days. In analogy to spleen and kidney, one can hypothesize that such a prolonged administration may have led to an expansion of MMC area sizes in the hepatopancreas. This event implies that hepatopancreas, besides exerting metabolic functions, may represent an additional defense organ in the fish, thus contributing to their longer survival. One can postulate that IFN-γ release in the hepatic milieu may also originate from the contingent of hepatopancreatic macrophages and MMCs, likely potentiating immune protective function. In general terms, IFN-γ is a product of Th1 cells, innate lymphoid cells 1 and macrophages in response to antigenic stimulation mostly related to intracellular pathogen cell wall components [40-42]. Particularly, IFN-γ potentiates the microbicidal activity of macrophages, also restoring the fusion of phagosome with lysosome, otherwise abrogated by *Mycobacterium tuberculosis*, as an example of intracellular organism [43, 44].

In the light of these considerations, it seems that macrophage functions, in the context of hepatopancreas MMCs, may be enhanced by virtue of polyphenol-induced effects. In this regard, we have provided previous evidence on the ability of Negroamaro red wine polyphenols to *in vitro* induce IFN-γ release from normal human lymphomonocytes [45].

With special reference to polyphenol concentrations in the liver, values at 323 days reach those detected in untreated fish. However, in treated animals at 273 days polyphenol concentration is lower than that detected in controls. All together, these data suggest that polyphenol utilization in the liver is maximally expressed at 273 days of dietary regimen, which ultimately leads to the expansion of hepatopancreas detected at the end of the treatment.

In muscles of treated fish, no variations of polyphenol content are observed when compared to control fish. This may depend on the different protective function performed by muscles in comparison to the liver where a more elevated accumulation of MMCs occurs.

Of note, treated sea bass in the interval of time between day 273 and day 323 underwent less mortality in aquaculture in comparison to untreated fish, likely in virtue of polyphenol-dependent potentiation of immune response, even including the contribution of the hepatopancreatic MMCs (unpublished observation).

Conclusion and future perspectives

Farmed fish represent a great source of food and their consumption has tremendously increased globally. However, aquacultures may represent contaminated environments at different degree and according to geographic areas. Microorganisms and various pollutants account for fish mortality in aquacultures and, thus, special feeds to prolong survival of healthy fish are required. With special reference to microbial infections, many attempts have been made to fortify fish immune responsiveness, *e.g.,* using vaccines. However, the current trend is represented by the supplementation to fish of dietary products to combat infections. In fact, many dietary compounds exert beneficial effects on fish health and, in addition, fish meat, when consumed by humans, may represent an additional source of nutrients. Particularly, polyphenols are very much available as a special feed for fish because of abundance of waste products from red grape (skin, seeds) or from extra virgin olive oil and olive leaf tree whose beneficial effects have been demonstrated in *in vitro* and *in vivo* experimental and clinical settings [46-48]. Also in view of the reduced mortality in treated fish, one can envisage a diminished use of antibiotics, commonly added to aquacultures [49]. Finally, as also reported in wild fish, liver MMCs seem to represent appropriate biomarkers to evaluate fish healthy status [50, 51]. Therefore, further studies will be aimed at the functional evaluation of MMCs in hepatopancreas in farmed and wild fish. Finally, the use of polyphenols in

aquaculture needs to be evaluated also under pathological circumstances such as stressful conditions (overcrowding) or bacterial, parasitic and viral infections.

List of abbreviations

BD= Bile duct;

EP= Exocrine pancreatic cells;

H&E= Hematoxylin-Eosin;

IFN= Interferon;

IL= Interleukin;

MMCs= Melano-macrophages centers;

Perox= Peroxidase methods;

Conflict of Interests

The authors declare that they have no competing interests.

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