



## Partial dietary fishmeal replacement with full-fat or defatted superworm (*Zophobas morio*) larvae meals modulates the innate immune system of gilthead seabream, *Sparus aurata*

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### ARTICLE INFO

#### Keywords:

Dietary insects  
Hematology  
Immunology  
Antibacterial activity  
Inflammation

### ABSTRACT

Full-fat and defatted superworm *Zophobas morio* larvae meals were used in a feeding trial of 540 gilthead seabream, *Sparus aurata*, juveniles (3.4 g initial weight) in order to assess their immunomodulatory effects. Six isonitrogenous (52 %) and isoenergetic (21 MJ/Kg) diets were formulated where the fishmeal of the control diet was replaced by full-fat *Z. morio* meal at 5 % and 10 % (FF5 and FF10) or defatted *Z. morio* meal at 10, 20 or 30 % (LF10, LF20 and LF30). Fish were kept in 18 glass tanks (125 L) within a closed recirculation seawater system and fed to satiation twice a day, 6 days per week for 100 days. LF20 significantly increased the percentage of neutrophils compared to a control fish-fed fishmeal (FM)-based diet. The dietary *Z. morio* meal immunomodulated the fish as expressed through an increased complement-associated bacterial killing (FF5 and FF10), nitric oxide production (LF30), and a tendency for an increased activity of lysozyme (FF5, LF20 and LF30) and of myeloperoxidase (FF5 and LF20), while trypsin inhibition was significantly suppressed by dietary FF5 and all 3 LF-containing diets compared to control FM-fed fish. These findings suggest that dietary *Z. morio* can immunomodulate gilthead seabream but further infectious challenge studies will have to be performed to determine if these effects are translated into an increased resistance to diseases.

### 1. Introduction

Gilthead seabream (*Sparus aurata*) is among the most important marine fish species reared in Europe with an annual world production of around 280,000 tons in 2022 (FAO, 2022). The use of fishmeal (FM) as the major protein source in aquafeeds is however problematic because of the increasing demands facing a stagnant production and depletion of wild fish stocks. Up to recently, alternatives to FM mainly consisted of protein-rich plant meals, but those compete with human and animal nutrition and contain anti-nutrient factors with a strong environmental impact (Kokou and Fountoulaki, 2018) that may also have adverse secondary effects on fish growth, reproduction, intestinal morphology, gut microbiota, immune status and health (Colombo, 2020). Other alternatives such as animal by-products are therefore under scrutiny, but consumers seem reluctant to eat animals fed with blood, bone, or feather

meals due to the several recent food crises and severe subsequent restrictions on their use in animal feed (Jedrejek et al., 2016). Due to their diversity, nutritive quality, sustainability, and recent approval for use in European aquafeed, insect meals have attracted much research interest (Alfiko et al., 2022). Given the high nutritional and environmental value of insects, they may become a favourite alternative to be used in animal feed in the future if their price, availability and composition stability problems are overcome. Together with aquaculture by-products, insects have been shown to have a great potential to be included in aquafeeds (Henry et al., 2015b; Hua et al., 2019). Among the insect species used thus far, most studies have focused on yellow mealworm, *Tenebrio molitor* and black soldier fly, *Hermetia illucens* (Gasco et al., 2020). However, other species mostly overlooked thus far could also be suitable for FM replacement. The giant mealworm, *Zophobas morio* (ZM) is a large tenebrionid beetle species with a high nutritive value that is

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<https://doi.org/10.1016/j.aqrep.2022.101347>

Received 17 June 2022; Received in revised form 7 September 2022; Accepted 24 September 2022

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commonly reared as feed for birds and reptiles (Rumbos and Athanassiou, 2021). It is generally considered as a pest of grain and flour (Alves et al., 2021) but it can be raised on a low-nutritive plant substrate efficiently converting it into high-value lipids and proteins with good levels of essential amino acids (Jabir et al., 2012; Rumbos and Athanassiou, 2021). The large size of ZM larvae may represent an advantage to increase the yield of insect meal available for aquafeeds. It has been successfully included in the diet of Nile tilapia, *Oreochromis niloticus*, at levels of 7.5–22.5 % inclusion thus replacing 25–75 % of the dietary FM (Jabir et al., 2012). In juvenile rainbow trout, *Onchorhynchus mykiss*, 5.5 % and 11 % of defatted ZM successfully replaced successfully 10–20 % of the dietary FM but 16.5 % and 22 % of defatted ZM replacing 30–40 % of dietary FM significantly affected fish growth (Hosseini Shekarabi et al., 2021). The fact that insects usually have a high oil content and are poor in omega-3 poly-unsaturated fatty acids which may represent a problem for fish nutrition and the quality of the final product, underlines the need to determine maximal dietary inclusion levels of full-fat (FF) and defatted (or low-fat, LF) insect meal devoid of negative effects on fish growth or health. Beyond their use as a FM alternative, insects may also represent an asset through the promotion of fish health as shown in a recent review (Gasco et al., 2020). They can thus be considered as nutraceuticals (Dietz and Liebert, 2018). Indeed, a recent study showed the effects of 10 % dietary inclusion of hydrolyzed ZM meal (56 % FM replacement) on the gut histology, microbiota and immune parameters of sea trout (*Salmo trutta trutta*) (Mikotajczak et al., 2020). The effect of dietary FF ZM at levels as high as 30 % was investigated on the growth, body proximal composition, haematological and innate immune responses of Nile tilapia (*O. niloticus*) (Alves et al., 2021) while the effect of defatted ZM was tested on the fish growth and haematology of Asian sea bass (*Lates calcarifer*) (Prachom et al., 2021). These recent developments motivated the present investigation on the effects of dietary Z. morio, used either as a FF or a LF larvae meal, on the health parameters of gilthead seabream.

## 2. Materials and methods

### 2.1. Experimental protocol-ethics aspects

Juveniles gilthead seabream were transferred from a licensed commercial hatchery (Breeding Code: GR06FISH0008) to the experimental aquarium facilities (EL-43BIO/exp-01) of the Aquaculture Laboratory of the Department of Ichthyology and Aquatic Environment at the University of Thessaly (Volos, Greece). All fish handling processes were performed according to the EU guidelines on the protection of animals used for scientific purposes (Directive 2010/63/EU). The research complied with the commonly-accepted '3Rs' and the ARRIVE (Animal Research: Reporting of in vivo Experiments) guidelines and was endorsed for experiments using live animals. Before the experimentation, the protocol and necessary documents were prepared by FELASA accredited scientists and were approved by the Department's Ethics Committee and the competent authority (Decision N°16048-01.08.2019). During the experimentation, a supervision protocol was applied by the person responsible for the protocol and the Welfare Committee member of the Department. A FELASA-accredited scientist was responsible for the supervision of care, procedures, samplings, anaesthesia and euthanasia of experimental fish.

### 2.2. Insect meal

ZM larvae were raised on a mixture of wheat bran (90 %) and egg layer hens feed (10 %). Late-instar larvae were collected, dried at 60 °C for 12 h and milled, to obtain a FF ZM meal that consisted (as fed) of 11.9 % moisture, 41.0 % crude protein, 39.7 % crude lipid, and 27.2 kJ/g gross energy. Another batch of the FF ZM was then defatted using petroleum ether extraction at 40 °C for 3 h (Horwitz and Latimer, 2016) to produce a LF ZM meal containing 5.4 % moisture, 69.4 % crude

protein, 3.8 % crude lipid, and 21.7 kJ/g gross energy.

### 2.3. Fish, diets and sampling

The 540 fish were distributed in 18 glass tanks (125 L) where they acclimated to the closed recirculation seawater system for 10 days before the start of the experiment when fish reached  $3.40 \pm 0.31$  g of mean initial body weight.

The five insect-based diets were formulated based on the control diet containing 61 % FM (65.5 % crude protein, Köster Marine Proteins GmbH, Hamburg, Germany) so that the FM-issued protein of the control diet was replaced by 5 or 10 % of FF ZM-protein (diets FF5 and FF10), or by 10, 20 or 30 % of LF ZM-protein (diets LF10, LF20 and LF30) corresponding to 4.9 % and 9.7 % of FF ZM and 5.8 %, 11.6 % and 17.5 % of LF ZM dietary inclusion, respectively in the 5 experimental diets. These inclusion levels enabled the formulation of 6 isonitrogenous (52 % crude protein) and isoenergetic (21 MJ/kg) diets.

Each experimental diet was supplied to triplicate tanks stocked with *S. aurata* to apparent satiation twice a day, 6 days per week for a period of 100 days. Mortality did not exceed 5 % in any of the experimental groups. Water quality parameters were monitored routinely with water temperature being maintained at  $21.0 \pm 1.0$  °C, pH at  $8.0 \pm 0.3$ , salinity at  $33 \pm 0.3$  g/L, dissolved oxygen at  $> 6.5$ /L, total ammonia-nitrogen at  $< 0.1$  mg/L, and photoperiod at 12:12 hr (light:darkness).

At the end of the feeding period of 100 days, 5 fish per tank (15 fish per dietary treatment) with a 40 g mean final body weight were fasted for 24 h and anaesthetized with tricaine methane-sulfonate (150 ppm). Blood samples were obtained from the caudal vein using non-heparinized syringes fitted with 23 G needles. A 200 µl of blood aliquot was heparinized for the haematological assessment. The remaining non-heparinized blood was left to clot and centrifuged. The collected serum was stored at  $-80$ °C until immunological analyses were performed.

### 2.4. Haematological parameters

Haematological parameters were assessed in heparinized whole blood. A total number of erythrocytes (RBC) and leucocytes (WBC) were manually counted using a Neubauer haemocytometer (Hausser Scientific, USA), while haematocrit (Ht) was determined using heparinized microhaematocrit capillaries centrifuged at 14490 g for 10 min. Differential leukocyte counts were performed on blood smears stained with May Grünwald & Giemsa (Sigma-Aldrich, Germany), counting 100 cells.

### 2.5. Immunological parameters

The serum antibacterial activity of lysozyme was assessed as described previously (Kokou et al., 2012) but kinetics were assessed at 450 nm every minute for 20 min. The antibacterial activity of the serum against a luminescent strain of *E. coli* was assessed as described previously (Henry et al., 2015a) in a 384-wells microplate on 20 µl of serum incubated with 10 µl of a solution of 100 mM  $Mg^{2+}Ca^{2+}$  for 30 min at 25 °C which then received 30 µl of *E. coli* (grown in LB medium at 37 °C to exponential phase and adjusted to  $OD_{450\text{ nm}} = 0.4$  after blank correction) and 40 µl of LB medium. Bacterial growth was followed for 20 h at 25 °C by a luminometer (GeniosPro, Tecan, Austria). The anti-protease activity was determined using azocasein as a substrate and trypsin as a standard (Henry and Fountoulaki, 2014). Nitric oxide was determined using the Griess reagent (Henry et al., 2015a) modified for use in 384-wells microplates by incubating 60 µl of serum or standard  $NaNO_2$  solution diluted with 30 µl 2.5 %  $H_3PO_4$  and 10 µl of Griess reagent at room temperature for 10 min before assessing the optical density at 550 nm. The myeloperoxidase activity was determined as described previously (Kokou et al., 2012; Henry et al., 2015a). The ceruloplasmin activity was determined using p-phenylenediamine as a substrate as described previously (Henry and Fountoulaki, 2014).

2.6. Statistical analyses

Normality of population and homogeneity of variances were checked using the Kolmogorov-Smirnov and Levene tests, respectively. Concerning the myeloperoxidase activity and the nitric oxide concentration, data were transformed using Ln to obtain homogenous variances. All data are presented as mean ± standard error (S.E.M.). Mean values were compared among treatments using a One-Way analysis of variance (ANOVA) and Tukey's t-test were performed when appropriate. When homogeneity of variances was not achieved, Welch correction was applied to the One-Way ANOVA and Duncan tests were applied to the data. The differences were considered to be significant at  $p < 0.05$ .

3. Results

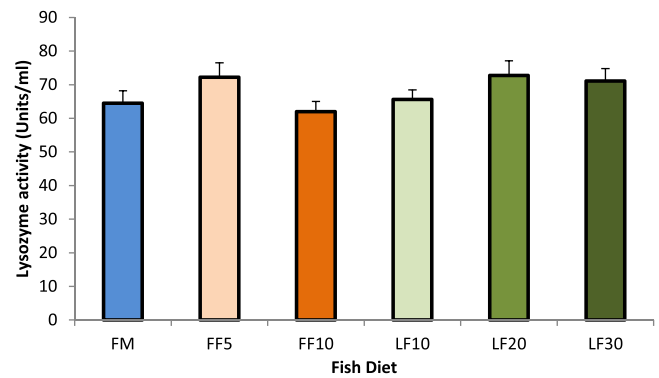
The haematological parameters of *S. aurata* fed with the experimental diets are shown in Table 1. The inclusion of dietary ZM larvae meal did not affect the values of Ht or RBC and WBC counts, although WBC counts tended to be increased in fish fed FF5, LF20 and LF30 compared to control fish. According to the differential leukocyte count, lymphocytes and neutrophils represented the 2 main cell types in the sampled fish. While no significant changes were observed in the counts of lymphocytes and eosinophils, neutrophil percentages were significantly higher in fish fed the LF20 diet compared to control fish and monocyte percentages were significantly lower in fish fed the LF20 diet compared to fish fed the FF10 diet.

The serum lysozyme activity was not significantly affected by the inclusion of ZM meal in the diet of gilthead seabream (Fig. 1). The serum killing of *E. coli* was significantly increased in fish fed the FF5 and FF10 compared to fish fed the FM-based control diet (Fig. 2). It was also increased in all groups of fish fed the LF ZM meal but not significantly so (Fig. 2). The time necessary for the complement complex assembly or for killing 50 % of the bacteria did not vary significantly between dietary treatments (data not shown). The anti-protease activity was significantly affected in fish fed both the full-fat (FF5) and defatted (LF20 and LF30) insect meals (Fig. 3). The serum nitric oxide concentration was significantly higher in fish fed 30 % of LF ZM meal (LF30) than in control fish fed the FM-based diet or in fish fed FF ZM (Fig. 4). The myeloperoxidase (Fig. 5) and ceruloplasmin (Fig. 6) activities of the fish serum did not show any significant difference between dietary treatments. However, a tendency for increased myeloperoxidase was noticed in fish fed FF5 and LF20 while ceruloplasmin activity tended to decrease in fish fed FF5, LF20 and LF30 compared to control fish-fed the FM-based diet.

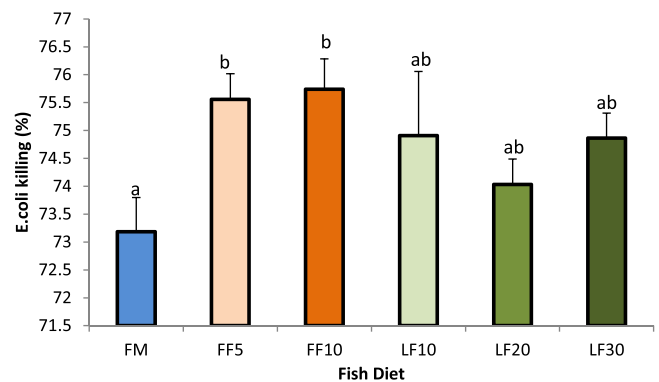
**Table 1**  
Haematological parameters of gilthead seabream fed the experimental diets for 100 days.

	FM	FF5	FF10	LF10	LF20	LF30
Ht (%)	28.93 ± 0.53	28.67 ± 1.25	25.87 ± 0.54	28.13 ± 1.07	26.47 ± 0.84	28.60 ± 0.63
RBC ( $10^6 \mu\text{l}^{-1}$ )	1.47 ± 2.18	1.59 ± 0.23	1.39 ± 0.18	1.49 ± 1.53	1.54 ± 1.31	1.45 ± 0.85
WBC ( $10^3 \mu\text{l}^{-1}$ )	59.04 ± 0.10	62.04 ± 1.19	58.91 ± 0.91	54.88 ± 0.50	59.65 ± 0.19	59.83 ± 0.75
Lymphocytes (%)	69.93 ± 2.94	62.47 ± 2.89	61.87 ± 5.48	61.07 ± 5.54	56.73 ± 4.93	58.86 ± 2.34
Neutrophil (%)	26.40 ± 2.50 <sup>a</sup>	33.30 ± 2.11 <sup>ab</sup>	34.87 ± 5.34 <sup>ab</sup>	35.00 ± 5.72 <sup>ab</sup>	41.47 ± 6.62 <sup>b</sup>	38.27 ± 2.81 <sup>ab</sup>
Eosinophil (%)	2.93 ± 0.55	3.87 ± 1.07	2.40 ± 0.20	3.30 ± 0.35	2.47 ± 0.18	2.33 ± 0.53
Monocytes (%)	0.73 ± 0.24 <sup>ab</sup>	0.33 ± 0.07 <sup>ab</sup>	1.00 ± 0.12 <sup>b</sup>	0.60 ± 0.20 <sup>ab</sup>	0.00 ± 0.00 <sup>a</sup>	0.53 ± 0.13 <sup>ab</sup>

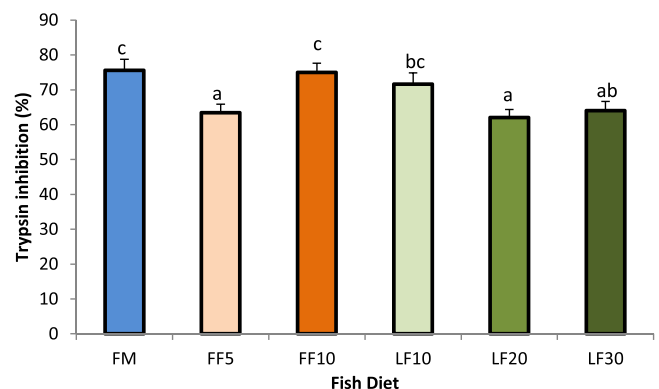
Data are presented as mean ± S.E.M. (n = 3). Different letters within the same row indicate a significant difference between diets (ANOVA;  $P < 0.05$ , n = 15). Abbreviations: Ht, haematocrit; RBC, red blood cells; WBC, white blood cells.



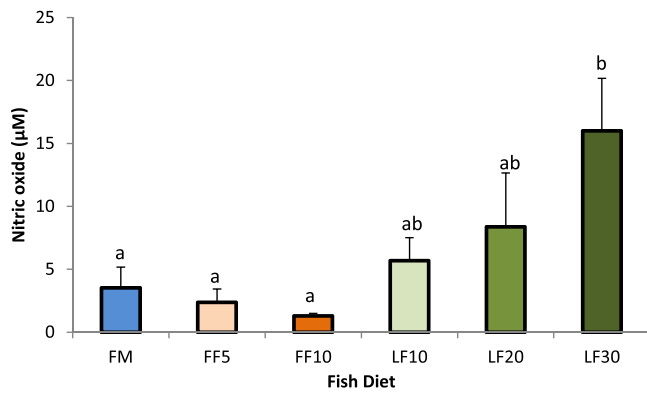
**Fig. 1.** Lysozyme activity of the serum of gilthead seabream fed for 100 days diets where the dietary protein was provided only by fishmeal (FM), or by 5 or 10 % full-fat *Z. morio* (FF ZM) meal (diets FF5 and FF10) or by 10, 20 or 30 % of defatted *Z. morio* (LF ZM) meal (diets LF10, LF20, and LF30). Bars represent mean ± S.E.M. (n = 15). There was no significant difference between dietary treatments (One-Way ANOVA,  $P > 0.05$ ).



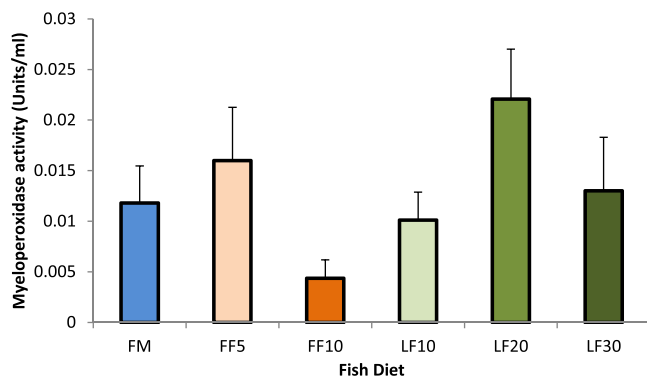
**Fig. 2.** Percentage of complement-mediated bacterial killing in the serum of gilthead seabream fed for 100 days diets where the dietary protein was provided only by fishmeal (FM), or by 5 or 10 % full-fat *Z. morio* (FF ZM) meal (diets FF5 and FF10) or by 10, 20 or 30 % of defatted *Z. morio* (LF ZM) meal (diets LF10, LF20, and LF30). Bars represent means ± S.E.M. (n = 15). Different letters represent a significant difference between dietary treatments (Welch-corrected ANOVA,  $P = 0.029$ ; Duncan t-test).



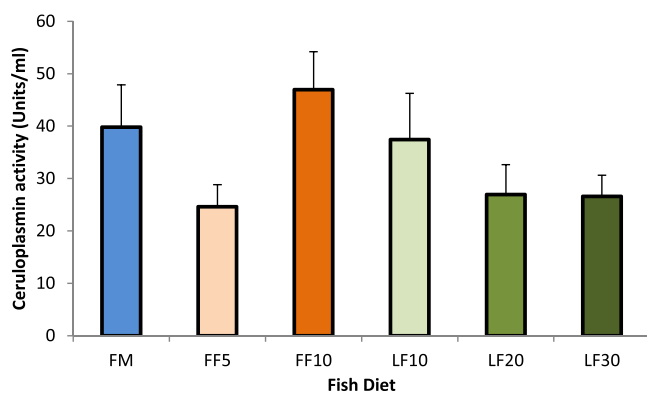
**Fig. 3.** Anti-protease activity of the serum of gilthead seabream fed for 100 days diets where the dietary protein was provided only by fishmeal (FM), or by 5 or 10 % full-fat *Z. morio* (FF ZM) meal (diets FF5 and FF10) or by 10, 20 or 30 % of defatted *Z. morio* (LF ZM) meal (diets LF10, LF20, and LF30). Bars represent means ± S.E.M. (n = 15). Different letters represent a significant difference between dietary treatments (One-Way ANOVA,  $P = 0.0005$ ; Duncan t-test).



**Fig. 4.** Nitric oxide concentration in the serum of gilthead seabream fed for 100 days diets where the dietary protein was provided only by fishmeal (FM), or by 5 or 10 % full-fat *Z. morio* (FF ZM) meal (diets FF5 and FF10) or by 10, 20 or 30 % of defatted *Z. morio* (LF ZM) meal (diets LF10, LF20, and LF30). Bars represent means  $\pm$  S.E.M. ( $n = 15$ ). Different letters represent a significant difference between dietary treatments (One-Way ANOVA of the ln-transformed data,  $P=0.011$ ; Duncan t-test).



**Fig. 5.** Myeloperoxidase activity of the serum of gilthead seabream fed for 100 days diets where the dietary protein was provided only by fishmeal (FM), or by 5 or 10 % full-fat *Z. morio* (FF ZM) meal (diets FF5 and FF10) or by 10, 20 or 30 % of defatted *Z. morio* (LF ZM) meal (diets LF10, LF20, and LF30). Bars represent means  $\pm$  S.E.M. ( $n = 15$ ). There was no significant difference between dietary treatments (Welch-corrected One-Way ANOVA of the ln-transformed data,  $P>0.05$ ).



**Fig. 6.** Ceruloplasmin activity of the serum of gilthead seabream fed for 100 days diets where the dietary protein was provided only by fishmeal (FM), or by 5 or 10 % full-fat *Z. morio* (FF ZM) meal (diets FF5 and FF10) or by 10, 20, or 30 % of defatted *Z. morio* (LF ZM) meal (diets LF10, LF20, and LF30). Bars represent means  $\pm$  S.E.M. ( $n = 15$ ). There was no significant difference between dietary treatments (One-Way ANOVA,  $P>0.05$ ).

#### 4. Discussion

All groups of fish promptly accepted the respective diets and had a similar feed intake and growth performance (Asimaki et al., 2020a and 2020b). Haematological parameters are essential tools as indicators of the physiological and physio-pathological changes in fish (Hrubic et al., 2000). A recent meta-analysis review has reported physiological levels of haematological parameters for *S. aurata* (Michail et al., 2022). Hematocrit and RBC counts in the present trial were relatively low compared to these suggesting that the fish were physiologically impaired. The WBC counts obtained in the present study were in the high range of the physiological values described in this meta-analysis (Michail et al., 2022). Blood profile has been acknowledged as a valuable tool to evaluate the effect of ingredient replacement on fish health status in different species (Shamna et al., 2017; Fawole et al., 2017). However, no data are available as far as ZM meal is concerned. According to the present results, blood parameters, such as RBC counts, Ht and WBC counts were not markedly altered among the various dietary treatments, indicating that dietary ZM larvae meal, either FF or LF, in the diet does not have negative effects on gilthead seabream's hematology, even at high dietary inclusion level. The same was true in the study of ZM inclusion in the diet of Nile tilapia (Alves et al., 2021) or Asian sea bass (Prachom et al., 2021). Similar results have been mentioned in the case of substitution of FM with other insect meals like BSF larval meal in the diet of Nile tilapia (*Oreochromis niloticus*), African catfish (*Clarias gariepinus*) and European sea bass (*Dicentrarchus labrax*) (Fawole et al., 2020; Adeoye et al., 2020; Abdel-Tawwaba et al., 2020, Tippayadara et al., 2021). WBC were significantly increased in African catfish (*C. gariepinus*) fed cricket meal (*Gryllus bimaculatus*) and fruit fly pupae (*Drosophila melanogaster*) (Okore et al., 2018; Taufek et al., 2018). Differential leucocyte counts, which are important parameters in the non-specific immunity of fish, revealed differences between the dietary groups. Concerning neutrophil counts, higher values were recorded in all ZM-fed groups compared to the FM group, indicating potential immunomodulating properties of the insect meal, as neutrophils contribute to the tissue's repair, to the killing mechanisms against pathogens and stimulation of macrophages, thus playing an important role in the immune response (Nathan, 2006). Neutrophil counts were also increased by 30 % of dietary ZM meals in Nile tilapia (Alves et al., 2021). This neutrophilia could be due to the potential similarities between insects and parasites' exoskeleton triggering the fish to increase neutrophil production to fight this perceived infection. The neutrophilia could also be considered a negative effect as it may reflect an infectious state of the fish fed the insect meal. However, no sign of infection was detected in the fish at any moment during the trial and mortality were very low in all experimental dietary treatments.

As observed with other insect meals, the present study showed that dietary ZM meal affects the fish immune system. Other dietary insects have been shown to immunostimulate various fish species: Yellow mealworm, *Tenebrio molitor*, immunostimulated pearl gentian grouper, (*Epinephelus lanceolatus* x *Epinephelus fuscoguttatus*, Song et al., 2018), European sea bass (*D. labrax*, Henry et al., 2018a), rainbow trout (*Oncorhynchus mykiss*, Henry et al., 2018b) and mandarin fish (*Siniperca scherzeri*, Sankian et al., 2018); BSF immunostimulated yellow catfish (*Pelteobagrus fulvidraco*, Xiao et al., 2018); dietary housefly (*Musca domestica*) pupae immunostimulated red seabream (*Pagrus major*, Ido et al., 2015) while silkworm pupae meal immunostimulated Jian carp (*Cyprinus carpio*, Ji et al., 2015) and cricket meal immunostimulated African catfish (*C. gariepinus*, Taufek et al., 2016, 2018). The various dietary insects studied seem to consistently stimulate serum lysozyme activity in the different fish species. The lysozyme activity is quite low as usually found in the serum of gilthead seabream compared to other fish species (Kokou et al., 2012; Henry et al., 2015a; Henry et al., 2020; Montero et al., 1999). Although not significantly so, dietary ZM tended to increase the lysozyme activity in fish fed FF5 or LF20 and LF30 in seabream in the present study, and in Nile tilapia when added at 30 %



(Alves et al., 2021) similarly to BSF in yellow catfish (Xiao et al., 2018) and *T. molitor* in European sea bass (Henry et al., 2018). The increase of lysozyme was significant in the case of African catfish fed cricket meal (Taufek et al., 2018). There was no significant effect on the lysozyme activity of *T. molitor*-fed rainbow trout (Henry et al., 2018b) or of hydrolyzed FF ZM-fed sea trout (Mikołajczak et al., 2020). Another important antibacterial component of the fish immune system is the complement cascade. In the present study the complement-associated *E. coli* killing was increased in all seabreams fed the ZM diets, being significantly higher in fish fed the FF meal. The haemolytic activity of complement was also increased in Nile tilapia fed 15 % FF ZM (Alves et al., 2021). The *E. coli* killing activity was not affected in European sea bass or rainbow trout fed TM but was slowed down in rainbow trout (Henry et al., 2018a and 2018b). ZM reduced the anti-protease activity in the serum of seabream, being significantly lower in some dietary groups (FF5, LF20 and LF30), but the opposite was true in European sea bass (Henry et al., 2018a) and rainbow trout (Henry et al., 2018b) fed *T. molitor*. LF ZM significantly increased the nitric oxide (NO) production in seabream sera as observed in the case of the LF30 diet. However, NO levels in seabream were relatively low compared to those found in European sea bass and rainbow trout where dietary *T. molitor* significantly decreased NO production (Henry et al., 2018a and 2018b). The myeloperoxidase activity which assesses the circulation of peroxide, a potent reactive oxygen species (ROS) tended to increase in some dietary groups of seabream (FF5 and LF20) as has been observed in rainbow trout feeding on *T. molitor*, where the activity was particularly high (Henry et al., 2018b). On the contrary, MPO decreased significantly in European sea bass fed *T. molitor* (Henry et al., 2018a). As in rainbow trout (Henry et al., 2018b), ceruloplasmin activity, which is linked to the inflammatory response (Das et al., 2018), tended to be reduced in fish fed FF5 and LF20 and LF30 diets in the present study suggesting an anti-inflammatory action of dietary ZM. This reduction of the ceruloplasmin activity was significant in European sea bass fed *T. molitor* (Henry et al., 2018a). Except for the reduced anti-protease activity and proportion of monocytes, most studied immunological parameters measured in the sera of gilthead seabream tended to show an increased activity with the dietary inclusion of ZM.

Many insects have shown antimicrobial and anti-inflammatory properties linked to bioactive substances such as antimicrobial peptides or to their richness in chitin and short-chain fatty acids such as lauric acid (Gasco et al., 2020). The immunomodulating activities observed in the present study could be due to the chitin content of the insects (Henry et al., 2015b). The closely related crustacean chitin has been shown to increase the lysozyme, complement and/or respiratory burst activities when included in the diet of various fish species (Mari et al., 2014; Esteban et al., 2001; Gopalakannan and Arul, 2006; Siwicki et al., 1994; Vahedi and Ghodrati-zadeh, 2011; Sakai et al., 1992) and could explain the increased antibacterial and myeloperoxidase activities of the present study. Moreover, some soluble acidic polysaccharides such as dipterose isolated from melon fly (*Bactrocera cucurbitae*) (Ohta et al., 2014), silkrose in Japanese oak silkworm (*Antheraea ayamamai*) (Ohta et al., 2016) and silkworm (*Bombyx mori*) (Ali et al., 2018) have been shown to activate nitric oxide production and production of proinflammatory cytokines in murine macrophages (Ohta et al., 2014, 2016; Ali et al., 2018). This could explain the present results concerning the increased NO production and the tendency for increased myeloperoxidase.

Furthermore, because insects often live in hostile environments, they have developed many antimicrobial compounds such as fatty acids or antimicrobial peptides, AMPs (Engel and Moran, 2013) which may be useful in prolonging the shelf-life of aquafeeds and may potentially immunostimulate fish or act indirectly or directly on the fish pathogens. A recent study showed that ZM contains fatty acids (octadecenoic acid, C16:0 and octadecadienoic acid, C18:0) and their methyl esters (Golebiowski et al., 2020) which have been shown to exhibit antibacterial activity (Zheng et al., 2005; Skalicka-Woźniak et al., 2010; Urbanek

et al., 2012; Golebiowski et al., 2013). Insect-produced AMPs include  $\alpha$ -helical cecropin-like and coprisin peptides, cysteine-rich defensin-like peptides, proline-rich peptides or glycine-rich peptides as reviewed recently (Veldkamp et al., 2021) or the antifungal gallerimycin produced by greater wax moth larvae, *Galleria mellonella* (Schuhmann et al., 2003). Peptides with both antifungal and antibacterial activities were isolated from *Musca domestica* (Fu et al., 2009; Hou et al., 2007). Three AMPs (coleoptericin, defensins B, and defensins C) have also been isolated from the close relative of ZM, *Zophobas atratus* and showed antibacterial activity against both Gram-positive and Gram-negative bacteria (Bulet et al., 1991). A recent study suggests that ZM contains a high diversity of antimicrobial molecules as shown by the multiple bands on SDS-PAGE analyses of *E. coli*-induced ZM hemolymph (Du et al., 2020). Interestingly, the killing activity of ZM hemolymph was shown against *E. coli* (Du et al., 2020) and could be related to the increased complement-mediated *E. coli* killing observed in the present study. AMPs are indeed not only directly antibacterial, but they also have an immunostimulating effect as exemplified by the insect-produced apidaecin, which was shown to increase the lysozyme activity of two species of carp (He et al., 2011; Zhou et al., 2008). The increased antibacterial activities (lysozyme and complement-mediated *E. coli* killing) of the fish-fed ZM obtained in the present study suggest that this insect either immunostimulates the fish or directly provides antibacterial compounds.

Also, many wild-caught insects or *Z. morio* fed on a basal diet enriched in carotenoids contain significant amounts of carotenoids which act as precursors of vitamin A and are involved in the coloration and reproduction of insectivores (Finke, 2015). Carotenoids may also act as strong anti-oxidants and have an anti-inflammatory role and may regulate the immune system of fish (Nakano and Wiegertjes, 2020). As carotenoids are lipid-soluble pigments, they may have been involved in the increased anti-*E. coli* activity of the serum of fish fed FF-ZM compared to all other dietary groups. It would be interesting in future studies to determine carotenoids and short-chain fatty acids levels in dietary insect meals to see if the antibacterial activity and other fish immune parameters are dose-related to these compounds.

Such dietary insect-triggered immunostimulation of various fish species has been linked to some protective effects against common fish diseases, such as yellow mealworm and common housefly offering some protection against *Edwardsiella tarda* to red seabream, *Pagrus major* (Ido et al., 2015, 2019), yellow mealworm slightly increasing resistance of pearl gentian grouper, *Epinephelus lanceolatus* x *Epinephelus fuscoguttatus* against *Vibrio harveyi* (Song et al., 2018), cricket meal offering some protection to African catfish, *C. gariepinus*, against *Aeromonas hydrophila* (Taufek et al., 2018), while maggot meal also enhanced resistance of black carp against *A. hydrophila* (Ming et al., 2013). Some of these studies showed very high protection efficacy. Concerning *Z. morio*, no study has yet investigated its effect on fish resistance to diseases but a recent study on Nile tilapia showed that fish fed ZM and injected with lipopolysaccharides from a Gram-negative bacterium to mimic bacterial infection showed significantly increased neutrophil and thrombocyte counts, lysozyme antibacterial activity and complement hemolytic activity suggesting a potential increased resistance of those fish against Gram-negative bacteria (Alves et al., 2021). Infectious challenges should be performed with dietary *Z. morio* to assess if the immunomodulation observed in the present study is translated into an increased resistance to diseases.

## 5. Conclusion

The present study showed the immune-modulating activity of dietary *Z. morio* meal. The different effects of LF and FF ZM on the antibacterial activity of the fish sera against *E. coli* suggested that lipid-bound compounds present in ZM meal may have either a direct antibacterial activity or stimulate the fish to increase their antibacterial activity. Moreover, the increased nitric oxide concentration, the neutrophilia and

decreased monocytes counts and trypsin inhibition observed in fish fed LF ZM compared to FF ZM or FM fed fish, suggested that dietary FF Z. morio meal may be more beneficial to Gilthead seabream than the defatted meal. The fat of Z. morio may bring some lipid-bound active or immunomodulating compounds that may be lost in the defatting process. It would be very interesting to use FF ZM meal in further challenge experiments to test if its dietary inclusion offers some protection of gilthead seabream against common bacterial (vibriosis, photobacteriosis) and/or parasitic (Monogeneans) infections.

## Funding

This study was part of the project coded MIS 5045804 (T6YBII-00076) that has been co-financed by the Hellenic Republic and the EU under the "Operational Programme Competitiveness, Entrepreneurship and Innovation - EPAnEK 2014-2020".

## CRediT authorship contribution statement

M.A.H. participated to the experimental design, performed the immunology analyses and wrote the MS. E.G. performed the haematological analyses and participated to the writing of the MS. A.A. and P.P. performed the nutritional experiment and data collection. E.F., E.M. and I.T.K. participated to the experimental design, data analyses and writing the MS. C.I.R. and C.G.A. provided the insect meal and participated to the writing of the MS.

## Declaration of Competing Interest

There was no conflict of interest.

## Data Availability

Data will be made available on request.

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