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Does dietary insect meal affect the fish immune system? The case of mealworm, Tenebrio molitor on European sea bass, Dicentrarchus labrax

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Abstract

Feeding small European sea bass, Dicentrarchus labrax, for 6 weeks with Tenebrio molitor larval meal showed significant anti-inflammatory responses (ceruloplasmin, myeloperoxidase and nitric oxide). Serum bacteriolytic activity against a Gram negative bacterium was not significantly affected by dietary Tenebrio, while both lysozyme antibacterial activity and serum trypsin inhibition usually linked to the anti-parasite activity of the fish, were significantly enhanced. The latter may be due to the similarities in the composition of the exoskeleton of parasites and insects that may therefore act as an immunostimulant potentially increasing the anti-parasitic activity. The addition of exogenous proteases significantly decreased both trypsin-inhibition and serum bacteriolytic activity probably through direct inhibition of the proteins responsible for these immune functions. Further investigation involving bacterial or parasitic challenges will be necessary to assess if the effects of dietary mealworm meal on the immune system observed in the present study are translated into an improved resistance to diseases.

Introduction

Yellow mealworm, Tenebrio molitor (TM), is a beetle that is considered a pest of grain and flour. Their larvae are easily raised on low-nutritive plant waste products. They are commercially produced to be used as pet food (birds and reptiles) or fishing baits. The recent EU commission regulation (2017/893-24/05/2017) to authorize the use of 7 insects (2 flies, 2 mealworms, 7 cricket species) in aquafeeds will further motivate the intensification of their production. They are rich in proteins (47e60%; up to 70% in defatted meal) and lipids (31e43%) and their amino acis and fatty acids profiles are suitable for inclusion in animal feeds (Makkar et al., 2014). Their use as a partial replacer of fishmeal (FM, 10e25% dietary inclusion) has been studied in poultry (Giannone, 2003; Klasing et al., 2000; Ramos-Elorduy et al., 2002; Wang et al., 1996) and more recently in fish: rainbow trout (Oncorhynchus mykiss) (Belforti et al., 2015; Gasco et al., 2014), African catfish (Clarias gariepinus) (Ng et al., 2001), common catfish (Ameiurus melas) (Roncarati et al., 2014), tilapia (Oreochromis nilotica) (de Haro et al., 2011), Gilthead seabream (Sparus aurata) (Piccolo et al., 2014) and European sea bass (Dicentrarchus labrax) (Gasco et al., 2016). At high dietary levels (25e50%), TM reduced fish growth and n-3 HUFA in fish fillets but at low dietary inclusion level (12.5e38%), fish growth was not affected for most of the fish species tested (Henry et al., 2015a) and 9% of dietary inclusion even improved the growth of African catfish (Ng et al., 2001). TM is therefore considered to be a good alternative for partial replacement of fishmeal in the diet of many fish species. Some components of insects such as silkose or dipterose have been shown to have immunostimulating activity in mammals (Ohta et al., 2014, 2016). In fish, low dietary levels of crustacean chitin have been shown to immunostimulate and to increase the disease

resistance of Gilthead seabream and common carp, Cyprinus carpio (Esteban et al., 2001; Gopalakannan and Arul, 2006).

Insects, including Tenebrio molitor, also contain varying amounts of chitin (Finke, 2007, 2013). Although insect chitin composition is slightly different from that of crustacean chitin (Henry et al., 2015a), it is reasonable to hypothesize that low levels of insect chitin may also have an immunostimulating activity in fish. However, very few studies have studied the effects of dietary insects on the immune system of fish. Two studies investigated the effect of low dietary levels of housefly pupae on the immune system and disease resistance of the red sea bream (Pagrus major) (Ido et al., 2015) and the black carp (Mylopharyngodon piceus) (Ming et al., 2013). The effects of higher dietary levels of insects used to replace large parts of the dietary fishmeal were only investigated in the case of silkworm pupae in Jian carp (Cyprinus carpio var. Jian) (Ji et al., 2013). Therefore, the present study aimed to assess the effect of the dietary inclusion of 25% of Tenebrio molitor larvae meal (corresponding to 36% of FM replacement) in absence or presence of exogenous enzymes on the immune system of European sea bass. This investigation was performed at the end of the digestibility trial described in a previous publication (Gasco et al., 2016).

Materials & Methods

Fish diet

A full fat Tenebrio molitor meal (TM) was used to prepare four isonitrogenous (53% CP) and isoenergetic (21.5 MJ.kg 1 DM) diets based on the control diet containing 70% fishmeal (CD). The three other diets contained 24.75% of Tenebrio molitor meal replacing 36% of FM without exogenous digestive enzymes (TMD), with proteases (Ronozyme ProAct) (TM-Prot) or with carbohydrases (xylanase and b-glucanases; Ronozyme MultiGrain) (TM-Carb) both obtained from DSM Animal Nutrition & Health (Heerlen, The Netherlands). The experimental feeds were prepared at the IMBBC laboratory using a pellet mill. The produced 1 mm-pellets were dried at 40 C and stored at 20 C. The feed formulation and proximate composition of these diets are available in our previous publication reporting the digestibility results (Gasco et al., 2016).

Experimental trial and sampling

European sea bass of 65.3 ± 5.7 g initial weight were distributed in 12 circular fiberglass tanks (15 fish per tank) of 270 L supplied with an open-circulation of borehole aerated seawater under natural photoperiod. Water conditions were maintained constant at 19.5 ± 0.5 C, 36‰ salinity and 6 ± 0.7 mg/L dissolved oxygen. Fish were fed the 4 experimental diets ad libitum twice a day for 6 weeks. At the end of the trial, 6 fish per tank were anaesthetized and bled by the caudal vein using a syringe without anti-coagulant. Blood samples were left to clot overnight at 4 C and centrifuged the next day to collect the sera. Serum samples were frozen at 80 C until immunological analyses were performed.

Immunological analyses

The myeloperoxidase activity, the nitric oxide concentration and the bacteriolytic (anti-Gram negative bacterium; E.coli) activities of the serum were measured as described before (Henry et al., 2015b). The ceruloplasmin and anti-protease (APA) activities (Henry and Fountoulaki, 2014) and the lysozyme antibacterial activity of the serum (Cotou et al., 2013) were assessed following the methods described before.

Statistical analysis

SPSS 13.0 software was used for all statistical analyses. Normality of data and homogeneity of variances were checked using Kolmogorov-Smirnov and Levene tests respectively. OneWay ANOVA or Kruskal-Wallis test were performed when appropriate. Pearson's parametric and Kendall's non-parametric bivariate correlations were assessed between all tested morphometric and immune parameters using corresponding values for each fish.

Results

As shown in our previous publication, the final fish weight was not affected significantly by the dietary treatment, while the digestibility of proteins was improved in TMD compared to the 3 other diets (Gasco et al., 2016). Hepato-Somatic Index was also improved by dietary TM supplemented or not by the exogenous enzymes (Gasco et al., 2016). Immunological parameters are presented in Figs. 1e4. The correlations between all morphometric and immunological parameters are presented in Table 1. The serum ceruloplasmin activity (Fig. 1), the serum myeloperoxidase activity (Fig. 2A) and nitric oxide serum concentration (Fig. 2B) were significantly decreased in fish fed TM-based diets with or without added enzymes compared to fish fed the FM-based diet. The antibacterial activity of serum against a Gram positive bacterium (Micrococcus luteus, lysozyme activity, Fig. 3A) did not show any significant difference between fish fed the different experimental diets, whereas the bacteriolytic activity against a Gram-negative bacterium (E.coli, bacteriolytic activity, Fig. 3B) was significantly lower in fish fed the insect diet supplemented with exogenous proteases compared to the 3 other diets (ANOVA, $P < 0.0001$). The anti-protease activity was increased in fish fed TMD and TM-carb compared to both control fish and fish fed TMprot (Fig. 4). It was strongly positively correlated to the lysozyme and to the morphometric indexes (Table 1).

Discussion

The incorporation of insect larvae into the fish diet is a relatively recent research theme and to our knowledge, only few studies of their effect on the immune system and antioxidant enzymes of the fish have been performed so far: Black carp (Mylopharyngodon piceus) fed with low doses (2.5%) of maggot (Musca domestica) for 60 days showed increased serum lysozyme, serum complement and liver SOD and CAT activities and reduced liver MDA suggesting an increased antibacterial activity and an antioxidant activity of the insect meal at low dietary dose (Ming et al., 2013). The insect meal also protected these fish against a bacterial challenge with Aeromonas hydrophila (Ming et al., 2013). A similar study in red seabream (Pagrus major) showed that the introduction of low doses (0.75 and 7.5%) of housefly (Musca domestica) pupae in the diet of red sea bream for 10 days showed significant increase of the phagocytic activity of peritoneal macrophages (Ido et al., 2015). Interestingly, 5% of dietary housefly pupae for 2 months did totally protect (100% survival) the fish against the bacterial pathogen Edwardsiella tarda while all control fish died 12 days after the bacterial challenge (Ido et al., 2015). The protective effect of dietary insects was hypothesized to be either direct through the secretion of antimicrobial peptides by the insect (Fu et al., 2009; Hou et al., 2007; Imamura et al., 1999; Schuhmann et al., 2003) or indirect through the stimulation of the fish immune system by chitin (Esteban et al., 2000, 2001; Lee et al., 2008) or by other insects components (Ido et al., 2015). Polysaccharides such as silkrose isolated from silkmoth pupae or dipterose isolated from melon fly pupae have been shown to immunostimulate mammalians (innate immune response, pro-oxidant and pro-inflammatory, TLR4 signaling pathway) (Ohta et al., 2014, 2016). The first hypothesis involving the secretion of antimicrobial peptides by the insects seemed however unlikely as dead insects do not secrete any AMPs. The indirect effect through the immunostimulation of the fish was therefore more likely.

The present study looked at the effects of much higher dietary levels of insect meal on the fish immune system. The results showed strong reduction of nitric oxide production and activities of the myeloperoxidase

and ceruloplasmin in the serum of the fish. These 3 parameters have been linked to the anti-inflammatory activity (Ceron and Martinez-Subiela, 2004; Dorward et al., 2012; Hodgkinson et al., 2015; Loria et al., 2008; Sahoo et al., 2013).

As shown in our previous publication, dietary mealworm affects the fatty acid profile of the fish: the total polyunsaturated fatty acids (PUFA) and more particularly the total omega 6 fatty acids in the fish fillets were significantly increased whereas omega 3 fatty acids were significantly reduced in fish fed TM compared to control-fed fish thus reducing significantly the Sn3/Sn6 fatty acid ratio (Gasco et al., 2016). The fatty acids composition of the immune cell membranes may also be affected by dietary mealworm and thus the functioning of these cells as shown in European sea bass fed different oil sources (Farndale et al., 1999; Sargent et al., 1999). As precursors of eicosanoids, highly unsaturated fatty acids (HUFA) can also indirectly modulate many immune functions (Kinsella and Lokesh, 1990; Montero et al., 2004). Dietary HUFA has been shown to alter phagocytic activity in rainbow trout (Puangkaew et al., 2004) or immunoglobin titers in Atlantic salmon (Bell et al., 1996) but not lysozyme, complement, respiratory burst and/or antiprotease activities (Bell et al., 1996; Puangkaew et al., 2004). In largemouth bass (Micropterus salmoides) also, lysozyme and complement activity were not affected by total replacement of dietary FO by vegetable oil (canola oil) (Subhadra et al., 2006). Likewise, the antibacterial activities against both Gram negative and Gram positive bacteria were not affected significantly by the dietary insect meal in the present study. On the contrary, in other fish species, lysozyme and/or complement were affected by dietary vegetable oils which affected the fatty acid composition of the fish fillet (Kenari et al., 2011; Montero et al., 2008, 2010).

The low level of HUFA is not the only characteristic of the insect meal that can affect the immune system of the fish. Its chitin content may also modulate the fish immune system. Previous studies incorporating crustacean chitin in the fish diet have generally shown an immunostimulating activity at low inclusion level (0.01e1%) with increased complement and/or respiratory burst activities (Esteban et al., 2001; Gopalakannan and Arul, 2006). Lysozyme stayed unchanged in Gilthead seabream (Sparus aurata) (Esteban et al., 2001) but was increased significantly in common carp (Cyprinus carpio) (Gopalakannan and Arul, 2006) by dietary crustacean chitin. An in vitro digestibility study showed that chitin represents 5.75% of the mealworm larvae meal (Marono et al., 2015). The TM-containing diets used in the present study would therefore contain a maximum of 1.45% of chitin which would be expected to increase both the oxidative burst and antibacterial activities. Surprisingly then, the present study showed a significant reduction of the myeloperoxidase activities and nitric oxide concentration in the serum accompanied by a significantly reduced ceruloplasmin activity when fish were fed insect larval meal in presence of enzymes or not. The discrepancy between the effect of dietary crustacean and insect chitin on the fish immune system may be due to the difference in composition: insect chitin is a matrix of proteins, lipids and other compounds (Kramer et al., 1995), whereas crustacean chitin is encompassed in a matrix of proteins and minerals (mostly calcium) (Johnson and Peniston, 1982; No et al., 1989). Further study involving dietary purified insect chitin will be useful to assess the immunomodulating effect of insect chitin. The immunomodulating action of chitin is however very complex and it has been suggested to be size-dependent. For example, large chitin polymers are thought to be inert, smaller fragments pro-inflammatory while even smaller fragments may be anti-inflammatory (Lee et al., 2008). All these make it impossible to conclude about the role of the insect content in chitin in the present modulation of the fish immune system.

In the present study, lysozyme did not show any significant difference between dietary treatments but a tendency for improved activity in fish fed TMD and TM-Carb compared to fish fed CD and TM-Prot was observed. This tendency became significant concerning the trypsin inhibition activity which represents the capacity of the fish to counteract the mechanisms put in place by the pathogens to escape the fish immune system, i.e. production of protease to evade immune mechanisms such as the complement activity. The anti-protease activity is therefore usually considered to be correlated with the anti-parasitic activity of the fish immune system (Henry et al., 2015b). The similarities between the composition of insects and parasites

exoskeleton suggest that the dietary insects may potentially act as an immunostimulant that may increase the fish anti-parasitic defense. Further study is therefore strongly encouraged because parasitic diseases have a heavy economic toll on the aquaculture of European sea bass in the Mediterranean.

The addition of exogenous dietary enzymes did not affect significantly the immunological parameters tested except for the serum bacteriolytic activity and trypsin-inhibition activities which were significantly decreased in presence of dietary proteases. Dietary proteases may have directly inhibited some proteins responsible for these immune functions. The use of exogenous enzymes such as proteases is therefore not recommended especially since it also paradoxically reduced the protein digestibility of the TM-containing diet. As discussed in our previous publication (Gasco et al., 2016), the effect of such enzymes may reduce the beneficial effects of the microbiome on both the digestive and immune systems of the fish.

The present study showed for the first time the effect of dietary Tenebrio molitor meal on the immune system of fish. The antiinflammatory and immunomodulating effect of dietary insect larval meal on European sea bass was hypothesized to be indirect through:

- the immuno-stimulating and anti-inflammatory effect of chitin, $ the low Sn3/Sn6 PUFA composition of fish which may alter the immune cell membrane composition and functioning,
- the affected HUFA, as precursors of eicosanoids, may also affect some immune functions and/or
- the similarities between the composition of insects and parasites exoskeleton thus potentially acting as an immunostimulant potentially increasing the fish anti-parasitic defense.

Conflict of interest

The authors declare that there are no conflicts of interest.

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FIGURES

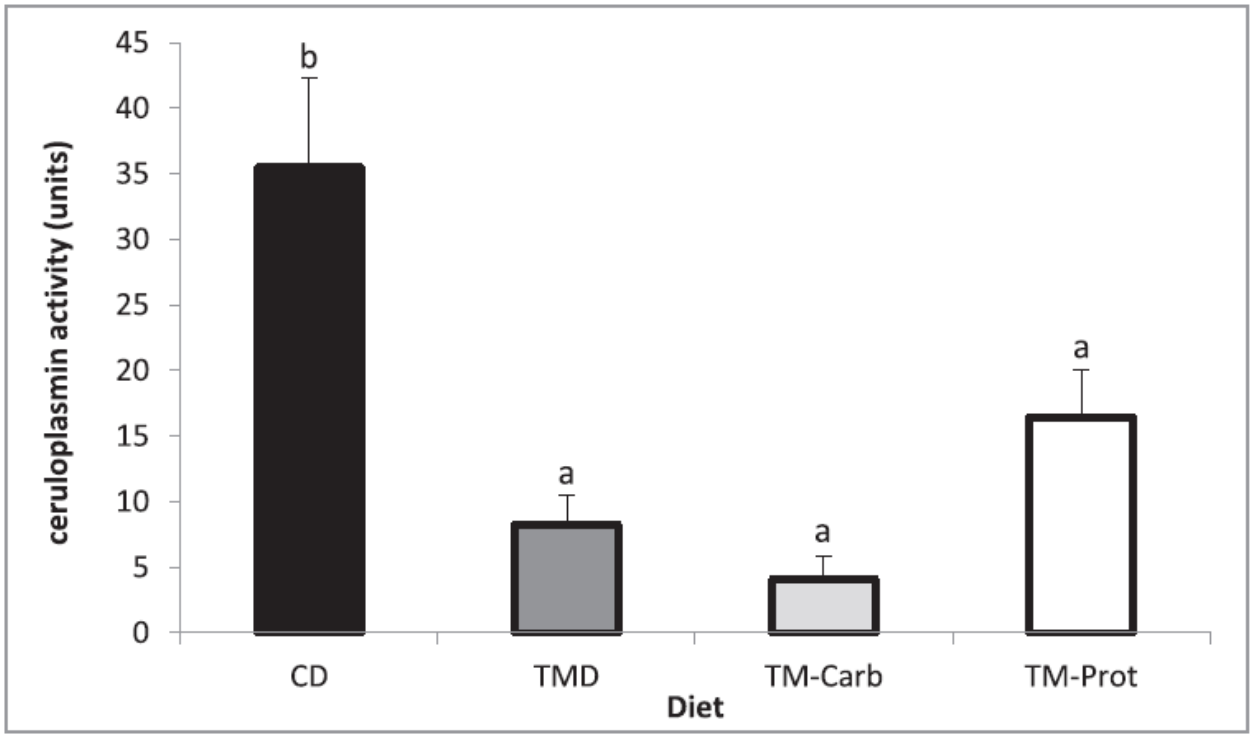

Fig. 1. Serum ceruloplasmin activity in European sea bass fed the 4 experimental diets. Different Latin letters show significant differences between dietary groups. (ANOVA, $P = 0.00003$). Bars represent mean ± S.E.M. $n = 18$. CD stands for control diet; TMD stands for *Tenebrio molitor* diet; TM-Carb and TM-Prot are the insect-containing diets enriched in carbohydrases and proteases respectively.

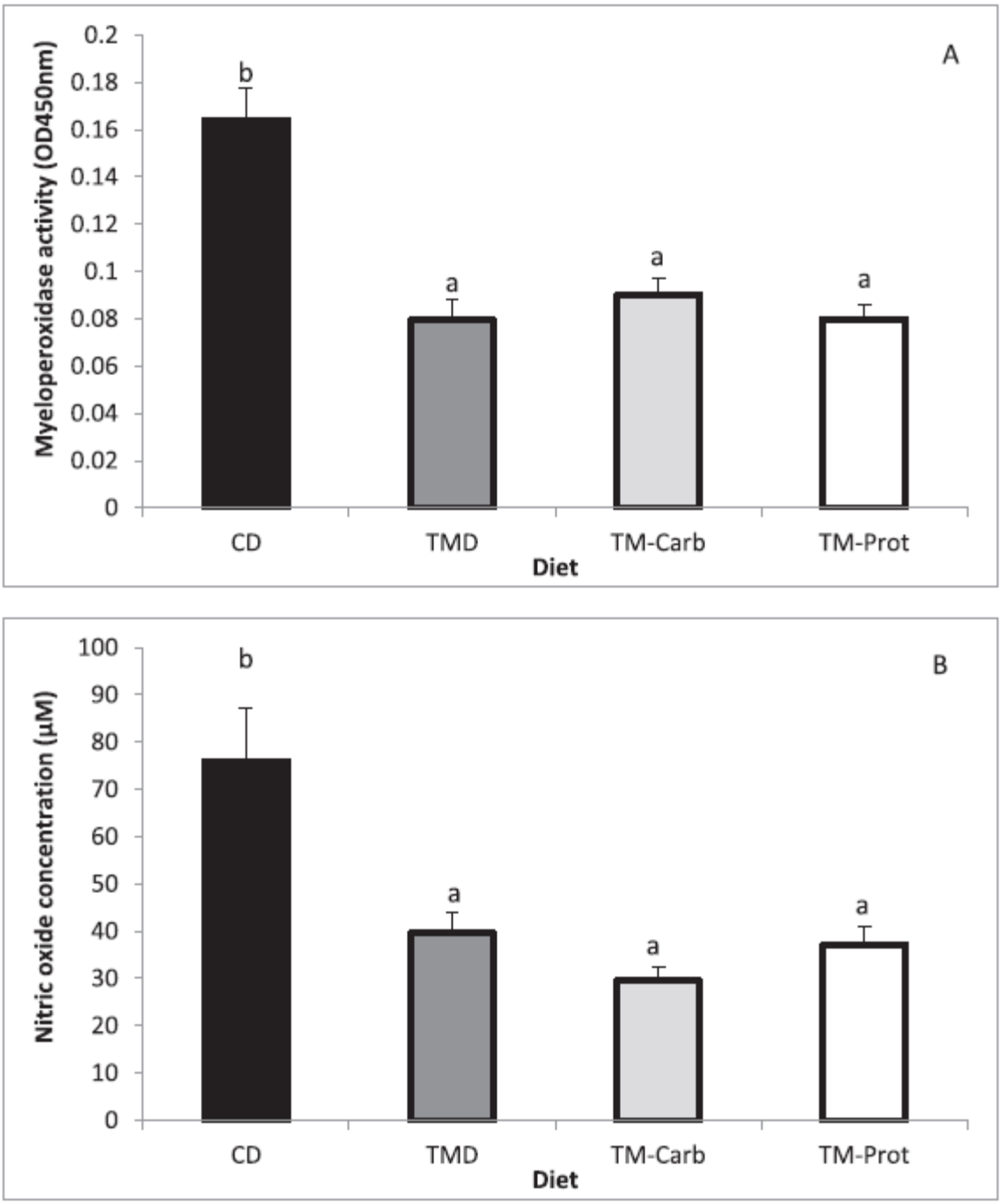

Fig. 2. Serum oxidative activity A) myeloperoxidase activity B) nitric oxide concentration (µM) in European sea bass fed the 4 experimental diets. Different Latin letters show significant differences between dietary groups (ANOVA, 3A: $P = 10^{-8}$; 3B: $P = 10^{-5}$). Bars represent mean ± S.E.M. n = 18. CD stands for control diet; TMD stands for *Tenebrio molitor* diet; TM-Carb and TM-Prot are the insect-containing diets enriched in carbohydrases and proteases respectively.

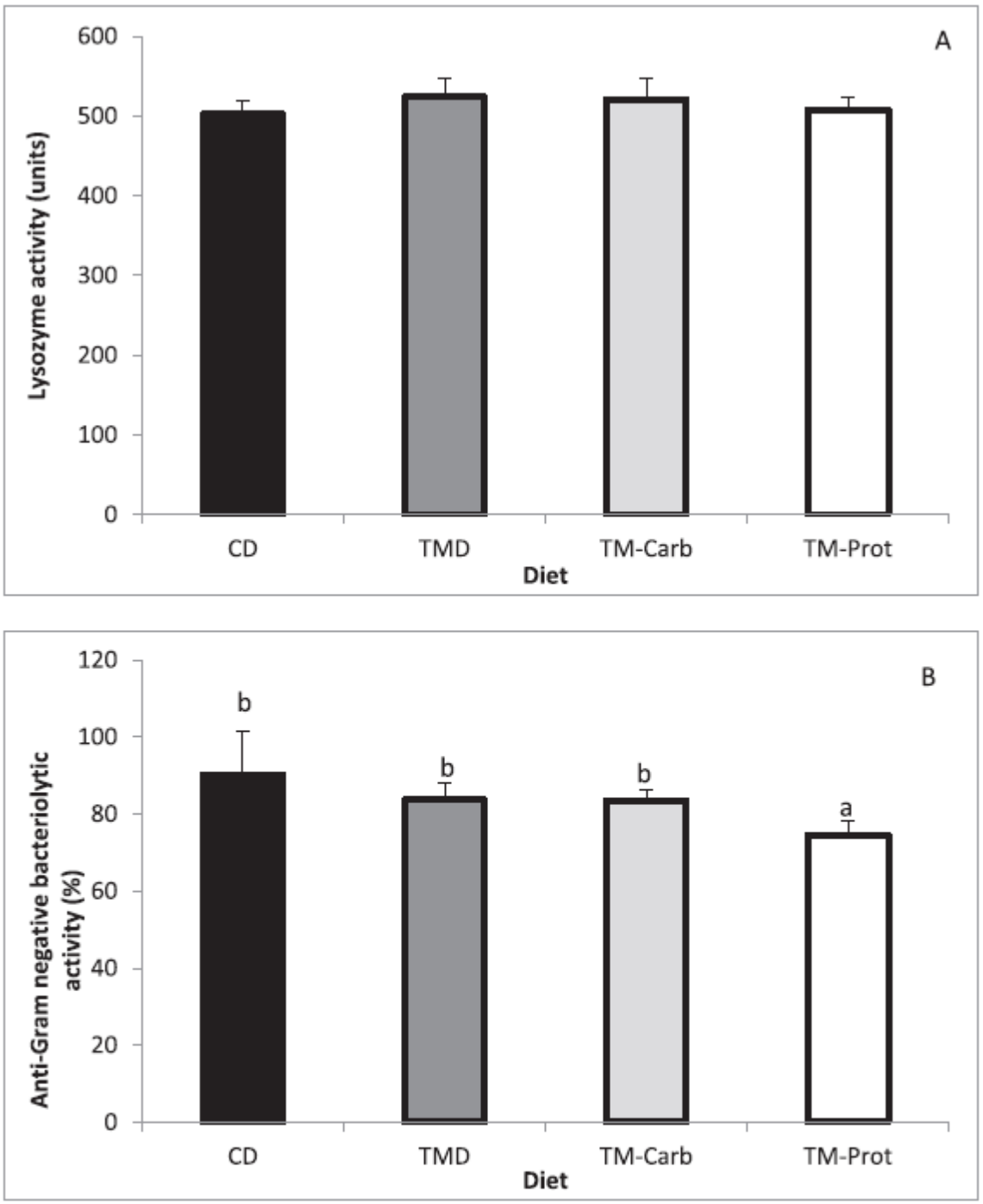

Fig. 3. Serum antibacterial activity of A) anti-Gram positive bacterium (Lysozyme activity) and B) Anti-Gram negative bacteriolytic activity in European sea bass fed the 4 experimental diets. Different Latin letters show significant differences between dietary groups (ANOVA, P = 0,00002). Bars represent mean ± S.E.M. n = 18. CD stands for control diet; TMD stands for *Tenebrio molitor* diet; TM-Carb and TM-Prot are the insect-containing diets enriched in carbohydrases and proteases respectively.

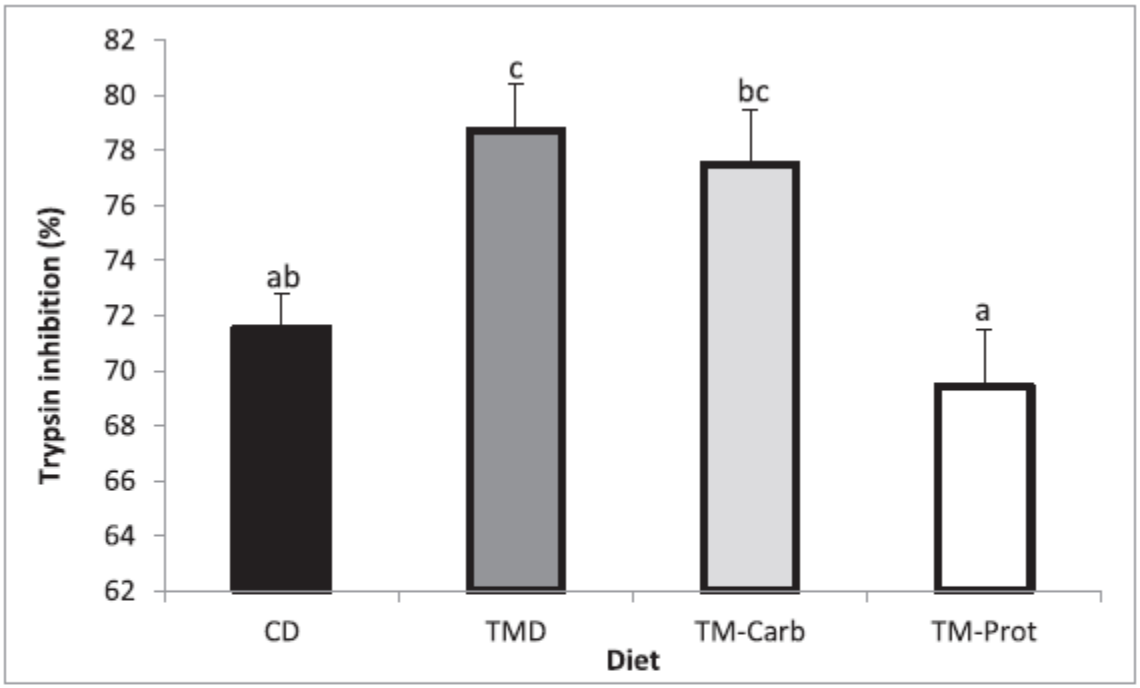

Fig. 4. Serum trypsin-inhibition activity in European sea bass fed the 4 experimental diets. Different Latin letters show significant differences between dietary groups. (ANOVA, $P = 0.0004$). Bars represent mean ± S.E.M. $n = 18$. CD stands for control diet; TMD stands for *Tenebrio molitor* diet; TM-Carb and TM-Prot are the insect-containing diets enriched in carbohydrases and proteases respectively.

Table 1
Parametric (Pearson-below diagonal) and non-parametric (Kendall-above diagonal) correlation tests between growth, morphometrics and immunological parameters. A "0" at the intersection of a column and a row denoted no significant correlation between the two parameters whereas a "+" described a positive correlation and a "−" a negative correlation between the 2 parameters. 1 symbol corresponded to a significance level of 90% ($P < 0.10$); 2 symbols to 95% ($P < 0.05$); 3 symbols to 99% ($P < 0.01$); 4 symbols to 99.9% ($P < 0.001$); and 5 symbols to 99.99% ($P < 0.0001$).

Pearson / Kendall	Weight	VSI	HSI	Lyso	Bact.	MPO	NO	Cerul	APA
Weight		0	0	0	−	0	0	0	0
VSI	0		++++	+	0	0	0	0	++
HSI	0	++++		+++	0	0	0	0	++++
Lysozyme	0	0	+++		0	−	0	−	+++
Bacteriolytic	−	0	0	0		0	++	0	+
Myeloperoxidase	0	0	0	0	++		+	+++++	0
Nitric Oxide	0	0	0	0	++	0		+++++	0
Ceruloplasmin	0	0	0	0	0	++	+		−
Trypsin inhibition	0	++	++++	+++	0	0	0	−	