Effect of partial dietary replacement of fishmeal by yellow mealworm (Tenebrio molitor) larvae meal on the innate immune response and intestinal antioxidant enzymes of rainbow trout (Oncorhynchus mykiss)

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Summary

The present study investigated the effects of 25 and 50% dietary inclusion of yellow mealworm, *Tenebrio molitor* (TM), larvae on the intestinal antioxidant enzymes, lipid peroxidation and immune system of rainbow trout, *Oncorhynchus mykiss*. Fish were fed for 90 days with a control diet (TM0) or with 2 test diets (TM25 and TM50) where 35 and 67% of the fishmeal was replaced by TM larvae meal respectively. At the end of the growth trial, humoral immune parameters were assessed in the sera of the fish and the activities of antioxidant enzymes were measured in intestine homogenates of the fish. The present results show a strong increase of the activity of the antioxidant intestinal enzymes and reduction of lipid peroxidation. Increased trypsin inhibition and faster antibacterial activity of the serum were also detected. These evidences could be due to the physiological similarities between the exoskeleta of parasites and insects. The present findings reinforce the previous results which revealed a positive effect of the dietary TM on rainbow trout growth and further confirm its adequacy for fishmeal partial replacement in aquafeed.

Keywords

Lysosyme; antibacterial activity; trypsin inhibition; superoxide dismutase; Gluthathione; catalase

Introduction

Rainbow trout is one of the most important aquaculture fish species in Europe but its feed heavily relies on fishmeal (FM) as the primary protein source. The limited availability of FM for the increasing demands for animal nutrition has motivated the search for sustainable alternative protein sources of plant and animal origins in the

last decades. Research has recently focussed on some insect species that offer good nutritional values for both fish and terrestrial animals and would represent an interesting and sustainable protein source. Processed animal protein derived from farmed insects have recently been authorized by the European Commission to be used in fish feed (Annexe II of regulation 2017/893 of 24 May 2017).

Yellow mealworm, *Tenebrio molitor* (TM), is one of these 7 authorized species. Itis a beetle that feeds ongrain, flour and their derived products. Its larvae are easily raised on low-nutritive plant and animal waste products. They are commercially produced to be used as pet food (birds and reptiles) or fishing baits. They are rich in proteins (47-60%) and lipids (31-43%) and their amino acids and fatty acids profiles are suitable for inclusion in animal feeds [1]. Their use as a partial replacer of conventional protein sources as soybean meal or fishmeal has been studied in poultry [2-5] and in several aquaculture fish species: African catfish (*Clarias gariepinus*) [6, 7], yellow catfish (*Pelteobagrus fulvidraco*) [8], tilapia (*Oreochromis niloticus*) [9], pearl gentian grouper (*Epinephelus lanceolatus x Epinephelus fuscoguttatus*) (Song et al., 2018), black bullhead (*Ameiurus melas*) [7], Gilthead seabream (*Sparus aurata*) [10], European sea bass (*Dicentrarchus labrax*) [11], rainbow trout (*Oncorhynchus mykiss*) [12, 13] and blackspot sea bream (*Pagellus bogaraveo*) [14]. At dietary inclusion level of 9-38% based on dry weight, fish growth was not affected for most of the fish species tested but high levels (25-43%) reduced fish growth and n-3 HUFA in fish fillets. Some insect meals have been suggested to have anti-oxidant activity through the stimulation of anti-oxidant enzymes by chitin or by other bioactive compounds [15].

Insect meals have been estimated to contain from 4.8 to 6.7% of chitin depending on the meal sample [16]. At low dietary levels (1-10%), crustacean chitin

immunostimulated and increased the disease resistance of Gilthead seabream and common carp [17, 18]. The effect of insect meal on fish immune system has rarely been investigated. For example, dietary maggot meal (25g/kg for 60 days) was shown to improve both lysozyme and complement activity of black carp (*Mylopharyngodon piceus*) and reduced significantly fish mortality due to *Aeromonas hydrophila* compared to fish fed a basal diet without insect [19]. The effect of low doses of dietary maggot was also studied on the immune system of red seabream, *Pagrus major*. The authors showed an increased leukocyte phagocytic activity and disease resistance against *Edwardsiella tarda* [20]. Recently Su et al. (2017) showed that a dietary inclusion level of at least 18% of TM meal could improve the immune response and the resistance of yellow catfish (*Pelteobagrus fulvidraco*) against *Edwardsiella ictaluri* without any negative effect on fish growth [8]. Concerning the effect on the fish immune system of high TM dietary doses as those used in growth performance studies , only a recent study performed in our facilities focused on the effect of TM on the immune system of European sea bass, *Dicentrarchus labrax* [21]. It showed an increase of the antiprotease activity but a decrease of myeloperoxidase activity, serum nitric oxide and ceruloplasmin activity.

The present study aims to assess the effect of the dietary inclusion of 25 and 50 % of *Tenebrio molitor* larvae meal (corresponding to 35 and 67% of FM replacement) on the immune response and the intestinal anti-oxidative enzyme activity of rainbow trout, *Oncorhynchus mykiss*.

Materials and Methods

Fish diets

Three experimental isonitrogenous and isoenergetic diets (45.2% crude proteins, 15.0% lipids, 91.5% dry matter) were formulated. They were obtained by includinggraded levels of TM larvae meal (commercial full-fat TM larvae meal from Gaobeidian Shannong Biology & Co Ltd, Shannong, China). The ingredients and proximate composition of TM0 (control diet) with no TM inclusion, TM25 containing 25% TM and TM50 containing 50% TM replacing 35% and 67% of FM respectively are described in details in Table 1. The ingredients were mixed with cod liver oil and water and pelleted using a 3.5mm die meat grinder. Pellets were dried at 50ºC for 48h and stored in a fresh dry dark room.

Fish and experimental design

Fish were obtained from a commercial farm (Troticoltura Bassignana, Beinette, Cuneo, Italy) and the growing trial was performed at the the experimental facility of the Department of Agricultural, Forest, and Food Sciences (Italy) as reported in Belforti et al [12]. Briefely, rainbow trout of 115.6 ± 14.0g initial weight were randomly distributed in twelve 400 l-fiberglass tanks (30 fish per tank; i.e. 8.6 – 23.6 kg/m^3 initial and final fish density) supplied by an artesian well to an open flow-through system delivering freshwater at 13±1ºC at a flow of 8 L min^{-1} under natura photoperiod. Dissolved oxygen was measured every 2 weeks (8.15 ± 0.55 mg L^{-1}). Fish were fed 6 days per week to visual satiety for 90 days with the 3 experimental diets (quadruplate tanks per diet). At the end of the trial, fish were weighed and blood samples were collected without heparin from the caudal vein of 3 fish per tank (12 fish per diet). After clotting overnight at 4ºC, blood was centrifuged (14000g for 10min) and serum was stored at -80ºC until immunological analyses were performed. Three fish were dissected from each tank (9 fish per diet) and intestine samples were

125 homogenised in 4 volumes of ice-cold 100 mM-Tris–HCl buffer containing 0.1 mM-
126 EDTA and 0.1 % (v/v) Triton X-100, pH 7.8. The procedure was performed on ice.
127 Homogenates were centrifuged at 30 000 g for 30 min at 4ºC and the resultant
128 supernatants were kept in aliquots and stored at -80ºC until use. The experimental
129 protocol was designed according to the guidelines of the European Directive
130 (2010/63/EU) on the protection of animals used for scientific purposes.

131

	TM0	TM25	TM50
Ingredients (g.kg^{-1})			
Herring Fishmeal (FF Skagen, Denmark)	750	490	250
Insect larvae meal (*Tenebrio molitor*)	0	250	500
Cod liver oil	80	39	0
Corn gluten meal	0	0	5
Grinded barley flakes	0	46	35
Wheat meal	63	58	58
Wheat bran	57	57	57
Gelatinised starch (D500)	30	40	75
Mineral premix*	10	10	10
Vitamin premix#	10	10	10
Proximate composition^			
Dry Matter (DM)	915	911	907
Proteins, g.kg^{-1}DM	452	446	448
Lipids, g.kg^{-1}DM	150	149	147
Ash, g.kg^{-1}DM	119	94	76
Gross Energy, MJ kg^{-1} DM	20.98	21.38	21.84

132 **Table 1: Diet formulation and proximate composition (from Belforti et al., 2015).**
133 * Mineral mixture (g or mg kg-1 diet): dicalcium phosphate, 500 g; calcium carbonate, 215 g; sodium salt 40, g; potassium
134 chloride, 90 g; magnesium chloride, 124 g; magnesium carbonate, 124 g; iron sulphate, 20 g; zinc sulphate, 4 g; copper sulphate,
135 3 g; potassium iodide, 4 mg; cobalt sulphate, 20 mg; manganese sulphate, 3 g; sodium fluoride, 1 g (purchased from Granda
136 Zootecnica, Cuneo, Italy).
137 # Vitamin mixture (U or mg kg-1 diet): DL-α tocopherol acetate, 60 U; sodium menadione bisulphate, 5 mg; retinyl acetate,
138 15,000 U; DL-cholecalciferol, 3000 U; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175
139 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 2000 mg (purchased
140 from Granda Zootecnica, Cuneo, Italy).
141 ^Values are reported as mean of duplicate analyses

142

143 *Activity of anti-oxidant enzymes*

144 All enzyme assays were carried out at 25ºC and the changes in absorbance
145 were monitored to determine the enzyme activity using a microplate reader (ELx808;
146 Bio-Tek Instruments, Winooski, Vermont, USA). The optimal substrate and protein
147 concentrations for measurement of maximal activity of each enzyme were established

in preliminary assays. The molar extinction coefficients used for H_2O_2 and NADPH were 0.039 and 6.22 cm/mM, respectively.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured by the ferricyto-chrome c method using xanthine/xanthine oxidase as source of superoxide radicals and the reaction monitored at 550 nm [22]. The reaction mixture consisted of 50 mM-potassium phosphate buffer (pH 7.8), 0.1 mM-EDTA, 0.1 mM-xanthine, 0.012 mM-cytochrome c and 0.025 IU/ml xanthine oxidase. Catalase (CAT, EC 1.11.1.6) activity was determined by measuring the decrease in H_2O_2 concentration at 240 nm according to Aebi (1984). The reaction mixture contained 50 mM-potassium phosphate buffer (pH 7.0) and 10 mM- H_2O_2 freshly added.

Glutathione peroxidase (GPX, EC 1.11.1.9) activity was measured as described before [23]. The oxidized glutathione (GSSG) generated by GPX was reduced by glutathione reductase, and NADPH oxidation monitored at 340 nm. The reaction mixture consisted of 50 mM-potassium phosphate buffer (pH 7.1), 1 mM-EDTA, 3.9 mM-reduced glutathione, 3.9 mM-sodium azide, 1 IU/ml GR, 0.2 mM-NADPH and 0.05 mM- H_2O_2.

Glutathione reductase (GR, EC 1.6.4.2) activity was assayed as described before [24], measuring the oxidation of NADPH at 340 nm. The reaction mixture consisted of 0.1 M-sodium phosphate buffer (pH 7.5), 1 mM-EDTA, 0.63 mM-NADPH and 0.16 mM-GSSG.

Glucose 6-phosphate dehydrogenase activity (G6PD, EC 1.1.1.49) was measured as described before [24], using a reaction mixture containing 50 mM-imidazole–HCl buffer (pH 7.4), 5 mM-MgCl2, 2 mM-NADP and 1 mM-glucose-6-phosphate.

Soluble protein concentration was determined using Bradford's method [25], using bovine serum albumin as standard .

Activity of SOD is reported in units of SOD/mg of protein, with one activity unit defined as the amount of enzyme necessary to produce 50 % inhibition of the ferricytochrome c reduction rate. The other enzyme activities are expressed as units (CAT) or milliunits (G6PD, GPX and GR) per mg of soluble protein, with one unit of enzyme activity defined as the amount of enzyme required to transform 1 μmol of substrate/min under the aforementioned assay conditions.

Lipid peroxidation

Concentration of thiobarbituric acid-reacting substances (TBARS) was determined according to Buege and Aust (1978). An aliquot of homogenate supernatant (100 μl) was mixed with 500 μl of a solution containing 15 % (w/v) tri-chloro-acetic acid, 0.375 % (w/v), thiobarbituric acid, 80 % (v/v) HCl 0.25 M and 0.01 % (w/v) butylated hydroxytoluene. The mixture was heated at 100ºC for 15 min and after cooling to room temperature centrifuged at 1500 g for 10 min. Absorbance of the supernatant was measured at 535 nm and compared with a blank (where intestine homogenate supernatant sample was replaced by Tris–HCl buffer containing EDTA and Triton X). Concentration was expressed as nmol malondialdehyde (MDA) /g of tissue, calculated from a calibration curve.

Immunological parameters

The myeloperoxidase activity [26], ceruloplasmin and antiprotease activities [27], antibacterial activities against Gram negative [28] and against Gram positive bacteria, [29], were assessed in fish sera following the methods described before. The assay to

measure serum lysozyme activity as indicator of the antibacterial activity against Gram positive bacteria was slightly modified to be optimized for rainbow trout: 10µl of serum were added to 200 µl of a solution of *Micrococcus luteus* at 750 µg/ml in distilled water and the linear decrease of the optical density at 450nm was recorded every minute for 8 minutes.

Statistical analysis

SPSS 21.0 software for Windows was used for all statistical analyses at a confidence level of 95%. Results are presented as means ± S.E.M.. Statistical analysis was done by two-way ANOVA after data were tested for normality by the Shapiro-Wilk test and homogeneity of variances by the Levene's test. Since interactions among factors were significant for the enzymatic activities, a one-way ANOVA and Tukey's multiple range post-hoc test were performed. For the immunological parameters, one-way ANOVA or Kruskal-Wallis test were performed when appropriate, i.e. when data did not comply with the normality assumption, followed by a Tukey's t-test.

Results

The effects of FM replacement by 25 and 50% TM on anti-oxidative enzymes of rainbow trout intestine are presented in Table 2. Superoxide dismutase activity was higher in the pyloric caeca than in the other intestinal tracts in all the experimental groups but no significant difference was detected between diets ($P>0.05$). It was significantly increased in the proximal and distal intestine of fish fed 25% TM and in the distal intestine of fish fed 50% TM compared to control fish fed fishmeal ($P<0.05$). The same observations were done for the catalase activity. Concerning the

gluthatione peroxidase and gluthatione reductase activities, fish fed both TM25 and TM50 showed significantly increased activities of these 2 enzymes compared to control fish in the proximal and/or distal intestine sections ($P<0.05$). Glucose 6-phosphate dehydrogenase activity was significantly increased in the proximal intestine of fish fed TM25 and in the distal intestine of fish fed TM25 and TM50 compared to fish fed the control diet ($P<0.05$). Lipid peroxidation was significantly reduced in the proximal and distal sections of the intestines of fish fed TM25 and TM50 ($P<0.05$).

Section	Diet	SOD	CAT	GPx	GR	G6PD	LPO
Pyloric caeca	**TM0**	452.0 ±30.3	ND	60.4 ±3.3$^{\alpha}$	37.7 ±2.0	26.5 ±2.2	55.7 ±4.2
	TM25	485.7 ±33.0		99.6 ±9.7$^{\beta}$	37.6 ±3.8	31.0 ±3.4	46.0 ±3.0
	TM50	571.1 ±50.8		108.5 ±6.5$^{\beta}$	30.5 ±5.0	26.8 ±2.4	55.9 ±4.0
Proximal Intestine	**TM0**	129.5 ±9.1^{a}	41.4 ±2.8^{a}	34.7 ±1.6^{a}	10.5 ±1.4^{a}	14.3 ±1.8^{a}	116.5 ±5.0^{b}
	TM25	275.7 ±35.6^{b}	144.0 ±9.5^{b}	61.5 ±4.5^{b}	25.2 ±3.1^{b}	23.4 ±0.4^{a}	49.2 ±3.7^{a}
	TM50	538.4 ±50.5^{c}	171.4 ±12.8^{b}	145.8 ±11.3^{c}	48.4 ±3.7^{c}	62.5 ±8.1^{b}	53.4 ±4.9^{a}
Distal Intestine	**TM0**	132.9 ±5.6^{A}	45.5 ±3.1^{A}	ND	8.6 ±1.4^{A}	14.3 ±3.0^{A}	95.1 ±4.7^{C}
	TM25	211.1 ±20.6^{B}	153.4 ±9.1^{B}		17.0 ±2.9^{B}	57.3 ±4.2^{B}	65.4 ±3.8^{B}
	TM50	166.4 ±17.1^{AB}	44.6 ±5.1^{A}		16.6 ±1.1^{B}	34.5 ±7.2^{C}	47.4 ±4.2^{A}

Table 2: Intestinal antioxidant enzymes and lipid peroxidation. Values are expressed as Mean ± S.E. CAT and SOD are expressed as U mg protein^{-1}. GPx, GR and G6PD are expressed as mU mg protein^{-1}. Lipid peroxidation (LPO) is expressed as nmol malondialdehyde (MDA) g tissue^{-1}. ND indicates "not determined" because the recorded value was below the detection limit. For each intestine section, different letters (greek for pyloric, latin lowercase for proximal and latin capital for distal intestine) in the same column indicate significant differences between diets ($P<0.05$). (n= 9). Indicate the significance of CAT, SOD, GPx, GR and G6PD

The results concerning the immune status of the rainbow trout fed diets containing FM or TM for 90 days are presented in figures 1-6: the antibacterial activity of serum against Gram-positive (Fig.1) and Gram-negative bacteria (Fig.2), the ceruloplasmin activity (Fig.3), the nitric oxide concentration (Fig.4), the myeloperoxidase activity (Fig.5) and the trypsin inhibition (Fig.6).

No significant difference between different dietary groups was obtained in ceruloplasmin activity (Fig.3) or nitric oxide concentration (Fig.4). It was also the

case of the antibacterial activity against Gram positive and against Gram negative bacteria (Fig.1 and 2A). However, the latter was significantly faster in fish fed the insect meal TM25 and TM50 compared to control fish (Fig.2B, ANOVA, P=0.0075). The trypsin inhibition and the myeloperoxidase activity of serum were significantly higher in fish fed TM50 compared to fish fed TM25 but none differed significantly from the control fish (Fig.6).

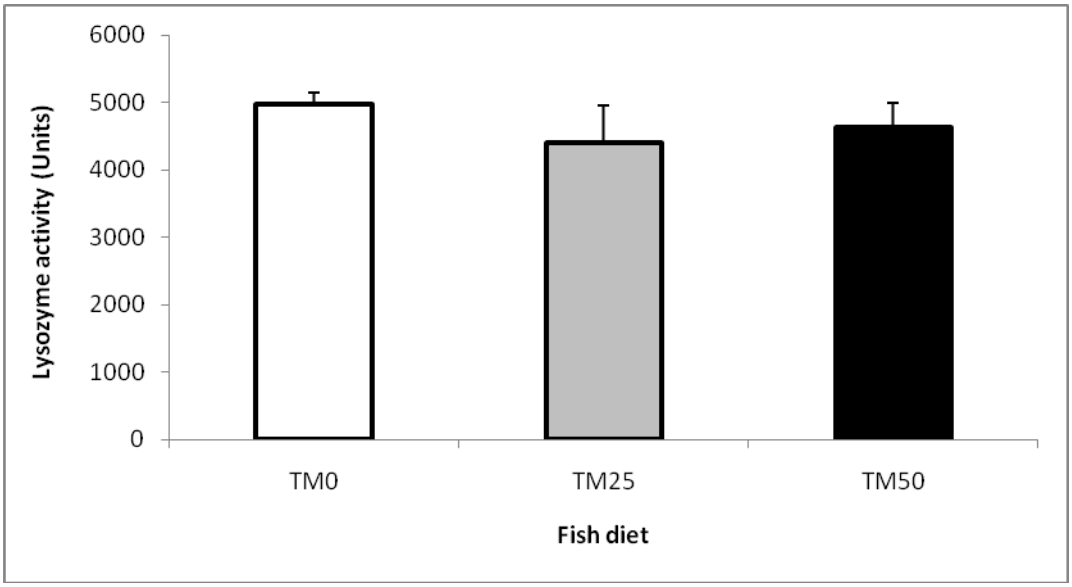

Fig.1: Antibacterial activity against Micrococcus luteus, due to the lysozyme contained in the serum of fish fed the control diet (TM0) or TM25 and TM50 diets. Bars represent the mean ± S.E.M. n=12.

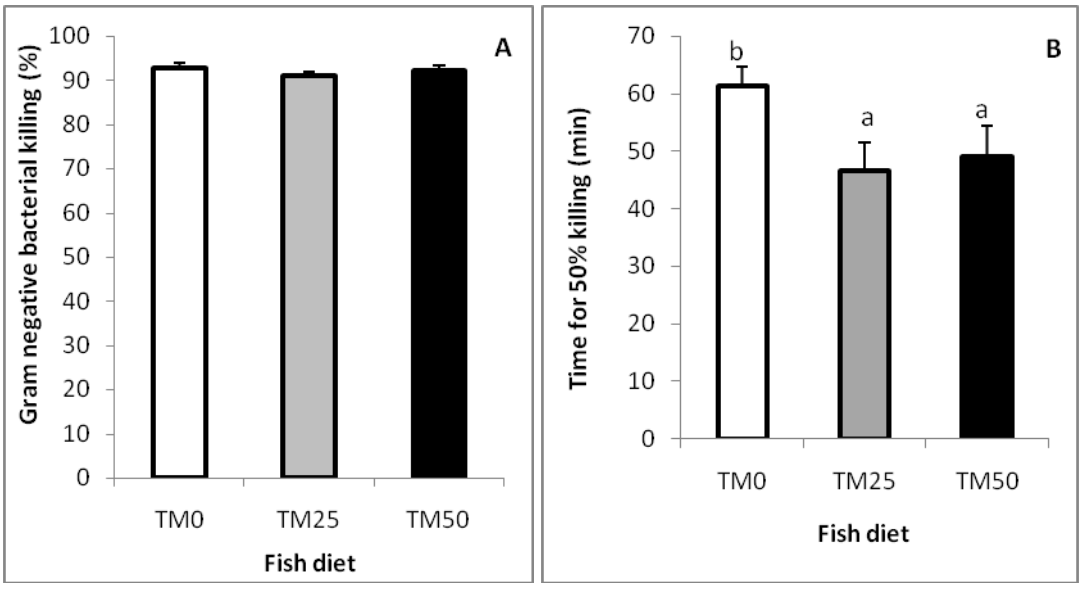

Fig.2: Bacteriolytic activity against *E.coli*, in the serum of fish fed the control diet (TM0) or TM25 and TM50 diets. A) antibacterial activity, B) Time for the killing of 50% of the bacteria.

Bars represent the mean ± S.E.M. Latin letters represent significant differences between dietary treatments (A, ANOVA, P>0.05; B, ANOVA, P=0.0075, Tukey's t-test). n=12.

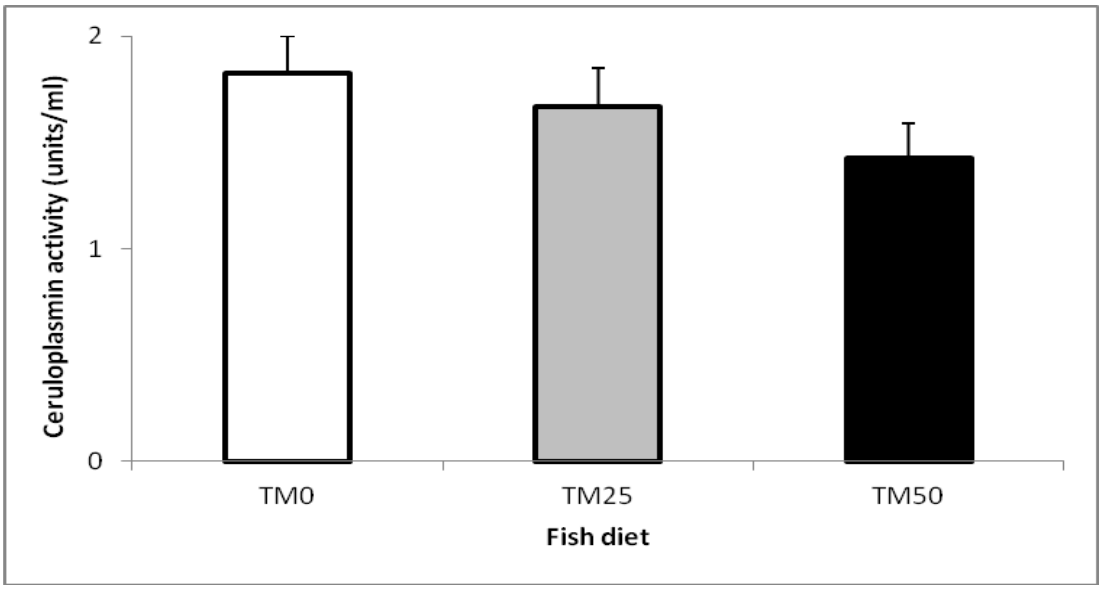

Fig.3: Ceruloplasmin activity in the serum of rainbow trout fed the control diet (TM0) or TM25 or TM50 diets. Bars represent the mean ± S.E.M. n=12.

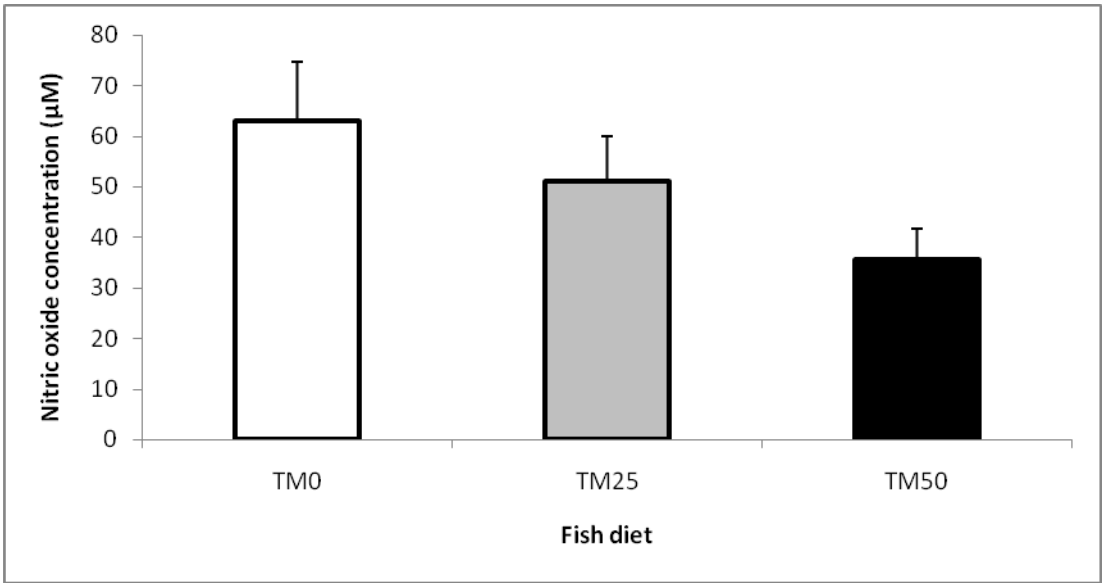

Fig.4: Nitric oxide concentration (µM) in the serum of rainbow trout fed the control diet (TM0) or TM25 or TM50 diets. Bars represent the mean ± S.E.M. n=12.

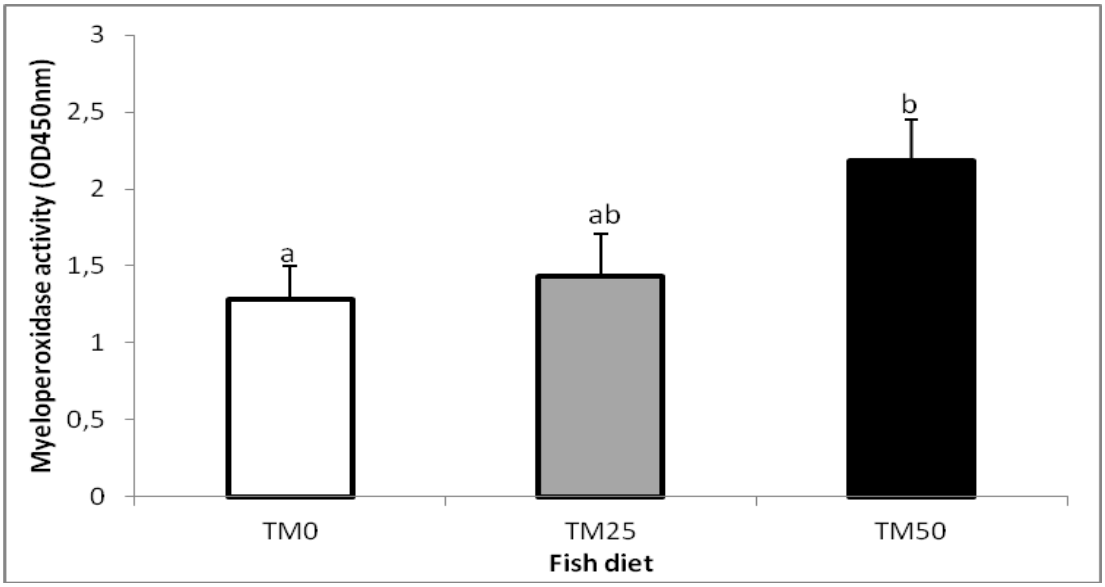

Fig.5: Myeloperoxidase activity in the serum of rainbow trout fed the control diet (TM0) or TM25 or TM50 diets. Bars represent the mean ± S.E.M. Latin letters represent significant differences between dietary treatments (ANOVA, P=0.031, Tukey's t-test). n=12.

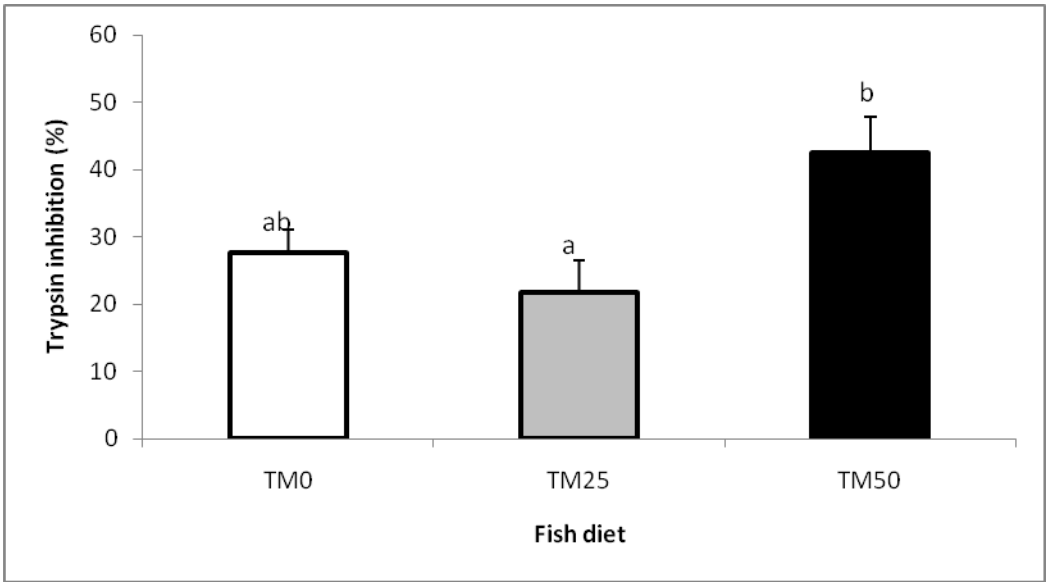

Fig.5: Trypsin inhibition in the serum of rainbow trout fed the control diet (TM0) or TM25 or TM50 diets. Bars represent the mean ± S.E.M. Latin letters represent significant differences between dietary treatments (ANOVA, P=0.010, Tukey's t-test). n=12.

Discussion

Due to the limited supply of marine resources and increasing demands of fishmeal for use in animal feed, last decades have seen the research in fish nutrition focus on alternatives to fishmeal. Although plant proteins are good candidates, they may compete with human and/or land animal nutrition for arable land. More sustainable

alternatives with low environmental footprint, such as insect proteins [30], are investigated and may provide a diversification of the possible ingredients for animal feed. Their potential as ingredients in fish and animal feed has been reviewed [1, 31] demonstratingthat dietary inclusion of some insects succeeded in replacing part of the dietary fishmeal to sustain animal growth. In rainbow trout feed in particular, partial replacement of FM was tested with frozen mosquitoes (*Culex pipiens*) [32], black soldier fly (*Hermetia illucens*) [33-36], silkworm (*Bombyx mori*) [37] and finally by yellow mealworm (*Tenebrio molitor*) [12, 13] was shown to be possible.

The effect of the inclusion of insect meals in the fish diet on intestinal anti-oxidant enzymes has been studied in several fish species. Our results showed a clear and significantly increased activity of all selected anti-oxidative enzymes (SOD, CAT, G6DP) and a strongly reduced lipid peroxidation (MDA) in the proximal and distal intestine sections of rainbow trout fed yellow mealworm supplemented diets, confirming that TM has the capacity to enhance the fish antioxidant defence system in the proximal and distal parts of the fish intestine. Moreover, GPx was the only enzyme studied here that was affected as early as the pyloric area with a significant increase of its activity in fish fed TM compared to control fish. To our knowledge, there are no other studies of fish fed insect meal that measured G6PD, GPx or GR that could be compared with the present results. Concerning SOD and CAT, similar findings were previously observed in fish sera, plasma and liver. Yellow catfish fed TM-containing diets showed significantly increased plasma SOD activity and decreased plasma MDA contents compared to control fish [8]. On the contrary, liver SOD decreased and MDA increased in pearl gentian grouper fed TM [38]. Liver SOD and CAT activities were increased by dietary maggot meal in black carp [19]. The same trend was also found for CAT activities in the serum of Jian carp fed with diets

316 containing defatted black soldier fly (*Hermetia illucens*) larvae meal [39], in the liver
317 of African catfish fed with diets including cricket meal [40] and in the liver of carp
318 fed maggot meal supplemented diets [41]. The anti-oxidant boosting activity of
319 dietary insects could be due to their chitin content or to other bioactive compounds as
320 suggested by Ngo and Kim (2014). These substances, i.e. chitin [17, 42, 43], silkrose
321 or dipterose [20, 44, 45] could also have immunostimulating activity. The effect of
322 insect meal on the fish immune system has thus far been widely overlooked. Only two
323 studies have investigated the effect of low doses (0.75-7.5%) of common fly larvae
324 (*Musca domestica*) on the immune system and resistance to bacterial diseases of black
325 carp (*Mylopharyngodon piceus*) or red seabream (*Pagrus major*) [19, 20]. They
326 showed improved lysozyme and complement activity in carp, increased leukocyte
327 phagocytic activity in red seabream and improved disease resistance in both fish
328 species. Recently, larger dietary doses were tested in European sea bass [21] and in
329 yellow catfish [8]. The dietary inclusion level of at least 18% of TM meal improved
330 the immune response and the resistance of yellow catfish (*Pelteobagrus fulvidraco*)
331 against *Edwardsiella ictaluri* without any negative effect on fish growth [8]. In
332 European sea bass, a dietary dose of 25% showed an anti-inflammatory effect of
333 dietary mealworm, *Tenebrio molitor*, together with an immunostimulating effect
334 possibly due to the similarities between the exoskeleton of insects and parasites [21].
335 Similarly, the present study looked at the effects of large doses (25, 50% of dietary
336 inclusion corresponding to 35 and 66% fishmeal replacement) of dietary mealworm
337 (*T. molitor*) on the immune response of rainbow trout (*Oncorhynchus mykiss*). The
338 results showed that although the antibacterial activity of the fish tended to be slightly
339 reduced by the inclusion of insects in the diet, the activity against Gram-negative
340 bacteria was significantly more rapid in fish fed TM than that of control fish. In

341 European sea bass, lysozyme activity against Gram-positive bacteria was not affected
342 whereas the anti-Gram negative activity was significantly reduced by the dietary
343 inclusion of 25% of *T. molitor* [21]. In a previous study in our facilities, the lysozyme
344 activity of fish fed 60% of TM was significantly increased compared to FM-fed
345 rainbow trout (data not published). The inflammatory response assessed through the
346 quantification of serum ceruloplasmin and nitric oxide also tended to be reduced by
347 the dietary insect in a dose-dependent manner. Compared to other fish species,
348 ceruloplasmin activity was particularly low suggesting a good health status of
349 rainbow trout in the present study. A strong and significantreduction of serum nitric
350 oxide, ceruloplasmin and myeloperoxidase activity has been detected in European sea
351 bass fed 25% of TM compared to control fish [21]. On the contrary, in the present
352 research, serum myeloperoxidase activity in was significantly increased in rainbow
353 trout fed TM50 compared to fish fed TM0 and TM25. This activity was also
354 significantly increased in rainbow trout previously fed a diet containing 60% of TM
355 compared to FM-fed rainbow trout (data not published). The myeloperoxidase activity
356 (MPO) is much stronger in rainbow trout than it is in European sea bass and may play
357 a more important role in the protection of this fish species. MPO is not only linked to
358 the inflammatory process but is also directly correlated to the respiratory burst activity
359 as it detoxifies hydrogen peroxide produced by the fish circulating blood cells. The
360 increase of MPO activity in fish fed TM diets may also be linked to the concomitant
361 increased activity of anti-oxidative enzymes recorded in the intestine. Furthermore, a
362 significant increase of trypsin inhibition was detected in rainbow trout fed 50% of TM
363 and 60% of TM in our previous study (data not published) compared to control fish.
364 This finding was also observed in European sea bass fed at dietary insect dose of
365 25%. This inhibition of the trypsin activity is much stronger in European sea bass than

in rainbow trout. An enhanced anti-protease activity together with a greater rapidity of the serum killing of *E.coli* have been linked in the past to the anti-parasitic arsenal of fish [28] and a similarly increased activity observed in the present study in rainbow trout fed TM suggested the immunostimulating activity of mealworm larvae,which could be due to chitin or to the similarities of the exoskeleton of parasites and insects.. However, further investigations are needed to evaluate the effect of TM on other immune parameters and on disease resistance to better understand the how this ingredient can influence the health of rainbow trout.

Aknowledgements

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References

[1] Makkar HPS, Tran G, Heuze V, Ankers P. State-of-the-art on use of insects as animal feed. Anim Feed Sci Technol 2014; 197(0):1-33. http://dx.doi.org/10.1016/j.anifeedsci.2014.07.008.

[2] Biasato I, De Marco M, Rotolo L, Renna M, Dabbou S, Capucchio MT, et al. Effects of dietary *Tenebrio molitor* meal inclusion in free-range chickens. J Anim Physiol Anim Nutr 2016; 100:1104-12. 10.1111/jpn.12487.

[3] Biasato I, Gasco L, De Marco M, Renna M, Rotolo L, Dabbou S, et al. Effects of yellow mealworm larvae (*Tenebrio molitor*) inclusion in diets for female broiler chickens: implications for animal health and gut histology. Anim Feed Sci Technol 2017; 234:253–63.

[4] Biasato I, Gasco L, De Marco M, Renna M, Rotolo L, Dabbou S, et al. Yellow mealworm larvae (*Tenebrio molitor*) inclusion in diets for male broiler chickens: effects on growth performances, welfare, gut morphology and histological findings. Accepted in Poultry Science 2018.

[5] Bovera F, Loponte R, Marono S, Piccolo G, Parisi G, Iaconisi V, et al. Use of *Tenebrio molitor* larvae meal as protein source in broiler diet: effect on growth performances, nutrient digestibility and carcass and meat traits. J Anim Sci 2016; 94:639–47.

[6] Ng W-K, Liew F-L, Ang L-P, Wong K-W. Potential of mealworm (*Tenebrio molitor*) as an alternative protein source in practical diets for African catfish, *Clarias gariepinus*. Aquac Res 2001; 32(S1):273-80.

[7] Roncarati A, Gasco L, Parisi G, Terova G. Growth performance of common catfish (*Ameiurus melas* Raf.) fingerlings fed Tenebrio molitor meal diet. Journal of insects Food Feed 2015; 1(3):233-40. 10.3920/JIFF2014.0006: 10.3920/JIFF2014.0006.

[8] Su J, Gong Y, Cao S, Lu F, Han D, Liu H, et al. Effects of dietary Tenebrio molitor meal on the growth performance, immune response and disease resistance of yellow catfish (*Pelteobagrus fulvidraco*). Fish Shellfish Immunol 2017; 69:59-66.
[9] Sanchez Muroz MJ, de Haro C, Sanz A, Trenzado CE, Villareces S, Barroso FG. Nutritional evaluation of *Tenebrio molitor* meal as fishmeal substitute for tilapia (*Oreochromis niloticus*) diet. Aquac Nutr 2016; 22:943-55. 10.1111/anu.12313.
[10] Piccolo G, Iaconisi V, Marono S, Gasco L, Loponte R, Nizza S, et al. Effect of *Tenebrio molitor* larvae meal on growth performance, *in vivo* nutrients digestibility, somatic and marketable indexes of gilthead sea bream (*Sparus aurata*). Anim Feed Sci Technol 2017; 226:12-20.
[11] Gasco L, Henry M, Piccolo G, Marono S, Gai F, Renna M, et al. *Tenebrio molitor* meal in diets for European sea bass (*Dicentrarchus labrax* L.) juveniles: Growth performance, whole body composition and in vivo apparent digestibility. Anim Feed Sci Technol 2016; 220:34-45. http://dx.doi.org/10.1016/j.anifeedsci.2016.07.003.
[12] Belforti M, Gal F, Lussiana C, Renna M, Malfatto V, Rotolo L, et al. *Tenebrio molitor* meal in rainbow trout (*Oncorhynchus mykiss*) diets: effects on animal performance, nutrient digestibility and chemical composition of fillets. Ital J Anim Sci 2015; 14(4170):670-6. 10.4081/ijas.2015.4170.
[13] Iaconisi V, Bonelli A, Pupino R, Gai F, Parisi G. Mealworm as dietary protein source for rainbow trout: Body and fillet quality traits. Aquaculture 2018; 484(Supplement C):197-204. https://doi.org/10.1016/j.aquaculture.2017.11.034.
[14] Iaconisi V, Marono S, Parisi G, Gasco L, Genovese L, Maricchiolo G, et al. Dietary inclusion of Tenebrio molitor larvae meal: Effects on growth performance and final quality treats of blackspot sea bream (Pagellus bogaraveo). Aquaculture 2017; 476(Supplement C):49-58. https://doi.org/10.1016/j.aquaculture.2017.04.007.
[15] Ngo DH, Kim SK. Antioxidant effects of chitin, chitosan and their derivatives. Adv Food Nutr Res 2014; 73:15-31.
[16] Marono S, Piccolo G, Loponte R, Di Meo C, Attia YA, Nizza A, et al. *In vitro* crude protein digestibility of *Tenebrio molitor* and *Hermetia illucens* insect meals and its correlation with chemical composition traits. Ital J Anim Sci 2015; 14:338-49.
[17] Esteban MA, Cuesta A, Ortuño J, Meseguer J. Immunomodulatory effects of dietary intake of chitin on gilthead seabream (*Sparus aurata* L.) innate immune system. Fish Shellfish Immunol 2001; 11(4):303-15.
[18] Gopalakannan A, Arul V. Immunomodulatory effects of dietary intake of chitin, chitosan and levamisole on the immune system of Cyprinus carpio and control of Aeromonas hydrophila infection in ponds. Aquaculture 2006; 255(1-4):179-87.
[19] Ming J, Ye J, Zhang Y, Yang X, Wu C, Shao X, et al. The influence of maggot meal and l-carnitine on growth, immunity, antioxidant indices and disease resistance of black carp (*Mylopharyngodon piceus*). J Chin Cereals Oils Assoc 2013; 28(2):80-6.
[20] Ido A, Iwai T, Ito K, Ohta T, Mizushige T, Kishida T, et al. Dietary effects of housefly (*Musca domestica*) (Diptera: Muscidae) pupae on the growth performance and the resistance against bacterial pathogen in red sea bream (*Pagrus major*) (Perciformes: Sparidae). Appl Entomol Zool 2015; 50(2):213-21. 10.1007/s13355-015-0325-z.
[21] Henry MA, Gasco L, Chatzifotis S, Piccolo G. Does dietary insect meal affect the fish immune system? The case of mealworm, Tenebrio molitor on European sea bass, Dicentrarchus labrax. Dev Comp Immunol 2018; 81:204-9. https://doi.org/10.1016/j.dci.2017.12.002.
[22] McCord JM, Fridovich I. Superoxide dismutase: an enzymic function for erythrocuprein. J Biol Chem 1969; 244:6049-55.
[23] Flohé L, Günzler WA. Assay of glutathione peroxidase. Methods Enzymol 1984; 105:115-21.
[24] Morales AE, García-Rejón L, de la Higuera M. Influence of handling and/or anaesthesia on stress response in rainbow trout. Effects on liver primary metabolism. Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology 1990; 95:87-93.
[25] Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye-binding. Anal Biochem 1976; 72:248-54.
[26] Kokou F, Rigos G, Henry M, Kentouri M, Alexis M. Growth performance, feed utilization and non-specific immune response of gilthead sea bream (*Sparus aurata* L.) fed graded levels of a bioprocessed soybean meal. Aquaculture 2012; 364:74-81. 10.1016/j.aquaculture.2012.08.009.
[27] Henry M, Fountoulaki E. Optimal dietary protein/lipid ratio for improved immune status of a newly cultivated Mediterranean fish species, the shi drum *Umbrina cirrosa*, L. Fish Shellfish Immunol 2014; 37(2):215-9. http://dx.doi.org/10.1016/j.fsi.2014.02.005.

[28] Henry MA, Nikoloudaki C, Tsigenopoulos C, Rigos G. Strong effect of long-term *Sparicotyle chrysophrii* infection on the cellular and innate immune responses of Gilthead sea bream, *Sparus aurata*. Dev Comp Immunol 2015; 51(1):185-93. http://dx.doi.org/10.1016/j.dci.2015.03.010.
[29] Henry MA, Alexis MN, Fountoulaki E, Nengas I, Rigos G. Effects of a natural parasitical infection (*Lernanthropus kroyeri*) on the immune system of European sea bass, *Dicentrarchus labrax* L. Parasite Immunol 2009; 31(12):729-40. 10.1111/j.1365-3024.2009.01150.x.
[30] van Huis A, Oonincx DGAB. The environmental sustainability of insects as food and feed. A review. Agronomy for Sustainable Development 2017; 37(5):43. 10.1007/s13593-017-0452-8.
[31] Henry M, Gasco L, Piccolo G, Fountoulaki E. Review on the use of insects in the diet of farmed fish: Past and future. Anim Feed Sci Technol 2015; 203:1-22. http://dx.doi.org/10.1016/j.anifeedsci.2015.03.001.
[32] Ostaszewska T, Dabrowski K, Kwasek K, Verri T, Kamaszewski M, Sliwinski J, et al. Effects of various diet formulations (experimental and commercial) on the morphology of the liver and intestine of rainbow trout (*Oncorhynchus mykiss*) juveniles. Aquac Res 2011; 42(12):1796-806. 10.1111/j.1365-2109.2010.02779.x.
[33] Renna M, Schiavone A, Gai F, Dabbou S, Lussiana C, Malfatto V, et al. Evaluation of the suitability of a partially defatted black soldier fly (Hermetia illucens L.) larvae meal as ingredient for rainbow trout (Oncorhynchus mykiss Walbaum) diets. Journal of Animal Science and Biotechnology 2018; 8(1). 10.1186/s40104-017-0191-3.
[34] Sealey WM, Gaylord TG, Barrows FT, Tomberlin JK, McGuire MA, Ross C, et al. Sensory Analysis of Rainbow Trout, Oncorhynchus mykiss, Fed Enriched Black Soldier Fly Prepupae, Hermetia illucens. J World Aquacult Soc 2011; 42(1):34-45. http://dx.doi.org/10.1111/j.1749-7345.2010.00441.x.
[35] St-Hilaire S, Sheppard C, Tomberlin JK, Irving S, Newton L, McGuire MA, et al. Fly prepupae as a feedstuff for rainbow trout, *Oncorhynchus mykiss*. J World Aquacult Soc 2007; 38(1):59-67. http://dx.doi.org/10.1111/j.1749-7345.2006.00073.x.
[36] Bruni L, Pastorelli R, Viti C, Gasco L, Parisi G. Characterization of the intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) fed with Hermetia illucens (black soldier fly) partially defatted larva meal as partial dietary protein source. Aquaculture 2018; 487:56-63.
[37] Dheke S, Gubhaju RS. Growth response of rainbow trout (*Oncorhynchus mykiss*) on substitution of shrimp meal by different protein sources. Nepalese Journal of Zoology 2013; 1(1):24-9.
[38] Song SG, Chi SY, Tan BP, Liang GL, Lu BQ, Dong XH, et al. Effects of fishmeal replacement by Tenebrio molitor meal on growth performance, antioxidant enzyme activities and disease resistance of the juvenile pearl gentian grouper (Epinephelus lanceolatus male x Epinephelus fuscoguttatus female). Aquac Res 2018; 49(6):2210-7. 10.1111/are.13677.
[39] Li S, Ji H, Zhang B, Zhou J, Yu H. Defatted black soldier fly (*Hermetia illucens*) larvae meal in diets for juvenile Jian carp (*Cyprinus carpio* var. Jian): Growth performance, antioxidant enzyme activities, digestive enzyme activities, intestine and hepatopancreas histological structure. Aquacult Fish Manage 2017; 477:62-70.
[40] Taufek NM, Aspani F, Muin H, Raji AA, Razak SA, Alias Z. The effect of dietary cricket meal (Gryllus bimaculatus) on growth performance, antioxidant enzyme activities, and haematological response of African catfish (Clarias gariepinus). Fish Physiol Biochem 2016; 42(4):1143-55. 10.1007/s10695-016-0204-8.
[41] Ogunji JO, Nimptsch J, Wiegand C, Schulz C, Rennert B. Effect of housefly maggot meal (magmeal) diets on catalase, and glutathione S-transferase in the liver and gills of carp *Cyprinus carpio* fingerling. International Aquatic Research 2011; 3:11-20.
[42] Lee CG, Da Silva CA, Lee JY, Hartl D, Elias JA. Chitin regulation of immune responses: an old molecule with new roles. Curr Opin Immunol 2008; 20:684–91.
[43] Esteban MA, Mulero V, Cuesta A, Ortuño J, Meseguer J. Effects of injecting chitin particles on the innate immune response of gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol 2000; 10(6):543-54.
[44] Ohta T, Kusano K, Ido A, Miura C, Miura T. Silkrose: A novel acidic polysaccharide from the silkmoth that can stimulate the innate immune response. Carbohydr Polym 2016; 136:995-1001.
[45] Ohta T, Ido A, Kusano K, Miura C, Miura T. A Novel Polysaccharide in Insects Activates the Innate Immune System in Mouse Macrophage RAW264 Cells. PLoS ONE 2014; 9(12):e114823. 10.1371/journal.pone.0114823.