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Effect of partial dietary replacement of fishmeal by yellow mealworm (*Tenebrio molitor*) larvae meal on the innate immune systems 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

50 last decades. Research has recently focussed on some insect species that offer good
51 nutritional values for both fish and terrestrial animals and would represent an
52 interesting and sustainable protein source. Processed animal protein derived from
53 farmed insects have recently been authorized by the European Commission to be used
54 in fish feed (Annexe II of regulation 2017/893 of 24 May 2017).
55 Yellow mealworm, *Tenebrio molitor* (TM), is one of these 7 authorized species. Itis a
56 beetle that feeds ongrain, flour and their derived products. Its larvae are easily raised
57 on low-nutritive plant and animal waste products. They are commercially produced to
58 be used as pet food (birds and reptiles) or fishing baits. They are rich in proteins (47-
59 60%) and lipids (31-43%) and their amino acids and fatty acids profiles are suitable
60 for inclusion in animal feeds [1]. Their use as a partial replacer of conventional
61 protein sources as soybean meal or fishmeal has been studied in poultry [2-5] and in
62 several aquaculture fish species: African catfish (*Clarias gariepinus*) [6, 7], yellow
63 catfish (*Pelteobagrus fulvidraco*) [8], tilapia (*Oreochromis niloticus*) [9], pearl
64 gentian grouper (*Epinephelus lanceolatus x Epinephelus fuscoguttatus*) (Song et al.,
65 2018), black bullhead (*Ameiurus melas*) [7], Gilthead seabream (*Sparus aurata*) [10],
66 European sea bass (*Dicentrarchus labrax*) [11], rainbow trout (*Oncorhynchus mykiss*)
67 [12, 13] and blackspot sea bream (*Pagellus bogaraveo*) [14]. At dietary inclusion
68 level of 9-38% based on dry weight, fish growth was not affected for most of the fish
69 species tested but high levels (25-43%) reduced fish growth and n-3 HUFA in fish
70 fillets. Some insect meals have been suggested to have anti-oxidant activity through
71 the stimulation of anti-oxidant enzymes by chitin or by other bioactive compounds
72 [15].
73 Insect meals have been estimated to contain from 4.8 to 6.7% of chitin depending on
74 the meal sample [16]. At low dietary levels (1-10%), crustacean chitin

75 immunostimulated and increased the disease resistance of Gilthead seabream and
76 common carp [17, 18]. The effect of insect meal on fish immune system has rarely
77 been investigated. For example, dietary maggot meal (25g/kg for 60 days) was shown
78 to improve both lysozyme and complement activity of black carp (*Mylopharyngodon*
79 *piceus*) and reduced significantly fish mortality due to *Aeromonas hydrophila*
80 compared to fish fed a basal diet without insect [19]. The effect of low doses of
81 dietary maggot was also studied on the immune system of red seabream, *Pagrus*
82 *major*. The authors showed an increased leukocyte phagocytic activity and disease
83 resistance against *Edwardsiella tarda* [20]. Recently Su et al. (2017) showed that a
84 dietary inclusion level of at least 18% of TM meal could improve the immune
85 response and the resistance of yellow catfish (*Pelteobagrus fulvidraco*) against
86 *Edwardsiella ictaluri* without any negative effect on fish growth [8]. Concerning the
87 effect on the fish immune system of high TM dietary doses as those used in growth
88 performance studies , only a recent study performed in our facilities focused on the
89 effect of TM on the immune system of European sea bass, *Dicentrarchus labrax* [21].
90 It showed an increase of the antiprotease activity but a decrease of myeloperoxidase
91 activity, serum nitric oxide and ceruloplasmin activity.
92 The present study aims to assess the effect of the dietary inclusion of 25 and 50 % of
93 *Tenebrio molitor* larvae meal (corresponding to 35 and 67% of FM replacement) on
94 the immune response and the intestinal anti-oxidative enzyme activity of rainbow
95 trout, *Oncorhynchus mykiss*.
96
97 **Materials and Methods**
98
99 *Fish diets*

100 Three experimental isonitrogenous and isoenergetic diets (45.2% crude proteins,
101 15.0% lipids, 91.5% dry matter) were formulated. They were obtained by
102 includinggraded levels of TM larvae meal (commercial full-fat TM larvae meal from
103 Gaobeidian Shannong Biology & Co Ltd, Shannong, China). The ingredients and
104 proximate composition of TM0 (control diet) with no TM inclusion, TM25 containing
105 25% TM and TM50 containing 50% TM replacing 35% and 67% of FM respectively
106 are described in details in Table 1. The ingredients were mixed with cod liver oil and
107 water and pelleted using a 3.5mm die meat grinder. Pellets were dried at 50ºC for 48h
108 and stored in a fresh dry dark room.

109

110 *Fish and experimental design*

111 Fish were obtained from a commercial farm (Troticoltura Bassignana, Beinette,
112 Cuneo, Italy) and the growing trial was performed at the the experimental facility of
113 the Department of Agricultural, Forest, and Food Sciences (Italy) as reported in
114 Belforti et al [12]. Briefely, rainbow trout of 115.6 ± 14.0g initial weight were
115 randomly distributed in twelve 400 l-fiberglass tanks (30 fish per tank; i.e. 8.6 – 23.6
116 kg/m^3 initial and final fish density) supplied by an artesian well to an open flow-
117 through system delivering freshwater at 13±1ºC at a flow of 8 L min^{-1} under natura
118 photoperiod. Dissolved oxygen was measured every 2 weeks (8.15 ± 0.55 mg L^{-1}).
119 Fish were fed 6 days per week to visual satiety for 90 days with the 3 experimental
120 diets (quadruplate tanks per diet). At the end of the trial, fish were weighed and blood
121 samples were collected without heparin from the caudal vein of 3 fish per tank (12
122 fish per diet). After clotting overnight at 4ºC, blood was centrifuged (14000g for
123 10min) and serum was stored at -80ºC until immunological analyses were performed.
124 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

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

150 Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured by the
151 ferricyto-chrome c method using xanthine/xanthine oxidase as source of superoxide
152 radicals and the reaction monitored at 550 nm [22]. The reaction mixture consisted of
153 50 mM-potassium phosphate buffer (pH 7.8), 0.1 mM-EDTA, 0.1 mM-xanthine,
154 0.012 mM-cytochrome c and 0.025 IU/ml xanthine oxidase. Catalase (CAT, EC
155 1.11.1.6) activity was determined by measuring the decrease in H_2O_2 concentration at
156 240 nm according to Aebi (1984). The reaction mixture contained 50 mM-potassium
157 phosphate buffer (pH 7.0) and 10 mM- H_2O_2 freshly added.

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

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

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

172 Soluble protein concentration was determined using Bradford's method [25],
173 using bovine serum albumin as standard .
174 Activity of SOD is reported in units of SOD/mg of protein, with one activity
175 unit defined as the amount of enzyme necessary to produce 50 % inhibition of the
176 ferricytochrome c reduction rate. The other enzyme activities are expressed as units
177 (CAT) or milliunits (G6PD, GPX and GR) per mg of soluble protein, with one unit of
178 enzyme activity defined as the amount of enzyme required to transform 1 µmol of
179 substrate/min under the aforementioned assay conditions.
180
181 *Lipid peroxidation*
182 Concentration of thiobarbituric acid-reacting substances (TBARS) was
183 determined according to Buege and Aust (1978). An aliquot of homogenate
184 supernatant (100 µl) was mixed with 500 µl of a solution containing 15 % (w/v) tri-
185 chloro-acetic acid, 0.375 % (w/v), thiobarbituric acid, 80 % (v/v) HCl 0.25 M and
186 0.01 % (w/v) butylated hydroxytoluene. The mixture was heated at 100ºC for 15 min
187 and after cooling to room temperature centrifuged at 1500 g for 10 min. Absorbance
188 of the supernatant was measured at 535 nm and compared with a blank (where
189 intestine homogenate supernatant sample was replaced by Tris–HCl buffer
190 containing EDTA and Triton X). Concentration was expressed as nmol
191 malondialdehyde (MDA) /g of tissue, calculated from a calibration curve.
192
193 *Immunological parameters*
194 The myeloperoxidase activity [26], ceruloplasmin and antiprotease activities [27],
195 antibacterial activities against Gram negative [28] and against Gram positive bacteria,
196 [29], were assessed in fish sera following the methods described before. The assay to

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

202

203 *Statistical analysis*

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

213

214 **Results**

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

222 gluthatione peroxidase and gluthatione reductase activities, fish fed both TM25 and

223 TM50 showed significantly increased activities of these 2 enzymes compared to

224 control fish in the proximal and/or distal intestine sections ($P<0.05$). Glucose 6-

225 phosphate dehydrogenase activity was significantly increased in the proximal

226 intestine of fish fed TM25 and in the distal intestine of fish fed TM25 and TM50

227 compared to fish fed the control diet ($P<0.05$). Lipid peroxidation was significantly

228 reduced in the proximal and distal sections of the intestines of fish fed TM25 and

229 TM50 ($P<0.05$).

Section	Diet	SOD	CAT	GP_X	GR	G6PD	LPO
Pyloric caeca	**TM0**	452.0 ±30.3		60.4 ±3.3$^{\alpha}$	37.7 ±2.0	26.5 ±2.2	55.7 ±4.2
	TM25	485.7 ±33.0	ND	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}		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}	ND	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}

230 **Table 2: Intestinal antioxidant enzymes and lipid peroxidation. Values are expressed as Mean ± S.E. CAT and SOD are**
231 **expressed as U mg protein^{-1}. GP_X, GR and G6PD are expressed as mU mg protein^{-1}. Lipid peroxidation (LPO) is**
232 **expressed as nmol malondialdehyde (MDA) g tissue^{-1}. ND indicates "not determined" because the recorded value was**
233 **below the detection limit. For each intestine section, different letters (greek for pyloric, latin lowercase for proximal and**
234 **latin capital for distal intestine) in the same column indicate significant differences between diets ($P<0.05$). (n= 9).** Indicate
235 the significance of CAT, SOD, GPx, GR and G6PD
236
237
238 The results concerning the immune status of the rainbow trout fed diets containing

239 FM or TM for 90 days are presented in figures 1-6: the antibacterial activity of serum

240 against Gram-positive (Fig.1) and Gram-negative bacteria (Fig.2), the ceruloplasmin

241 activity (Fig.3), the nitric oxide concentration (Fig.4), the myeloperoxidase activity

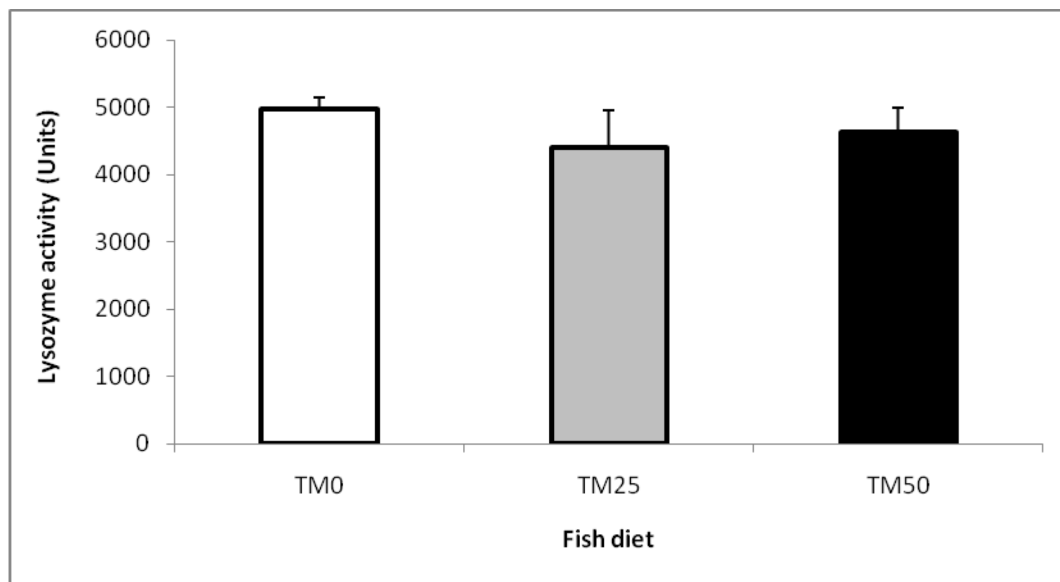
242 (Fig.5) and the trypsin inhibition (Fig.6).

243 No significant difference between different dietary groups was obtained in

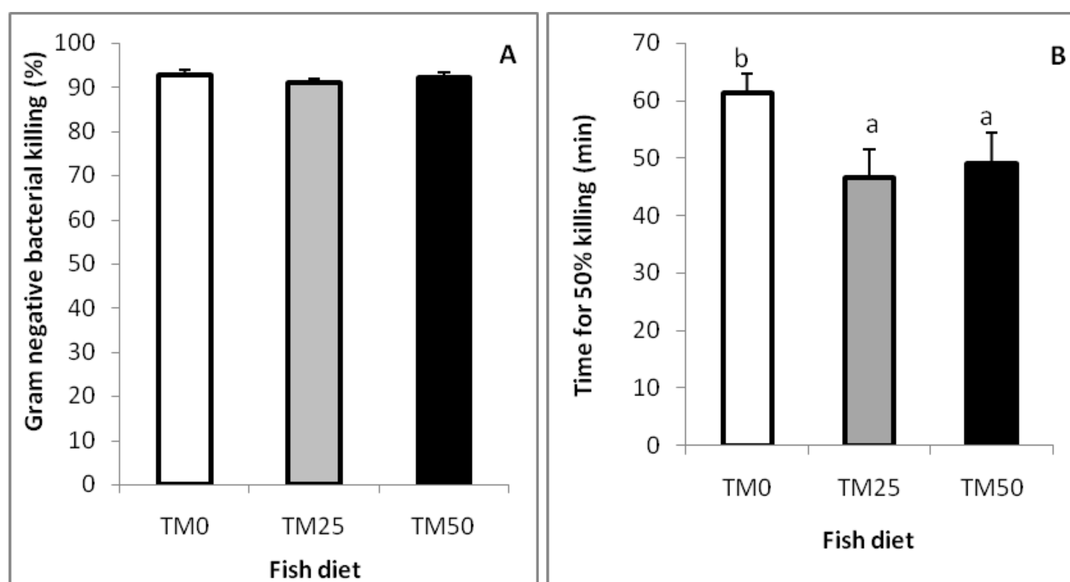
244 ceruloplasmin activity (Fig.3) or nitric oxide concentration (Fig.4). It was also the

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

251

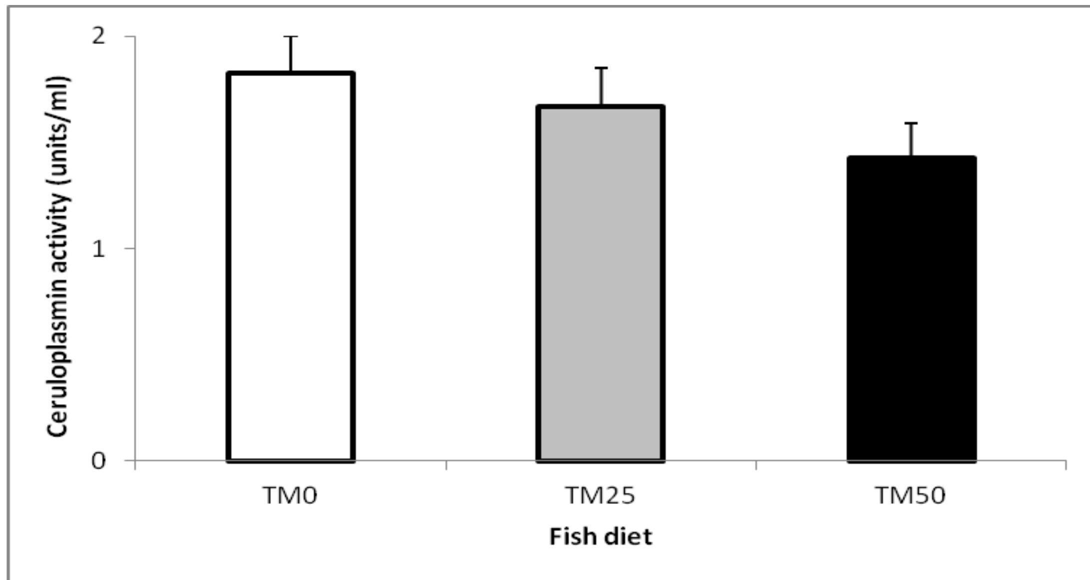


252
253 **Fig.1: Antibacterial activity against Micrococcus luteus, due to the lysozyme contained in the**
254 **serum of fish fed the control diet (TM0) or TM25 and TM50 diets. Bars represent the mean ±**
255 **S.E.M. n=12.**
256

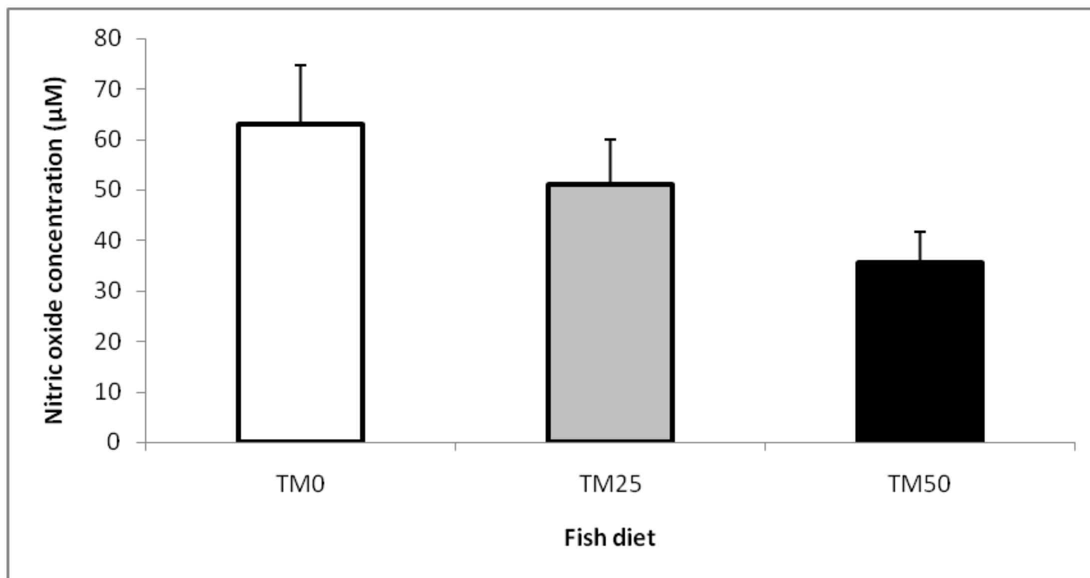


257
258
259 **Fig.2: Bacteriolytic activity against *E.coli*, in the serum of fish fed the control diet (TM0) or**
260 **TM25 and TM50 diets. A) antibacterial activity, B) Time for the killing of 50% of the bacteria.**

261 **Bars represent the mean ± S.E.M. Latin letters represent significant differences between dietary**
262 **treatments (A, ANOVA, P>0.05; B, ANOVA, P=0.0075, Tukey's t-test). n=12.**
263
264

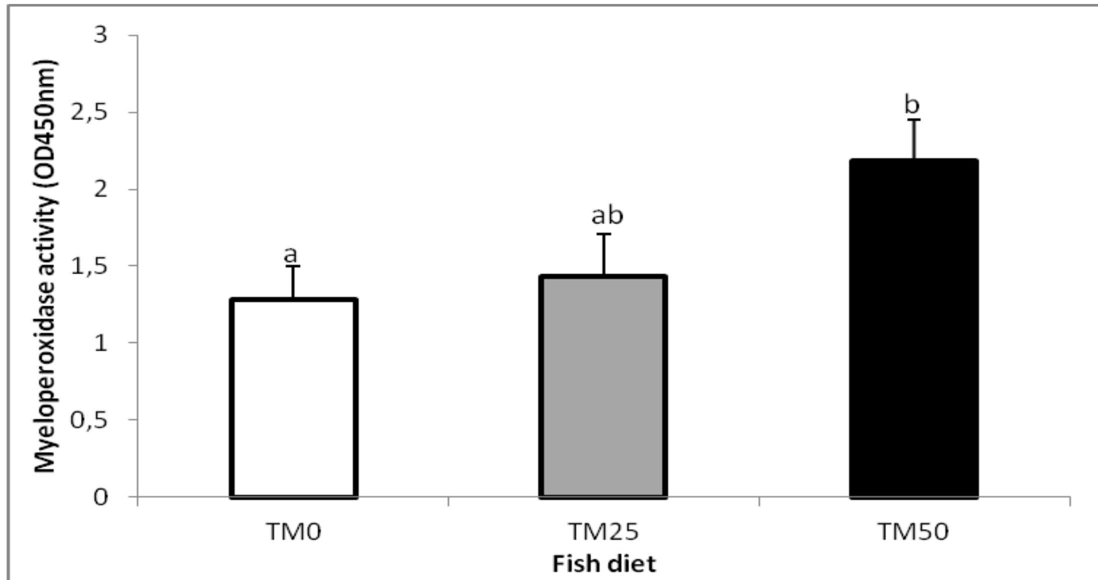


265
266 **Fig.3: Ceruloplasmin activity in the serum of rainbow trout fed the control diet (TM0) or TM25**
267 **or TM50 diets. Bars represent the mean ± S.E.M. n=12.**
268

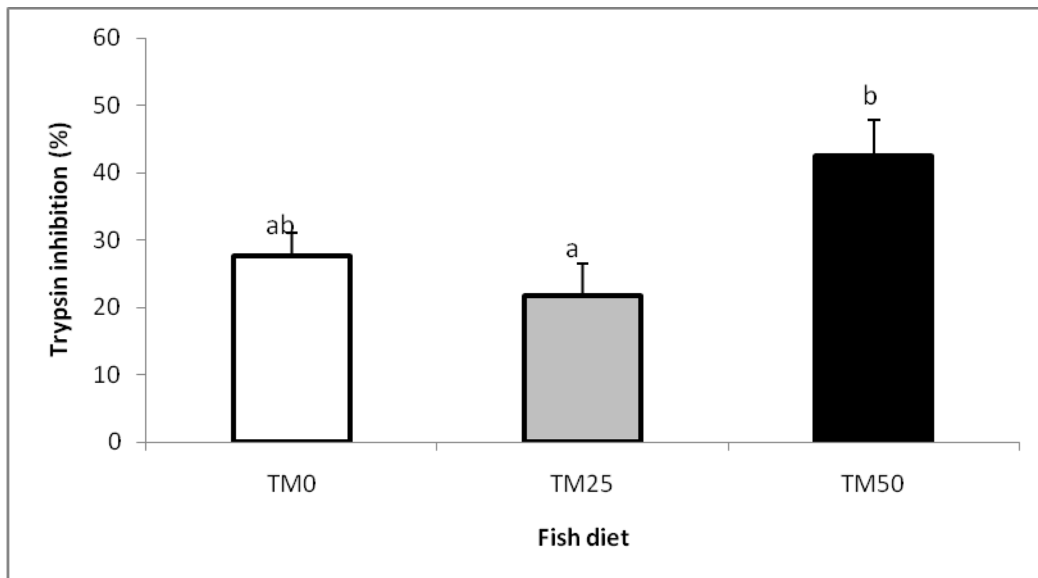


269
270 **Fig.4: Nitric oxide concentration (µM) in the serum of rainbow trout fed the control diet (TM0)**
271 **or TM25 or TM50 diets. Bars represent the mean ± S.E.M. n=12.**
272
273
274

275



276 **Fig.5: Myeloperoxidase activity in the serum of rainbow trout fed the control diet (TM0) or**
277 **TM25 or TM50 diets. Bars represent the mean ± S.E.M. Latin letters represent significant**
278 **differences between dietary treatments (ANOVA, P=0.031, Tukey's t-test). n=12.**
279



280
281 **Fig.5: Trypsin inhibition in the serum of rainbow trout fed the control diet (TM0) or TM25 or**
282 **TM50 diets. Bars represent the mean ± S.E.M. Latin letters represent significant differences**
283 **between dietary treatments (ANOVA, P=0.010, Tukey's t-test). n=12.**
284
285

286 # Discussion

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

291 alternatives with low environmental footprint, such as insect proteins [30], are
292 investigated and may provide a diversification of the possible ingredients for animal
293 feed. Their potential as ingredients in fish and animal feed has been reviewed [1, 31]
294 demonstratingthat dietary inclusion of some insects succeeded in replacing part of the
295 dietary fishmeal to sustain animal growth. In rainbow trout feed in particular, partial
296 replacement of FM was tested with frozen mosquitoes (*Culex pipiens*) [32], black
297 soldier fly (*Hermetia illucens*) [33-36], silkworm (*Bombyx mori*) [37] and finally by
298 yellow mealworm (*Tenebrio molitor*) [12, 13] was shown to be possible.
299 The effect of the inclusion of insect meals in the fish diet on intestinal anti-oxidant
300 enzymes has been studied in several fish species. Our results showed a clear and
301 significantly increased activity of all selected anti-oxidative enzymes (SOD, CAT,
302 G6DP) and a strongly reduced lipid peroxidation (MDA) in the proximal and distal
303 intestine sections of rainbow trout fed yellow mealworm supplemented diets,
304 confirming that TM has the capacity to enhance the fish antioxidant defence system in
305 the proximal and distal parts of the fish intestine. Moreover, GPx was the only
306 enzyme studied here that was affected as early as the pyloric area with a significant
307 increase of its activity in fish fed TM compared to control fish. To our knowledge,
308 there are no other studies of fish fed insect meal that measured G6PD, GPx or GR that
309 could be compared with the present results. Concerning SOD and CAT, similar
310 findings were previously observed in fish sera, plasma and liver. Yellow catfish fed
311 TM-containing diets showed significantly increased plasma SOD activity and
312 decreased plasma MDA contents compared to control fish [8]. On the contrary, liver
313 SOD decreased and MDA increased in pearl gentian grouper fed TM [38]. Liver SOD
314 and CAT activities were increased by dietary maggot meal in black carp [19]. The
315 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

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

374

375

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