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Garlic extract on physio-biochemical responses of *M. rosenbergii*

1 **Effect of garlic (*Allium sativum*) extract on growth, enzymological and biochemical**
2 **responses, and immune related gene expressions in giant freshwater prawn (*Macrobrachium***
3 ***rosenbergii*)**

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35

36 **Abstract**

37 In the current study, growth performance, biochemical constituents of muscle, activities of
38 enzymes in the haemolymph, and expressions of immune-related genes were evaluated in the giant
39 freshwater prawns *Macrobrachium rosenbergii* fed diets supplemented with aqueous garlic
40 (*Allium sativum*) extract at 0, 5, 10, and 20 g/kg (w/w) for 60 days. At the end of the feeding trial,
41 weight gain and specific growth rate were significantly improved in garlic-fed prawn groups
42 compared with the control ($P < 0.05$). Moreover, feed conversion ratio was significantly lower in
43 the garlic-fed groups than in the control ($P < 0.05$). Activities of catalase (CAT), superoxide
44 dismutase (SOD) and glutathione peroxidase (GSH-px) in the hepatopancreas, activities of alanine
45 aminotransferase (ALT), aspartate aminotransferase (AST), and levels of albumin and total protein
46 in the hemolymph were significantly increased in the garlic treatments ($P < 0.05$). Furthermore,
47 garlic supplemented diets improved muscle biochemical profiles, particularly contents of crude
48 protein and total ash, and up-regulations of immune deficiency (IMD) and heat shock proteins
49 (HSP70) gene expression ($P < 0.05$). Therefore, garlic has positive effects on growth performance
50 and physio-biochemical responses of *M. rosenbergii*, and thus, it can be used as an additive for
51 stress resistance and as a growth promoter in sustainable aquaculture.

52

53 **Keywords:** Garlic supplementation, Growth, HSP70, IMD, Prawn aquaculture

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62 **Introduction**

63 The prevalence of diseases is one of the most important limiting factors in the development of
64 aquaculture practices (Stentiford et al., 2012). Although synthetic drugs are key to control disease
65 outbreaks in aquaculture, their multiple negative impacts on the host, the environment, and even

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66 human health has fueled the search of alternative more sustainable alternatives (Rico et al., 2013;
67 Lieke et al., 2020; Reverter et al., 2020). Natural immunostimulant components, including the use
68 of bioactive plant components, are therefore arising as promising sources to prevent and treat
69 disease outbreaks in aquaculture (Van Hai, 2015; Pourmozaffar et al., 2019; Tamadoni Jahromi et
70 al., 2021). Garlic (*A. sativum*) is known as an "all-healing" herb (M Abu Elala, 2016). Garlic has
71 been used for many years as a traditional medicine and a food additive to improve human's
72 physical health and to fight some diseases (Srivastava et al., 2012). In aquatic animals, garlic has
73 been found to increase in growth performance through improved appetite, gastrointestinal motility,
74 and stimulation of digestive enzymes (Lee et al., 2012). Additionally, immune responses in aquatic
75 animals including *Oncorhynchus mykiss* (Mohebbi et al., 2012; Adineh et al., 2020), *Oreochromis*
76 *niloticus* (Aly et al., 2010), *Rutilus rutilus* (Ghehdarijani et al., 2016), *Huso huso* (Gholipour
77 Kanani et al., 2014), *Carassius auratus* (Dadgar et al., 2019) and *Litopenaeus vannamei* (Samadi
78 et al., 2016) were enhanced following oral garlic administration. Garlic possesses wide spectrum
79 of antimicrobial, antiviral, and antifungal activities against aquaculture-relevant pathogens such as
80 *Aeromonas hydrophila* (Nya et al., 2009), spring viremia (Karimi Pashaki et al., 2020), *Vibrio*
81 *alginolyticus*, *V. harvey*, *V. anguillarum* (Natasya-Ain et al., 2018), and *Yersinia ruckeri*
82 (Zaefarian et al., 2017). Some of the previously reported health benefits of garlic are ascribed to
83 their organosulphur compounds (thiosulfinates), and especially allicin (diallyl thiosulfate). For
84 example, Breyer et al. (Breyer et al., 2015) suggested that allicin can inhibit pathogen infection
85 (*Aeromonas salmonicida*) through improvement of host (*Oncorhynchus mykiss*) immunological
86 parameters, including phagocytic activity, lysozyme activity and antibody production. Moreover,
87 garlic contains other valuable ingredients such as vitamins (C, B, and A), linoleic acid, silicates,
88 iodine salts, flavonoids, and other thiosulfinate compounds (allyl methyl thiosulfonate, 1-propenyl
89 allyl thiosulfonate, ajoene, γ -L-glutamyl-S-alkyl-L- cysteine) that might also display beneficial
90 effects on aquatic animals (Adineh et al., 2020).

91 After fish and molluscan shellfish Farming, crustacean farming is the most important aquaculture
92 practice in many countries (Yearbook, 2020). The giant freshwater prawn, *M. rosenbergii*, is a
93 particularly esteemed species in aquaculture due to its fast growth, big sizes and its habitat
94 versatility. Although it is mainly cultured in Asian countries (Southern and South-Eastern), this
95 species has also been introduced in many other regions and its farming is now globally widespread
96 (Yearbook, 2020). Up to now, whether dietary supplementation of garlic extract could enhance the

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97 physiological parameters and molecular responses of giant freshwater prawn is still unknown.
98 Thus, the purpose of the present work was to evaluate the effect of garlic extract on some
99 physiological and biochemical parameters, immune related genes and growth performance in giant
100 freshwater prawn.

101 2. Materials and methods

102 2.1. Preparation of garlic extract

103 Fresh garlic bulbs were purchased from a local farm in Kermanshah, Iran. Peeled garlic cloves
104 (100 g) were chopped and then blended with 200 ml distilled water for 3 min. The solution was
105 centrifuged at 10,000 rpm for 5 min. The supernatant was removed and was passed through a
106 Whatman filter paper (42µm). The obtained extract was kept at -20⁰ C.

107 2.2. Experimental design

108 The *M. rosenbergii* post-larvae (PL-12, ranging from 1.0 to 1.3 cm in length and 1.1 to 1.3g) were
109 obtained from a local commercial shrimp farm located in Ghasr-e-Shirin city (Kermanshah
110 province, Iran). A total of 360 PL were randomly distributed in twelve (30 PL/tank) 25 L
111 (70x40x40) aerated tanks with dechlorinated water and were acclimatized to the experimental
112 conditions for ten days. Four groups of prawns were assigned in triplicate for 60 days. Three groups
113 were fed with experimental diets consisting of a basal diet supplemented with garlic extract (5, 10,
114 and 20 g/kg of garlic extract) (Breyer et al., 2015), and one group was fed with the control diet
115 (i.e., basal diet, (Table 1). The water temperature, dissolved oxygen and pH were measured daily
116 using aWagtech portable temperature, oxygen and pH meter (Berkshire, UK). Uneaten food and
117 faeces were removed daily. Water was exchanged (50%) in daily manner. Shrimps were fed daily
118 at 3% of body weight, and during the experimental period they were fed at 08:00, 13:00, 17:00
119 and 23:00.

120 2.3 Sample collection

121 All animal experiments and animal protocols were conducted in accordance with National Ethical
122 Framework for Animal Research in Iran. At the end of experiment, three prawns per tank (9 per
123 treatment) were anesthetized with clove oil, 100 mg/l (Ranjit Kumar et al., 2013) and sacrificed.
124 First, the whole prawns were weighted. Then muscle tissues from abdominal segments were

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125 immediately dissected for analysis of their proximate composition. Hepatopancreas were
126 collected, weighed and stored at -80°C until further antioxidant enzyme and gene expression
127 analysis (Akbari et al., 2019). Haemolymph was withdrawn from the ventral sinus of prawns at
128 the end of experimental period using 1-ml tuberculin syringe with 24-gauge needle and were used
129 in the analysis of different biochemical enzymes. The samples were centrifuged at 12000 g for 20
130 min and supernatants were collected and stored at -20°C until use.

131 2.4 Survival, growth and nutritional indices analysis

132 At the end of experiment, growth parameters and survival (S) were calculated as described by
133 Zhou et al. (Zhou et al., 2007) and Akbari (Akbari et al., 2020)

134 Survival (S) (%) = final prawn number/initial prawn number × 100

135 Weight gain (WG) (%) = {(Final body weight (FBW)– Initial body weight (IBW)) / IBW} × 100

136 Specific growth rate (SGR%)={ln FBW - ln IBW} / Experiment period} ×100

137 Food conversion ratio (FCR) = Total feed intake (g) / Total wet weight gain (g)

138 2.5. Muscle biochemical constituent

139 A standard method (AOAC, 2003) was used to measure crude protein, crude lipid, moisture, and
140 ash contents of the prawn muscle and experimental diets. The samples were analyzed for crude
141 protein (Kjeldahl method, Kjeltec 2100 Distillation Unit, Hoganäs, Sweden), crude fat (Soxhlet
142 extraction), moisture (24 h at 105 °C) and ash content (4h at 550 °C). (Jahanbakhshi et al., 2012;
143 Akbari et al., 2018).

144 2.6. Antioxidant enzyme assays

145 Frozen hepatopancreas samples were homogenized in a phosphate buffer (0.1 mol /l, pH 7.2) on
146 ice. Homogenates were centrifuged at 1000×g for 15 min at 4°C. After being centrifuged, the
147 supernatant was extracted and placed in new tubes (1–1.5 mL) and then frozen at -20 °C up to the
148 time of analysis. The activities of CAT, GSH-Px, and SOD enzymes were determined according
149 to the Zehra and Khan (2019) procedure. Antioxidant enzyme activities were expressed in units
150 per milligram of protein (U/mg protein).

151 2.7. Haemolymph biochemical parameters

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152 The haemolymph samples were mixed with an anticoagulant solution (100 mM sodium citrate, 10
153 mM tris-HCl, 250 mM sucrose, pH 7.2) (Ranjit Kumar et al., 2013). The aspartate aminotransferase
154 (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) activities and albumin
155 concentration were measured using detection kits (Pars Azmoon Co. Iran) and an autoanalyzer
156 (Hitachi 917, Japan) (Tamadoni Jahromi et al., 2020). Total protein concentration was measured
157 according to the Lowry method (Lowry et al., 1951).

158

159 2.8. RNA extraction

160 For RNA extraction, samples were immediately frozen in liquid nitrogen and stored at -80°C
161 until analyzed. Biozol reagent (Bio flux; China) was used for extracting total RNA from
162 hepatopancreas samples according to the manufacturer's instructions. Quality of RNA was
163 estimated by electrophoresis on ethidium bromide staining and a 1.5% agarose gel. The RNA's
164 quantity and quality was measured with a nanodrop spectrophotometer at a wavelength of 260/280
165 nm. Samples with RNA ratios greater than 1.8 (at 260/280 nm) were used for further experiments.
166 In accordance with the manufacturer's protocol, supprime script RT premix (2 \times) cDNA synthesis
167 kit (GeNet BIO Inc, South Korea) was used to synthesis the first strand of cDNA (Pourmozaffar
168 et al., 2017).

169 2.9. Relative mRNA expression of immune-related genes

170 The transcript expression levels were determined by Real-time Polymerase Chain Reaction (RT-
171 PCR, Bio-Rad, USA). Table 2 exhibits the primers used for amplification of heat shock proteins
172 (HSP70), immune deficiency (IMD), and β -actin (housekeeping gene). In accordance with
173 standard protocols, 10 μl of SYBR Green qPCR Master Mix (1 \times) (Fermentase, Lithuania), 0.2 μl
174 of forward and reverse specific primers (100 nM), 10 ng of cDNA template, and 6.40 μl nuclease
175 free water to final volume of 20 ml were used (Pourmozaffar et al., 2017). PCR reaction mixtures
176 were subjected to the following thermal profile: 95 $^{\circ}\text{C}$ for 5 min, followed by 40 cycles of 10 s at
177 95 $^{\circ}\text{C}$, and 10 s at 54 $^{\circ}\text{C}$ and 10 s at 72 $^{\circ}\text{C}$. The $2^{-\Delta\Delta\text{CT}}$ method was used in calculating the fold
178 changes in HSP70 and IMD relative mRNA expression. The β -actin gene was used to normalize
179 the expression levels of the target genes.

180 2.10. Statistical analysis

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181 Results were analyzed statistically using One-way analysis of variance (ANOVA) and Duncan's
182 post hoc test was performed to determine the significant differences from each other. Normality
183 and homogeneity were tested using the Kolmogorov–Smirnov and the Levene's tests, respectively.
184 $P < 0.05$ was considered significant difference. Statistical analysis was performed using SPSS
185 software (version 20).

186

187 **3. Results**

188 **3.1. Growth performance**

189 Table 3 presents the growth performance of *M. rosenbergii* PL fed with garlic extracts. There was
190 no mortality in the experiment. Final body weight for all groups was in the range 9.53- 16.43 g
191 and significant differences were observed among the groups ($P > 0.05$). Significant increase ($P <$
192 0.05) in FBW, WG, and SGR was observed in treated groups in a dose-dependent manner
193 compared with control group. A significant reduction in FCR was observed in the treated groups
194 compared to control group ($P < 0.05$). Highest and lowest weight gains and SGR values were
195 observed in the control group and 20 g/kg garlic extract respectively. Giant freshwater prawn fed
196 with a diet containing garlic extract at 20 g/kg showed the highest FBW, WG and SGR, and the
197 lowest FCR ($P < 0.05$).

198 **3.2. Muscle biochemical constituent**

199 The muscle composition of prawn's carcass fed the experimental diets are presented in (Table 4).
200 The highest concentration of protein was recorded in prawns fed with diets containing 20 g/kg
201 garlic extract ($P < 0.05$). The lipid content decreased with increasing garlic extract levels in the
202 diets ($P < 0.05$). The highest lipid content (9.82 ± 0.45 %) in muscle tissue was observed in the
203 control group ($P < 0.05$). Significant increase in ash content was observed in *M. rosenbergii* fed
204 with diets containing garlic extract ($P < 0.05$). Prawns fed with diets enriched with garlic extracts
205 had lower average values of moisture content but no significant difference was observed among
206 the groups ($P > 0.05$).

207 **3.3. Antioxidant enzymes**

208 The antioxidant enzyme activities of *M. rosenbergii* fed with different garlic extracts are shown in
209 Table 5. SOD, CAT, and GSH-Px activities of prawn fed the extract-containing diets were

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210 significantly higher than those of shrimp fed the control diet ($P < 0.05$). Moreover, the activity of
211 these enzymes was highest in the 20 and 10 g/kg garlic groups, respectively, which was also
212 significantly higher than in control and 5 g/kg garlic groups ($P < 0.05$).

213 3.4. Haemolymph biochemical parameters

214 Haemolymph enzymes' activity of prawn fed with experimental diets are presented in Table 6.
215 ALT, AST, ALP activities and total protein and albumin levels increased with the increase of garlic
216 levels in the diets ($P < 0.05$). The highest ALT, AST, and ALP activities were observed in prawns
217 fed with 20g/kg garlic group ($P < 0.05$). Similar trends were observed in the levels of total protein
218 and albumin in prawn hemolymph ($P < 0.05$).

219 3.5. Related immune gene expression

220 Expression of the HSP70 and IMD genes was significantly up-regulated in garlic groups ($P < 0.05$)
221 (Fig 1 and Fig 2). Moreover, the highest expression of IMD and HSP70 genes was observed in
222 prawns fed a diet containing 20 g/kg garlic extract ($P < 0.05$).

223

224 4. Discussion

225 In the present study, growth parameters of *M. rosenbergii* were significantly increased when fed
226 with a garlic extract supplemented diet. These results are in accordance with previous reports in *L.*
227 *vannamei* (Labrador et al., 2016; Samadi et al., 2016; Kumar et al., 2019), *O. mykiss* (Nya et al.,
228 2009; Etyemez Büyükdeveci et al., 2018; Adineh et al., 2020), *R. rutilus* (Ghehdarijani et al.,
229 2016), *Salmo caspius* (Zaefarian et al., 2017), *Mesopotamichthys sharpeyi* (Maniat et al., 2014),
230 *Lates calcarifer* (Talpur et al., 2012), *O. niloticus* (Metwally, 2009), *Cyprinus carpio* (Karimi
231 Pashaki et al., 2018), and *M. rosenbergii* (Poongodi et al., 2012). As proposed, this improvement
232 in growth performance could be related to higher digestive enzymes activities (such as lipase,
233 protease and amylase) as well as changes in intestinal microbiota (Shanthi et al., 2012;
234 Radhakrishnan et al., 2015; Etyemez Büyükdeveci et al., 2018). Supa-aksorn et al. (Supa-aksorn
235 et al., 2017), reported that amylase, lipase, and trypsin activities was higher in *O. niloticus* fed with
236 diets enriched with garlic extract (5 and 10 g/kg) than that of fish fed with a control diet. Likewise,
237 diets supplemented with garlic extract may act as appetizers, thereby increasing feed intake
238 (Poongodi et al., 2012; Platel et al., 2016). Labrador et al. (Labrador et al., 2016), found that Pacific

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239 white leg shrimp (*L. vannamei*) fed diets enriched with garlic powder (20, 40, and 60 g/kg) showed
240 the highest weight gain compared to other groups. In another study, the highest growth parameters
241 in *Tilapia zillii* fingerlings were recorded when fish fed a diet containing 20 g/kg garlic for 75 days
242 (Jegade, 2012). Allicin in garlic can enhance growth performance through stimulating intestinal
243 flora, improving digestive system, and enhancing energy utilization (Supa-aksorn et al., 2017).
244 The proximate body composition is often regarded as a suitable indicator of the physiological
245 condition of an organism (Kotiya et al., 2019). Our results showed that *M. rosenbergii* body
246 composition was significantly affected by the oral administration of garlic extracts. The crude
247 protein and ash of prawns fed control diet were notably ($P < 0.05$) lower than treated groups fed
248 garlic extracts. Samadi et al. (Samadi et al., 2016), showed positive effects of dietary garlic
249 supplementation on crude protein by *L. vannamei* fed with a garlic extract at 800 mg/kg, which is
250 consistent with results of the present study. Additionally, Adineh et al. (Sarhadi et al., 2020) found
251 that diets enriched with garlic extracts (10 g/kg) led to markedly increased crude protein and
252 markedly reduced crude fat in whole fish body. Similar results were documented in *O. niloticus*
253 and *T. zillii* fed with diets containing 30 and 10-30 g/kg garlic extract, respectively (Shalaby et al.,
254 2006; Ajiboye et al., 2016). It has been shown that garlic affects body protein metabolism caused
255 by hormonal regulation through raising protein metabolism or stimulating hormone secretion
256 (Srivastava et al., 2012). The fat value in this study was similar to Samadi et al. (Samadi et al.,
257 2016), Maniat and Ghotbeddin (Maniat et al., 2014), and Shalaby et al. (Shalaby et al., 2006)
258 reports. *M. rosenbergii* body fat decreased from 9.82 to 5.22 % with increases in dietary garlic
259 extract levels. Garlic administration may affect whole-body fat due to hepatic activities reduction
260 of cholesterogenic and lipogenic enzymes like fatty acid synthase, dehydrogenase and malic
261 enzyme which are caused to reducing of carcass lipid values (Yeh et al., 2001). In addition, some
262 organosulfur compounds in garlic such as diallyl-disulfide (allicin) and S-allyl cysteine may
263 prevent the synthesis of cholesterol, which could also lead to lower fat levels (Samadi et al., 2016).
264 Kim et al. (Kim et al., 2011) also showed that dietary addition of garlic oil has no notable difference
265 in moisture level of *L. vannamei*. Overall, it seems that dietary supplementation with garlic and its
266 derivatives (e.g., extracts, bioactive compounds) affect positively aquatic animals body
267 composition, however these effects are dependent on the level of garlic used, cultured species, and
268 the environmental conditions (Mahmoud et al., 2019).

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269 SOD/CAT system is the first defense mechanism against reactive oxygen species (ROS)
270 (Mahmoud et al., 2019). In the present study, antioxidant activities were higher in prawn fed diets
271 supplemented with garlic extract compared to control group. Prawn fed the diets containing 10 and
272 20 g/kg garlic diets exhibited the highest SOD, CAT and GSH-Px values compared with other
273 diets. These results are in accordance with earlier studies in Nile tilapia (*O. niloticus*) (Metwally,
274 2009; Mahmoud et al., 2019), common carp (*C. carpio*) (Naeiji et al., 2013; Yousefi et al., 2020)
275 and European seabass, *Dicentrarchus labrax* (Mosbah et al., 2018) and rainbow trout (*O. mykiss*)
276 (Mohebbi et al., 2012; Adineh et al., 2020) through enhancing the endogenous antioxidant
277 enzymatic mechanisms and counteracting the effects of ROS (Abdel-Tawwab et al., 2021). In
278 addition, Jahanjoo et al. (Jahanjoo et al., 2018) reported that the SOD activity was notably
279 increased in fish fed diets supplemented with 1% ginger or garlic. Garlic has been shown to
280 activate defense mechanisms and counteract infection through the production of superoxide anions
281 (Sahu et al., 2007). Sulphur-containing compounds (diallyl disulphide and s-allyl cysteine) and
282 flavonoids are two important types of antioxidant components within garlic, which might be
283 related to the higher antioxidant activities observed in prawns fed with garlic extracts (Sharma et
284 al., 2010).

285 Hepatic enzymes are commonly used as indicators of hepatopancreas yield, because these enzymes
286 have crucial roles in the interactions of carbohydrates, fats and amino acids (Hemre et al., 1996).
287 Higher values of AST and ALT may be therefore regarded as indicators of alterations in the
288 permeability of hepatopancreas tissue cells (Zeng et al., 2016). Our results showed that, ALP,
289 ALT, and AST activities were significantly higher in the 20 g/kg garlic group than in the other
290 groups. Similarly, Mohebbi et al. (Mohebbi et al., 2012) indicated that serum alanine
291 aminotransferase and aspartate aminotransferase levels in rainbow trout increased after fed with
292 40 and 50 g/kg garlic powder. Joseph et al. (Joseph et al., 1989) demonstrated that rats fed with a
293 garlic extract at 200 g/l had significant increase in hepatic enzyme activity compare to other
294 experimental groups after 10 days. Moreover, increase in hepatopancreas antioxidant activities
295 were accompanied by higher activity of haemolymph AST and ALT. The observed dose-
296 dependent increases in ALT, AST and ALP in treated shrimp could eventually suggest that
297 hepatopancreas functions were activated. When the lesions in the hepatopancreas were not aimed
298 to observe and in the view of the better growth, these alterations may be taken as an initial adaptive

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299 mechanism of prawns. ALT and AST increases were also observed in African catfish fed with
300 dietary clove (Adeshina et al. 2018).

301 Total protein and albumin levels were significantly increased in the 10 and 20 g/kg garlic groups
302 compared with other groups which are in agreement with Talpur and Ikhwanuddin (Talpur et al.,
303 2012) and Adineh et al. (Adineh et al., 2020) results. Higher levels of total protein and albumin
304 are often related to improved innate immunity following functional feed supplementation (Nya et
305 al., 2009; Pourmozafer et al., 2019). The S-allyl cysteine in garlic may play a crucial role in the
306 function of organs related to blood cell and immune system stimulation (Ndong et al., 2011).
307 Meanwhile, Nya and Austin (Nya et al., 2009) found that *O. mykiss* fed diets enriched with 1 and
308 10 g/kg garlic exhibited higher serum total protein than fish fed with control diet. The enhancement
309 of total protein level may be due to induction of antiprotease activity by garlic (Zaefarian et al.,
310 2017).

311 Our results showed that the expression of HSP70 and IMD genes in the hepatopancreas were
312 significantly increased in prawn fed with diets containing garlic extract compared to the control
313 group. HSP70 act as a molecular stress protein, which can contribute to the maintenance of cellular
314 homeostasis (Liu et al., 2016) and endogenous peroxidase activity for catalyzing the conversion
315 of ROS (Duan et al., 2018). Consistent with our results, previous literature showed the up-
316 regulation of related-immune genes following feeding Nile tilapia (*O. niloticus*) with diet
317 containing *Spirulina platensis* + garlic (M Abu Elala, 2016). Moreover, Kaleo et al. (Kaleo et al.,
318 2019) and Duan et al. (Duan et al., 2018) demonstrated that the expression of HSP70 gene in the
319 *M. rosenbergii* and *L. vannamei* was significantly up regulated after fed diets containing M.
320 oleifera (2.5 and 5 g/kg) and succinic acid (2.5, 5, and 10 g/kg), respectively. The up-regulation
321 of HSP70 is related to modulating cellular anti-stress reactions (Feder et al., 1999; Wan et al.,
322 2014). Based on the present study, there was a significant increase in expression of IMD gene
323 when prawns fed diets containing garlic extracts. IMD contributed shrimps innate immune
324 reactions (Li et al., 2013). The up-regulation of expression of IMD in *M. rosenbergii* may be
325 attributed to high concentrations of garlic extract, so that the prawn's body for resisting stress might
326 have to activate the IMD signaling pathway (Kaleo et al., 2019). As suggested, increase in IMD
327 and HSP-70 gene expressions could also support stress management in prawns fed with garlic. It
328 has been reported that an increase in HSP-70 gene expression in fish fed with plant-and probiotic-

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329 enriched diets was rather related to an anti-stress response (Abarike et al. 2020). Sung et al. (2012)
330 also reported that enhancing HSP-70 synthesis could protect common carp against ammonia-
331 related stress. Therefore in the present study, the general health of *M. rosenbergii* was found to
332 be improved by up-regulations of IMD and HSP-70 genes.

333 **Conclusion**

334 In summary, our results show some potential in using low-doses of garlic in prawn aquaculture as
335 a growth-stimulator and antioxidant promoter. In order to fully evaluate the positive/negative
336 potential of garlic-enriched diets, further research also needs to assess whether increased HSP-70
337 in garlic-fed shrimp would be beneficial in stressful conditions (e.g. shrimp exposed to pathogens
338 or to pollutants), or if in contrast garlic-treated shrimp would display exacerbated stress-responses.
339 In our study, despite increases in ALT and AST, treated prawn displayed higher growths and
340 higher antioxidant activities (CAT, SOD, GSH-px) suggesting that garlic enrichment, at least at
341 low doses, could potentially be beneficial. However, with the progressive increase in garlic
342 concentration, an important increase in the AST, ALP and ALT was observed, which could suggest
343 the presence of hepatic toxicity. However, further research is needed to explore whether a garlic-
344 enriched diet could cause histological changes in the hepatopancreas of *M. rosenbergii*.

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347 **Conflict of interest:** The authors declare no conflict of interest.

348 **Data availability:** The data that support the findings of this study are available on request from
349 the corresponding author.

350 **Ethics approval:** The authors confirm that the ethical policies of the journal, as noted on the
351 journal's author guidelines page, have been adhered to and the appropriate ethical review
352 committee approval has been received. The authors confirm that they have followed EU standards
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355 **References**

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- 357 Chinese herbs and a commercial probiotic *Bacillus* species improves hepato-immunological,
358 stress, and antioxidant parameters, and expression of HSP70 and HIF-1 α mRNA to hypoxia, cold
359 and heat stress in Nile tilapia, *Oreochromis niloticus*. *Aquaculture Reports.*, **18**, 100438.
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Table 1. Composition (g/kg) and proximate analysis of the experimental diets

Ingredients	Treatments			
	Control (Garlic-Free)	Diet 1 (5 g/kg Garlic)	Diet 2 (10 g/kg Garlic)	Diet 3 (20 g/kg Garlic)
Fish meal	460	460	460	460
Soy bean meal	260	260	260	260
Wheat bran	150	150	150	150
Corn flour	90	85	80	70
Garlic powder	0	5	10	20
Vegetable oil	30	30	30	3
Vit- min premix ^a	10	10	10	10
Proximate analysis				

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Crude protein	39.3	40.1	40.2	40.3
Crude fat	10.50	10.35	10.62	10.42
Crude fiber	4.5	4.8	4.2	4.6
Ash	13.3	13.7	14.1	14.3

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 638 ^aVitamins and mineral mixes: Vitamin A (10,000,000 IU), Vitamin D3 (3,000,000 IU), Vitamin E
 639 (10,000 IU), Vitamin B1 (400 mg), Vitamin B2 (1,200 mg), Vitamin B6 (1,200 mg), coated
 640 Vitamin C (25,000 mg), Folic acid (600 mg), Niacin (6,000 mg), Calcium pantothenate (10,000
 641 mg), Biotin (20,000 mcg), Choline Chloride (10,000 mg), Iron (12,000 mg), Copper (1,200 mg),
 642 Iodine (400 mg), Manganese (5,000 mg), Zinc (6,000 mg), Cobalt (20 mg), Selenium (20 mg)

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 644 **Table 2.** Primers used for the real-time PCR assay.
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Gene	qPCR primers, forward/reverse	Length (bp)	Amplicon (bp)	Sequence source
IMD*	CGACCACATTCTCCTCCTCCC	21	184	KR827675.1
	TTCAGTGCATCCACGTCCTC	21		
HSP70	TGACAAGGGTCGCCTCAGTA	20	158	EU884290.2
	CATTATCTTGTTGCGATCCTC	21		
β-actin	TCCGTAAGGACCTGTATGCC	20	96	AY651918.2
	TCGGGAGGTGCGATGATTTT	20		

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 648 **Table 3.** Growth performance of prawn fed with experimental diets.
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parameters	Treatments			
	Control (Garlic-Free)	Diet 1 (5 g/kg Garlic)	Diet 2 (10 g/kg Garlic)	Diet 3 (20 g/kg Garlic)
Mean initial body weight (g)	1.12 ± 0.02	1.10 ± 0.03	1.13 ± 0.01	1.12 ± 0.02
Mean final body weight (g)	9.51 ± 0.12 ^c	12.93 ± 0.31 ^b	13.15 ± 0.26 ^b	16.43 ± 0.41 ^a
Weight gain(g)	8.39 ± 0.11 ^c	11.83 ± 0.31 ^b	12.02 ± 0.47 ^b	15.31 ± 0.22 ^a
SGR ¹	4.73 ± 0.33 ^c	5.78 ± 0.32 ^b	5.82 ± 0.27 ^b	6.93 ± 0.31 ^a
FCR ²	2.12 ± 0.01 ^c	1.76 ± 0.02 ^b	1.73 ± 0.01 ^b	1.42 ± 0.03 ^a

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 651 Data are mean values of nine replicates expressed as mean ±SE. Data with different superscripts show
 652 significant differences ($P < 0.05$).
 653 ¹Specific growth rate
 654 ²Feed conversion ratio

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 656 **Table 4.** Muscle biochemical constituent of shrimp fed with experimental diets.
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parameters	Treatments			
	Control (Garlic-Free)	Diet 1 (5 g/kg Garlic)	Diet 2 (10 g/kg Garlic)	Diet 3 (20 g/kg Garlic)
Crude protein	13.2 ± 0.21 ^c	15.6 ± 0.11 ^b	16.1 ± 0.34 ^b	18.5 ± 0.42 ^a
Fat	9.82 ± 0.45 ^a	7.62 ± 0.52 ^b	7.41 ± 0.22 ^b	5.22 ± 0.17 ^c
Ash	3.82 ± 0.04 ^c	4.46 ± 0.01 ^b	4.58 ± 0.06 ^b	5.24 ± 0.05 ^a
Moisture	65.86 ± 0.18	65.82 ± 0.10	65.62 ± 0.15	65.59 ± 0.12

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659 **Table 5.** Effect of garlic extract on hepatopancreas antioxidant capacity of *M. rosenbergii* after 60 days.

Parameters	Treatments			
	Control (Garlic-Free)	Diet 1 (5 g/kg Garlic)	Diet 2 (10 g/kg Garlic)	Diet 3 (20 g/kg Garlic)
CAT (U/mg protein)	27.4 ± 1.32 ^c	33.5 ± 1.31 ^b	38.7 ± 1.62 ^a	39.2 ± 1.39 ^a
SOD (U/mg protein)	87.5 ± 1.42 ^c	98.2 ± 1.83 ^b	109.4 ± 1.59 ^a	110.3 ± 1.42 ^a
GSH-Px (U/mg protein)	25.3 ± 1.15 ^c	30.3 ± 1.25 ^b	35.1 ± 1.19 ^a	36.8 ± 1.61 ^a

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661 Data are mean values of nine replicates expressed as mean ± SE. Data with different superscripts
662 show significant differences ($P < 0.05$).

663

664 **Table 6.** Effect of garlic extract on haemolymph biochemical parameters of *M. rosenbergii* after 60 days.

Parameters	Treatments			
	Control (Garlic-Free)	Diet 1 (5 g/kg Garlic)	Diet 2 (10 g/kg Garlic)	Diet 3 (20 g/kg Garlic)
ALT (U/ml)	105.2 ± 1.24 ^c	113.3 ± 1.41 ^b	115.8 ± 1.67 ^b	133.2 ± 1.59 ^a
AST (U/ml)	60.1 ± 1.42 ^c	71.7 ± 1.10 ^b	73.6 ± 1.32 ^b	83.7 ± 1.70 ^a
ALP (U/ml)	192.2 ± 2.56 ^c	231.8 ± 3.17 ^b	233.5 ± 2.72 ^b	265.3 ± 3.27 ^a
Total protein (g/l)	23.3 ± 1.15 ^c	29.7 ± 1.28 ^b	38.8 ± 1.34 ^a	39.7 ± 1.41 ^a
Albumin (g/l)	3.3 ± 0.12 ^c	4.6 ± 0.22 ^b	5.7 ± 0.17 ^a	5.9 ± 0.27 ^a

665 Data are mean values of nine replicates expressed as mean ± SE. Data with different superscripts
666 show significant differences ($P < 0.05$).

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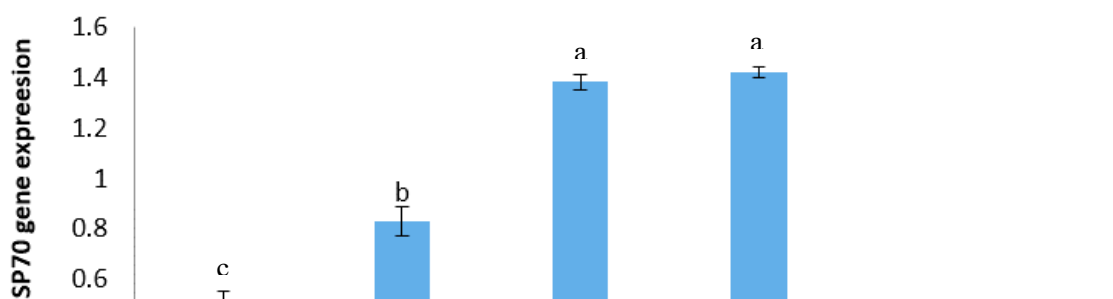
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Garlic extract on physio-biochemical responses of *M. rosenbergii*

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680 **Fig 1.** The relative gene expression of HSP70 in hepatopancreas of *M. rosenbergii* fed
681 experimental diets. Data are expressed as the mean SD. Different lowercase letters indicate
682 statistically significant differences between groups ($P < 0.05$).

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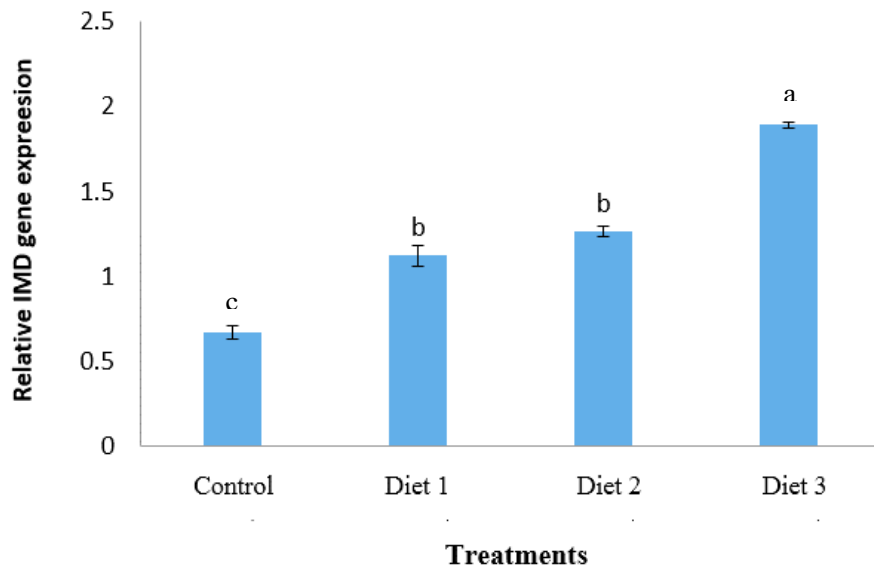
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692 **Fig 2.** The relative gene expression of IMD in hepatopancreas of *M. rosenbergii* fed experimental
693 diets. Data are expressed as the mean SD. Different lowercase letters indicate statistically
694 significant differences between groups ($P < 0.05$)