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**Development of immunity in rainbow trout (*Oncorhynchus mykiss*, Walbaum) to  
*Aeromonas hydrophila* after the dietary application of garlic**

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Running title: Immunity to *Aeromonas hydrophila*

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36 **Abstract**

37           The development and duration of immune protection against *Aeromonas*  
38 *hydrophila* infections with garlic as immunostimulant in rainbow trout *Oncorhynchus*  
39 *mykiss* was studied. Rainbow trout fingerlings of 14 g average weight were fed with 0  
40 g (= Control), 0.5 g and 1.0 g of garlic 100 g<sup>-1</sup> of feed for 14 days. Physiological  
41 factors, biochemical, immunological, hematological parameters and electrolyte  
42 indices were evaluated after a further 14, 21 and 28 days before challenge with  
43 *Aeromonas hydrophila*. Fourteen days after the cessation of feeding with garlic,  
44 mortality rates of 12 % (relative percent survival [RPS] = 86 %) and 16 % (RPS = 80  
45 %) were recorded in groups which received 0.5 g and 1.0 g of garlic 100 g<sup>-1</sup> of feed,  
46 respectively, compared to 84 % mortalities in the controls. The corresponding RPS 21  
47 days after ending the feeding regime was 75 % and 68, respectively. One week later,  
48 the RPS had dropped to 55% and 46% in the groups fed with 0.5 g and 1.0 g garlic  
49 100 g<sup>-1</sup> of feed, respectively.

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51 *Keywords:* Immunity; Immune defence mechanism; *Aeromonas hydrophila*, Rainbow  
52 trout.

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54 **1. Introduction**

55           The basis of this study was an investigation of the duration of protection and  
56 immunity against infection with *Aeromonas hydrophila* following the administration  
57 of garlic as a feed supplement to rainbow trout (*Oncorhynchus mykiss*, Walbaum)  
58 fingerlings. Certainly, the use of immunostimulants as dietary supplements is  
59 recognized to improve the non-specific defence mechanism in fish, thus providing  
60 resistance to infections [1; 2]. Interestingly, it has been argued that the fish innate  
61 immune system lacks memory, and as such the duration of beneficial

62 immunostimulant induced responses will inevitably be shorter than the specific or  
63 adaptive immune response [3]. Also, it has been considered that long-term exposure  
64 to immunostimulants lead to immune suppression and tolerance insofar as the immune  
65 system becomes de-sensitized thereby losing its sensitivity [4; 5]. However, the use of  
66 dietary garlic has certainly led to protection in fish against a range of bacterial fish  
67 pathogens, [6; 7 and 8]. Evidence suggests that garlic constituents provide suitable  
68 bases for new therapies because of their generalized antimicrobial and immunological  
69 properties [9]. The level of protection recorded in tilapia (*Oreochromis niloticus*)  
70 following challenge with *A. hydrophila* reflected the concentration used [7].

71 This study has sought to extend the earlier work [10], by examining the  
72 duration of protection of administration of garlic when administered orally, and by  
73 detailing the nature of the immunological and physiological responses in rainbow  
74 trout.

75

## 76 **2. Materials and methods**

### 77 *2.1. Fish*

78 Rainbow trout, of 14 g average wet weight, were obtained from a commercial  
79 fish farm in Scotland, and acclimatized in aerated free flowing dechlorinated water at  
80 12°C. The health status was examined immediately upon arrival in the aquaria and at  
81 14 days intervals thereafter [11]. One hundred and twenty (120) fish were randomly  
82 distributed into 3 experimental groups following a complete randomized design  
83 [CRD; 12], with each group to represent feed treatment of 0g (=Control), 0.5g and  
84 1.0g per 100g of feed.

85

### 86 *2.2. Feeding regimes*

87 Oven-dried garlic bulbs were obtained from a local supermarket, crushed  
88 using a household garlic press, sieved with the use of appropriate sized wire mesh

89 sifter and mixed with commercial fish feed (Biomar, Bio-optimal Start) to achieve 0 g  
90 (= control), 0.5 g and 1.0 g 100 g<sup>-1</sup> of feed. The modified feed was stored in screw cap  
91 bottles at room temperature until needed. The experimental fish groups were fed twice  
92 daily to satiation for 14 days. Fish were fed with standard commercial diet after the  
93 administration of garlic.

94

### 95 2.3. *Bacterial pathogen*

96 *A. hydrophila* (AE 57) was obtained from diseased Barramundi (*Lates*  
97 *calcarifer*), isolated on tryptone soya agar (Oxoid), identified biochemically and by  
98 DNA sequence homology, and maintained as stocks in 15% (v/v) glycerol at -70°C.  
99 For routine use, cultures were grown overnight on TSA at 28°C. Authenticity was  
100 verified after Austin and Austin [13]. Broth cultures were prepared in tryptone soya  
101 broth (TSB; Oxoid) with overnight incubation at 28°C. Then, the broths were  
102 centrifuged at 3000 x g for 10 min at 4°C, before the cells were washed twice in PBS  
103 (Oxoid) pH 7.4, and the pellets resuspended in fresh buffer. The concentration was  
104 adjusted to 10<sup>6</sup> cells ml<sup>-1</sup> as determined by means of a hemocytometer slide (Improved  
105 Neubauer Type, Merck) at a magnification of x400 on a Kyowa light microscope.

106

### 107 2.4. *Experimental challenge and measurement of immunological parameters*

108 Challenge of 20 fish from the experimental groups and the control was by i.p.  
109 injection with 0.1 ml<sup>-1</sup> suspension of *A. hydrophila* in 0.9 % (w/v) saline containing  
110 10<sup>6</sup> cells ml<sup>-1</sup>, 24 h after stopping feeding trials. Previous work had determined the LD  
111 50% to be 1.7 x 10<sup>5</sup> cells/ ml<sup>-1</sup>. Mortalities were monitored over 14 days, and any  
112 dead or moribund fish examined bacteriologically to confirm the presence of *A.*  
113 *hydrophila* [11]. The relative percentage survival (RPS) was calculated after [14].  
114 Thus as:

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116 1 – Mortality of treatment group / mortality of control group x100.

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118 Body weight, gutted weight, length (cm<sup>3</sup>) and condition factor (CF) were  
119 calculated as outlined by [15]. Sub-groups of 10 fish were used to determine growth  
120 performance in which the percentage weight gain and specific growth rate (SGR)  
121 were determined according to [16]. Thus:

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123  $\text{Wt. gain \%} = \text{Final wt} - \text{Initial wt.} / \text{Initial wt.} \times 100.$

124  $\text{SGR} = \text{Log}_e \text{ of final wt.} - \text{Log}_e \text{ of Initial wt.} / \text{No. of days.}$

125  $\text{FCR} = \text{Feed given (dry wt.)} / \text{Body wt. gain (wet wt.).}$

126  $\text{PER} = \text{Net wt. gain (wet wt.)} / \text{protein fed.}$

127  $\text{CF} = \text{gutted wt.} / \text{length} \times 100.$

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### 130 2.5. *Mode of action of the garlic*

131 Separate groups of 10 rainbow trout obtained from the experimental groups,  
132 fed with garlic, as before, and the Control were used to determine immune  
133 parameters. Thus, blood was collected by venepuncture, and transferred into vacuette  
134 tubes containing heparin as anticoagulant (Greiner) to prevent clotting. This blood  
135 was used for determination of hematocrit (Hct), hemoglobin (Hb) content, and total  
136 erythrocyte and leucocyte counts. For this, the blood was diluted to 10<sup>-2</sup> and 10<sup>-3</sup> in  
137 PBS, and the number of leucocytes and erythrocytes counted [17]. Duplicate blood  
138 samples were also collected and allowed to clot at room temperature for 2 h and  
139 refrigerated overnight at 4°C before the clotted blood was centrifuged at 3000 x g for  
140 10 min at 4°C, and the serum collected and stored at -70°C until used. Immune  
141 parameters such as the lysozyme activity, respiratory burst and serum peroxidase  
142 activities were determined following methods previously described [10]. Serum/blood

143 biochemical parameters were analysed using a Quantichrom™ kit (Bio Assay  
144 Systems, Hayward, CA, USA.). Serum total protein was estimated by a method based  
145 on an improved Bradford assay [18]. The OD of standard and test samples were  
146 measured against a blank in a microplate reader (Tecan, Männedorf, Switzerland) at  
147 OD<sub>595</sub>. Albumin content was estimated by the bromocresol green binding method  
148 [19], and absorbance was taken against a blank at OD<sub>620</sub> in a microplate reader  
149 (Tecan). Globulin content was calculated by subtracting albumin values from serum  
150 total protein. The albumin/globulin ratio was estimated by dividing albumin values by  
151 those of globulin [20].

152 Electrolytes, i.e. calcium (Ca<sup>++</sup>), magnesium (Mg<sup>++</sup>), sodium (Na<sup>+</sup>),  
153 potassium (K<sup>+</sup>) and ferrous iron (Fe<sup>+</sup>) ppm ml<sup>-1</sup>, were determined by flame emission  
154 photometry [21], using an automated system – Atomic Absorption Spectrometer  
155 (Perkin Elmer) with appropriate standards.

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## 157 2.6. Statistical analyses

158 Values for each parameter measured were expressed as arithmetic mean ±  
159 standard error (SE). Hematological and biochemical parameters were tested using  
160 one-way ANOVA, and a comparison of the mean values was done by using Duncan's  
161 multiple range tests [22], at the 5 % level of significance. The software programme  
162 SPSS (Version 14.0) for Windows was used.

163

## 164 3. Results

### 165 3.1. Fish growth

166 The physiological indices i.e. body weight, length, gutted weight, SGR and  
167 weight gain are shown in Table 1. Overall, the experimental groups did not differ  
168 significantly (P> 0.05) from each other in respect of body weight gain, length and  
169 gutted weight. The specific growth rate (SGR) of the fish 14 days after cessation of

170 feeding with garlic was  $1.2 \pm 0.1$  in the control group and  $1.2 \pm 0.1$  and  $0.9 \pm 0.3$  in the  
171 groups, which received 0.5 and 1.0 g garlic  $100 \text{ g}^{-1}$  of feed, respectively (Table 1). The  
172 feed conversion ratio (FCR) and protein efficiency ratio (PER) was also enhanced in  
173 experimental groups compared with the control (data not included).

174 The condition factor (CF) of fish receiving different doses of dietary garlic  
175 after withdrawal for 14, 21 and 28 days is shown in Table 3. However, CF was much  
176 lower 28 days after the ending of feeding with garlic.

177

### 178 3.2. Duration of protection

179 Experimental challenges at 14, 21 and 28 days after withdrawal of garlic  
180 supplemented diet led to a steady reduction in the level of protection in rainbow trout  
181 following challenge with *A. hydrophila* (Figure 1a, b and c). Thus 14 days after  
182 ending the administration of garlic dosed at  $0.5 \text{ g } 100 \text{ g}^{-1}$ , the RPS was 86%,  
183 decreasing to 75% and 68% after 21 and 28 days of dietary garlic treatments,  
184 respectively (Figure 1a, b and c). In comparison, 14 days after stopping feeding with  
185  $1.0 \text{ g } 100 \text{ g}^{-1}$ , the RPS was 80%, reducing to 55% after 21 days, and 46% at 28  
186 days. Generally after challenge, diseased fish displayed abdominal distension,  
187 necrosis, ascitic fluid and exophthalmia.

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### 189 3.3. Mode of action of garlic

190 Compared to the controls, the number of RBC and WBC was significantly  
191 ( $P < 0.05$ ) higher in experimental groups, which received 1.0 g of garlic  $100 \text{ g}^{-1}$  of  
192 feed, but not at the lower dose, at 14 days after use of the experimental diet.  
193 Thereafter, the number of RBC remained significantly higher than the controls Table  
194 2). Yet for WBC, the number of cells in the group fed with  $0.5 \text{ g } 100 \text{ g}^{-1}$  of  
195 feed was lower than the controls (Table 2). The use of garlic did not have any

196 significant effect ( $P < 0.05$ ) on Hb, although Hct was higher in some sampling period  
197 when compared with the controls, it was not statistically significant (Table 1).

198 Dietary garlic led to a negligible effect on the biochemical indices of the  
199 treatment groups (Figure 2). In particular, the serum total protein content remained  
200 similar to the controls throughout the experimental period (Figure 2).

201 The production of superoxide anion as a measure of the respiratory burst  
202 activity was significantly influenced ( $P < 0.05$ ) by dietary garlic (Figure 3).  
203 Furthermore, a significant ( $P < 0.05$ ) increase in respiratory burst activity, i.e.  
204  $0.3 \pm 0.4$  OD, was recorded in fish which received  $0.5 \text{ g garlic } 100 \text{ g}^{-1}$  feed, compared  
205 to  $0.2 \pm 0.0$  of the controls. Although respiratory burst activity 28 days after feeding  
206 with garlic was lower, the data were nevertheless higher than the controls (Figure 3).

207 There were significant ( $p > 0.05$ ) differences in serum lysozyme activity in  
208 the experimental groups, compared with the controls (Fig. 4). Moreover, the activity  
209 was 1780 and 1590 units  $/\text{ml}^{-1}$  in fish group which received 0.5 and 1.0 g garlic 100  
210  $\text{g}^{-1}$  feed respectively at 14 days post dietary garlic withdrawal as compared to 1100  
211 units  $/\text{ml}^{-1}$  in the control. In particular 2 weeks latter i.e. at 28 days, it decreases to  
212 867 and 820 units  $/\text{ml}^{-1}$  for groups which was fed 0.5 and 1.0 g garlic 100  $\text{g}^{-1}$  feed  
213 respectively, compared to 760 units  $/\text{ml}^{-1}$  in the control.

214 Use of garlic at  $0.5 \text{ g}$  and  $1.0 \text{ g } 100 \text{ g}^{-1}$  of feed had no significant ( $P < 0.05$ )  
215 effect on the serum peroxidase activity, as levels declined over the 28 day withdrawal  
216 period (Figure 4).

217 The  $\text{Ca}^+$  levels were higher in all groups during feeding with garlic, rather  
218 than afterwards, whereas the amounts of  $\text{Mg}^+$ ,  $\text{Fe}^{++}$ , K and  $\text{Na}^+$  reduced. In contrast,  
219 those of the controls remained high (Table 3).

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#### 225 **4. Discussion**

226 This study reinforces the view that garlic is beneficial for the control of *A.*  
227 *hydrophila* infection in rainbow trout, and thereby extends the previous study [10], by  
228 demonstrating the longer term memory effect after the cessation of the feeding  
229 regime. Of relevance to the present study, a previous investigation using brook trout,  
230 *Salvelinus fontinalis*, which were administered with chitosan by a 30-min immersion  
231 led to reduced protection 14 days afterwards [23]. Moreover by 21 days after  
232 concluding the administration of chitosan, there were not any significant differences  
233 in the levels of protection with the controls. Certainly, it has been argued that the  
234 long-term application of immunostimulants leads to immunosuppression and loss of  
235 effect of the compounds [24; 5]. Indeed, it is speculative whether or not a similar  
236 effect could have happened in this study.

237 Previous work using rainbow trout which received dietary garlic for 14 days  
238 treatment periods revealed protection against challenge with *A. hydrophila* and  
239 enhanced innate defence mechanisms, such as high oxidative radical production by  
240 serum neutrophils, proliferation of lymphocytic cells and phagocytic activity of the  
241 head kidney macrophage [10]. However, modulation of non-specific defence  
242 mechanisms in treated fish may have been chiefly by activation of the released of  
243 reactive oxygen species (ROS) by immune cells. This might explain the significant  
244 increase ( $P < 0.05$  %) in the respiratory burst activity of the neutrophils, measured by  
245 the reduction of NBT to formazan as indicator of superoxide anion ( $O_2^-$ ) production.  
246 This reactive oxygen species include superoxide radicals and hydrogen peroxide,  
247 which are known to be toxic to pathogenic bacteria [25; 26]. Moreover, the significant  
248 difference between the treatment and control groups was similar to the finding of [16],  
249 who observed a high NBT activity in rohu *Labeo rohita* juveniles fed with 0.4%

250 dietary yeast RNA. Comparable results were also obtained by [27], in *Cyprinus*  
251 *carpio* which received dietary nucleotide derived from yeast RNA. Moreover, similar  
252 reports of an increase in NBT activity over controls in rohu juveniles fed 0.1%, 0.5 %  
253 and 1.0 % of garlic [8].

254 Furthermore, serum Lysozymes activity plays a key role in the lyses of  
255 bacterial pathogens, activation of Phagocytosis and haemolytic complement activity.  
256 Serum Lysozymes activity presents a first line of defence mechanism, with lytic  
257 factors by preventing adhesion and colonization of bacterial pathogens. Thus,  
258 resulting in the prevention of infections and disease [28; 29]. In this present study, the  
259 serum lysozyme activity was higher in dietary garlic treated groups than the control  
260 14, 21 and 28 days after stopping supplemented feed administrations. Definitely such  
261 profound enhancement in this innate immune factor stem from dietary  
262 supplementation may have provided the observed protection against this pathogen.  
263 Similarly some authors [30], had also observed significantly enhanced Lysozymes  
264 activity after 1, 2 or 3 weeks treatments of Tilapia with medicinal plant *Eclipta alba*  
265 leaf extracts.

266 Certainly, the proliferation rate and number of lymphocytes produced is very  
267 important for the magnitude and duration of protection against disease [31]. This  
268 supports the view that the persistence of an immune activator may be a critical factor  
269 in maintaining long-term protection against disease causing situations. With garlic,  
270 various bioactive compounds have been found to exhibit immunological properties  
271 and are detectable in blood after oral uptake [32; 33 and 34]. Also in comparison to  
272 this study, the CF was reduced in the work involving rainbow trout reported by [14].  
273 Furthermore, it is noteworthy that CF has been regarded as a useful bio-indicator of  
274 stress [35], and is reflected in changes in energy budgets [36]. In the present study, it  
275 is possible that the deterioration in CF may be a consequence of disrupted metabolic  
276 processes, resulting from the withdrawal of garlic from the diet. Furthermore, the

277 changes in levels of blood electrolyte ions may be explained by the reduced energy  
278 metabolism as considered previously by [37]. It is interesting to note that similar  
279 results were documented in rainbow trout treated with central nervous seizure agents  
280 [38].

281 In conclusion, this study has affirmed that the protective effect of dietary  
282 garlic extends 28 days beyond the period of its application to rainbow trout.

283

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