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

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Abstract

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

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

1. Introduction

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

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

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

2. Materials and methods

2.1. Fish

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

2.2. Feeding regimes

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

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

2.3. Bacterial pathogen

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

2.4. Experimental challenge and measurement of immunological parameters

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

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

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118 Body weight, gutted weight, length (cm^3) 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 Wt. gain % = Final wt – Initial wt. /Initial wt. X 100.

124 SGR = Log_e of final wt. – Log_e of Initial wt. / No. of days.

125 FCR = Feed given (dry wt.) / Body wt. gain (wet wt.).

126 PER = Net wt. gain (wet wt.) / protein fed.

127 CF = gutted wt. / length x 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^{-2} and 10^{-3} 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^{o}C before the clotted blood was centrifuged at 3000 x g for
140 10 min at 4^{o}C, and the serum collected and stored at -70^{o}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

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

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

2.6. Statistical analyses

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

3. Results

3.1. Fish growth

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

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

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

3.2. Duration of protection

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

3.3. Mode of action of garlic

Compared to the controls, the number of RBC and WBC was significantly ($P < 0.05$) higher in experimental groups, which received 1.0 g of garlic 100 g^{-1} of feed, but not at the lower dose, at 14 days after use of the experimental diet. Thereafter, the number of RBC remained significantly higher than the controls Table 2). Yet for WBC, the number of cells in the group fed with 0.5 g garlic 100 g^{-1} of 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±0.4 OD, was recorded in fish which received 0.5 g garlic 100 g^{-1} feed, compared
205 to 0.2±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 /ml^{-1} in fish group which received 0.5 and 1.0 g garlic 100
210 g^{-1} feed respectively at 14 days post dietary garlic withdrawal as compared to 1100
211 units /ml^{-1} in the control. In particular 2 weeks latter i.e. at 28 days, it decreases to
212 867 and 820 units /ml^{-1} for groups which was fed 0.5 and 1.0 g garlic 100 g^{-1} feed
213 respectively, compared to 760 units /ml^{-1} in the control.
214 Use of garlic at 0.5 g and 1.0 g 100 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 Ca+ levels were higher in all groups during feeding with garlic, rather
218 than afterwards, whereas the amounts of Mg^{+}, Fe^{++}, K and Na^{+} reduced. In contrast,
219 those of the controls remained high (Table 3).
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4. Discussion

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

Previous work using rainbow trout which received dietary garlic for 14 days treatment periods revealed protection against challenge with *A. hydrophila* and enhanced innate defence mechanisms, such as high oxidative radical production by serum neutrophils, proliferation of lymphocytic cells and phagocytic activity of the head kidney macrophage [10]. However, modulation of non-specific defence mechanisms in treated fish may have been chiefly by activation of the released of reactive oxygen species (ROS) by immune cells. This might explain the significant increase ($P< 0.05$ %) in the respiratory burst activity of the neutrophils, measured by the reduction of NBT to formazan as indicator of superoxide anion (0_2-) production. This reactive oxygen species include superoxide radicals and hydrogen peroxide, which are known to be toxic to pathogenic bacteria [25; 26]. Moreover, the significant difference between the treatment and control groups was similar to the finding of [16], who observed a high NBT activity in rohu *Labeo rohita* juveniles fed with 0.4%

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

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

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

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

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

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