Effect of garlic (*Allium sativum*) extract on the growth and disease resistance of *Carassius auratus* (Linnaeus, 1758)

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ABSTRACT

*In-vitro* inhibitory activity of garlic (*Allium sativum*) extract was examined against *Pseudomonas* spp. and *Aeromonas* spp. isolated from diseased freshwater ornamental fish. The effect of garlic extract supplemented feed on the growth performance of freshwater ornamental fish, *Carassius auratus* was also investigated. The MIC 80 of garlic was found to be 0.40 – 0.41% and 0.99 – 1.43% against *Aeromonas* spp. and *Pseudomonas* spp., respectively. The feed containing garlic at a rate 1g / 100g significantly improved the weight gain and food conversion ratio of *C. auratus*. The garlic extract did not show any significant inhibitory effect on fish gut bacterial flora *in vivo* when administered through feed. On the other hand, it was able to improve the disease resistance of fish particularly to *Pseudomonas fluorescens* infection.

Introduction

With increasing intensification in commercial aquaculture, many products are being made available for aquaculture purpose with varying success rate. Use of antibiotic is a common practice in aquaculture to control bacterial fish diseases. The associated problem of antibiotic resistance in aquaculture has grown to be more complex (GESAMP, 1997) which necessitated the search for alternative drugs, preferably of herbal origin with no side effects to the host. Garlic (*Allium sativum*) has been receiving attention not only as a spice or a food, but also for treatment of many diseases (Anon, 1995). The antibacterial activities of garlic have been widely studied (Ankri *et al.*, 1999). The active inhibitory chemical of garlic is allicin or di-allyl thiosulphinic acid which is enzymatically released from a pre-cursor form when the garlic bulbs are crushed (Saleem and Al-Delaimy, 1982). Garlic treatment has been attempted against diverse infections and infestations in marine fish, notably cryptocaryosis, caused by *Cryptocaryon irritans* (Cortes-Jorge Jr, 2001). Although there are reports on the positive effects of garlic in controlling the parasitic diseases (Lun *et al.*, 1994; Ankri *et al.*, 1997), its effects on bacterial fish pathogens are meagre. This communication reports the *in-vitro* inhibitory effect of crude extract from
garlic bulbs on opportunistic bacterial pathogens of fish as well as its effect on the growth performance and disease resistance of freshwater ornamental fish, *Carassius auratus* when administered through feed.

**Materials and methods**

*Determination of MIC of antibiotics and garlic extract*

The bacterial isolates (n=4) used in this study were isolated from *Carassius auratus* and *Xiphophorus helleri*, diagnosed to have tail rot and fin rot, respectively on tryptic soy agar and identified to be *Aeromonas* and *Pseudomonas* as per the descriptions of Austin and Austin (1999). Fresh garlic (*Allium sativum*) bulbs were first sterilized with mercuric chloride at the concentration of 0.2% and washed repeatedly (five times) with sterile distilled water. The treated garlic bulbs were aseptically ground to fine paste and aqueous portion was collected by centrifugation at 5000 rpm for 20 minutes at 25°C. The aqueous portion was then used immediately as crude aqueous garlic extract for further experimentation. The MIC of garlic was determined following the technique of Kumar and Berwal (1998). The tubes containing antibiotic assay medium - 37 was supplemented with different levels of crude garlic extracts at concentration of 0, 0.5, 1.0, 1.5, 2.0 and 2.5%. The tubes were then inoculated separately with two strains each of *Aeromonas sp.* and *Pseudomonas sp.* at a concentration of 5.0 x 10⁶/ml. The initial optical density (OD) was noted at 620 nm in a spectrophotometer. The inoculated tubes and control were incubated at 30°C for 24 h. The growth of the organism was observed with the aid of the spectrophotometer in terms of turbidity. The difference between the final and initial OD was interpreted as the growth of bacteria, whereas comparison of the final readings with the control readings depicted the inhibitory effect of garlic on fish bacterial pathogens.

The MIC of garlic at an inhibition level of 80% was determined by plotting change in OD against the concentration of garlic. From the points on the curve depicting 20% growth compared with that of control (no garlic) lines were plotted to meet the corresponding point as y-axis. From the respective points on the curve, perpendicular lines were dropped to the x-axis. The point of intersection of perpendicular lines on the x-axis represented the concentration of garlic that inhibited 80% of the test organism and the concentration was referred as MIC 80.

*Experimental fish and growth condition*

The goldfish, *Carassius auratus* of size ranging from 1.32 to 1.78g were procured from a commercial goldfish-breeding unit in Howrah, West Bengal, India. The fish were disinfected by placing in 5-ppm potassium permanganate (KMnO₄) solution for 15 min and transferred to circular FRP tanks of 500 L capacity containing bore-well water. The bore-well water was filtered through a sand and gravel filter with charcoal bed of 20 cm thick and used immediately. The dead and morbid fish were removed immediately and the healthy ones were stocked @ 100 fish/tank. The fish were fed with commercial pellet feed containing crude protein 41%, crude fat 6%, crude fiber 3% and moisture 11% twice daily. Continuous aeration was provided. The temperature, pH and total hardness of the rearing water, determined as described in APHA/AWWA/WEF (1998), and were maintained at about 27°C, 7.60 and 220 ppm respectively. All the fish were
maintained in such condition for at least 10 days prior to experimentation. The wastes and faecal matter were siphoned out on every 3rd day. Prior to experimentation, 15 numbers each of healthy fish were transferred to six glass aquaria of size 60 cm L x 45 cm B x 30 cm H (G1, G2, G3 and C1, C2, C3) containing 35L filtered water and acclimatized for 3 days. All experimental fish were fed with basal pelleted feed @ 5% of the body weight daily in 2 split doses.

Preparation of experimental feed and feeding experiment

The garlic paste was admixed with basal pelleted feed at a rate 1g/100g feed using binding gel at 10 ml/100 g feed. In control feed, binder alone was added as in test feeds. The test and control feeds were air dried for 2 days and placed in airtight plastic containers separately at room temperature 26-32°C. During the experimental period of 60 days, fish of G1, G2 and G3 were fed with garlic feed and those of C1, C2 and C3 were fed with control feed at the rate of 5% of the body weight daily in 2 split doses. The wastes and faecal matter were siphoned out and 50% of the water was exchanged on every 3rd day. The fish were observed for mortality daily. The length (in mm) and weight (0.001g accuracy) of the fish were noted at regular intervals and the survival percentage, wet weight gain, feed conversion ratio (FCR) and specific growth rate (SGR) were determined as described below:

\[
\text{Total wet weight gain (g)} = (\text{Final wet weight at the end of the experiment}) + \text{Wet weight of dead fish}) - \text{Initial wet weight}
\]

Food conversion ratio on dry weight basis (g)

\[
\text{Total feed consumed (FCR)} = \frac{\ln W_f - \ln W_i}{\text{Days of culture}} 
\]

Specific growth rate = \(\frac{W_f - W_i}{W_i} \times 100\)

Collection and preparation of samples for bacteriology

The water samples for bacteriology were collected from a particular experimental tank throughout the experiment. The water samples were collected aseptically in sterile glass bottles of 250 ml capacity, closed with stoppers and analyzed immediately. The bacterial flora associated with the gut of fish were enumerated as described in Blanch et al. (1997). In brief, two fish were scooped out from the specified tank and transferred to sterile container on each sampling day. The fish were killed by placing ice cubes in sterile containers, dissected-out and the entire gut removed aseptically. Care was taken to prevent contamination from entrails. The gut tissues along with contents were aseptically transferred to pre-weighed sterile glass tube containing 10 ml physiological saline. The saline along with fish gut was weighed to get the weight of gut and its contents. The gut and contents were then macerated using a sterile glass rod and thoroughly mixed using a vortex mixer. The thoroughly macerated and vortexed gut samples and also the water samples were diluted by 10 fold serial dilution in saline to appropriate levels and used for the enumeration of different groups of bacteria immediately.
Bacteriological analysis

Spread plate technique was followed for the enumeration of total plate counts of both water and fish gut samples. Aliquots (0.1 ml each) of appropriately diluted water and gut samples were spread on to TSA plates in duplicate. Inoculated plates were incubated at 30±2°C for 48 h and the colonies counted. The enumeration of presumptive pseudomonads from water and gut samples was done by spread plating on Pseudomonas isolation agar. The enumeration of motile aeromonads was carried out by spread plating on starch ampicillin agar (SAA; Palumbo et al., 1985). The seeded SAA plates after 24 h of incubation were flooded with iodine solution. The ampicillin resistant, amylase positive and yellow colonies were counted as presumptive motile aeromonads. The enumeration of lactose fermenters and non-fermenters was carried out on MacConkey agar by spread plating. The red or pink colonies were counted as lactose fermenters; while the colourless colonies were counted as lactose non-fermenters. The most probable number (MPN) five tube technique was followed to enumerate total coliforms of both water and gut samples. Aliquots (1.0 ml) of appropriately diluted samples were inoculated into respective tubes containing 10 ml lactose broth and inverted Durham’s tube. The tubes were incubated at 30±2°C for 48 h and observed for gas formation. The number of positive tubes from each dilution was noted and referred the McCardy table to get the MPN total coliforms count/100 ml water and/or/g gut (APHA, 1992).

Bacterial challenge test

A fish pathogenic bacterium, Pseudomonas fluorescens from the collections of the Department of Fishery Pathology and Microbiology, which has been isolated from the ulcers of Carassius auratus, was used in the challenge experiment by immersion assay (Austin et al., 1995). This bacterium, maintained on tryptic soy agar (TSA) slant, was streaked on to TSA plate and incubated at 30±2°C for 24 h to get young culture. One or two young discrete colonies of the bacterium was aseptically picked, transferred to 10 ml tryptic soy broth (TSB) and incubated at 30±2°C for 24 h. This 24 h old culture was then transferred to 1000 ml TSB and re-incubated at 30±2°C for 24 h. The cells were harvested by centrifugation at 7500 rpm for 20 min at 25°C in a centrifuge. The cell pellets were washed twice by centrifugation with sterile physiological saline and finally re-suspended in 50 ml sterile saline and used immediately. A portion of the cell suspension was suitably diluted up to 10^8 in sterile saline and the number of cells/ml of suspension was determined by spread plating on TSA plates after incubation at 30±2°C for 48 h.

Ten fish each from garlic fed and control groups were introduced respectively into each of T1, T2, T3, T4 tanks containing 20 L filtered water. To facilitate infection, two or three scales were removed from five fish from each tank and reintroduced into the respective tanks. The cell suspension of P. fluorescens was inoculated into T1 and T3 tanks in such a way to get a level of >10^5 cells/ml rearing medium. The tanks T2 and T4 served as control for garlic fed and control groups respectively. The experiment was carried out for a period of 30 days and the fish were fed daily with basal diet on demand. The accumulated wastes and faecal matter were siphoned out on every 5th day. Mortality, external signs of infection and behavioural abnormalities were recorded.
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The growth performance and bacteriological parameters of groups fed on garlic supplemented feed and control feed were compared by t-test using Microsoft Excel Package.

**Results and discussion**

Fig. 1 depicts the growth of fish pathogens in different concentrations of garlic, which was observed at 620 nm. The higher the OD the greater was the number of viable microorganisms. About 90% growth inhibition of *Aeromonas* strains took place at a concentration of 0.46% garlic. At this concentration, only 50% growth inhibition was noticed in *Pseudomonas* spp. Almost 90% arrest of growth of *Pseudomonas* spp. was observed at concentration of 1.80% of garlic. The minimal concentration of garlic to inhibit two strains of *Aeromonas* was almost the same, whereas it varied with *Pseudomonas* strains. The results showed *Aeromonas* was more sensitive to garlic than *Pseudomonas*. The results of the effect of garlic-supplemented feed on the growth and survival of *C. auratus* are given in Table 1. The total wet weight gain in garlic fed group (36.53± 0.83 g) was significantly higher (P<0.001) than

Fig 1: Growth inhibition of bacterial fish pathogens by garlic

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Growth performance of Carassius auratus fed with garlic supplemented feed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth parameters</td>
<td>Garlic fed</td>
</tr>
<tr>
<td>Total wet weight gain (g)</td>
<td>36.53 ± 0.83&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean survival (%)</td>
<td>100 ± 0.00</td>
</tr>
<tr>
<td>Feed conversion ratio</td>
<td>3.19 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Specific growth rate</td>
<td>1.56 ± 0.05</td>
</tr>
</tbody>
</table>

Values sharing common superscripts within rows are significantly different.

a: t=11.03; df=4; P<0.0004; b: t=-4.0; df=4; P<0.016

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Survival and infectivity in experimentally infected C. auratus fed with garlic-supplemented feed</th>
</tr>
</thead>
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<tr>
<td>Treatment</td>
<td>Number survived Experimentally infected stock</td>
</tr>
<tr>
<td>Garlic fed</td>
<td>10</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>*</sup>Number of fish exhibited tail/fin rot in 30 days of experimental period infected with *Pseudomonas fluorescens* @ 3.90x 10<sup>5</sup> / ml.
Table 3 - Log counts of bacteria in the gut and rearing medium of Carassius auratus

<table>
<thead>
<tr>
<th>No. of days of culture</th>
<th>Total plate count, cfu/g</th>
<th>Motile aeromonads, cfu/g</th>
<th>Presumptive pseudomonads, cfu/g</th>
<th>Lactose non-fermenters, cfu/g</th>
<th>Lactose fermenters, cfu/g</th>
<th>Total coliforms MPN/g</th>
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</thead>
<tbody>
<tr>
<td>Gut</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>C</td>
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<tr>
<td>0</td>
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<td>8.20</td>
<td>7.58</td>
<td>7.53</td>
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<td>3.66</td>
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<tr>
<td>15</td>
<td>8.86</td>
<td>8.36</td>
<td>7.19</td>
<td>7.04</td>
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<td>4.22</td>
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<tr>
<td>30</td>
<td>8.83</td>
<td>8.77</td>
<td>7.78</td>
<td>7.85</td>
<td>4.02</td>
<td>4.04</td>
</tr>
<tr>
<td>45</td>
<td>8.90</td>
<td>8.78</td>
<td>7.85</td>
<td>7.91</td>
<td>3.95</td>
<td>4.28</td>
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<tr>
<td>60</td>
<td>8.83</td>
<td>8.77</td>
<td>6.48</td>
<td>7.29</td>
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<tr>
<td>Rearing water</td>
<td>Cfu/ml</td>
<td>Cfu/ml</td>
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<td>Cfu/ml</td>
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<td>4.64</td>
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<td>0.70</td>
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<tr>
<td>30</td>
<td>4.71</td>
<td>4.84</td>
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<td>3.95</td>
<td>&lt;1.00</td>
<td>&lt;1.00</td>
</tr>
<tr>
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<td>5.30</td>
<td>3.89</td>
<td>3.80</td>
<td>&lt;1.00</td>
<td>&lt;1.00</td>
</tr>
<tr>
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<td>5.32</td>
<td>4.95</td>
<td>4.00</td>
<td>4.00</td>
<td>&lt;1.00</td>
<td>&lt;1.00</td>
</tr>
</tbody>
</table>

MPN: Most Probable Number; G: Garlic fed group; C: Control group
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that of control group (30.89±0.31g) probably indicating the beneficial effect of garlic due to the supply of unknown growth factors. The food conversion ratio was also significantly better in garlic fed group than the control group (P<0.01). The specific growth rate and mean percent survival did not differ significantly between these two groups.

Garlic was able to improve the disease resistance particularly to P. fluorescens infection (Table 2), which was reported to cause disease in gold fish (Bullock, 1965). The survival percentage did not differ and both groups survived experimental bacterial infection. However, the fish showing signs of fin / tail rot were markedly more in control than in garlic fed group. Likewise, Robertson et al. (2000) observed less evidence of minor health problems such as fin and tail rot in probiotic fed group when challenged with a bacterial pathogen. As seen in Table 3, the total plate counts in the gut of all fish groups were always above log 8.0/g and in conformity with Ringo et al. (1995) who reported as much as 10⁴ cells/g in digestive tract of fish. The counts of lactose fermenters in the gut of garlic fed and control groups decreased from the initial count. But the decrease was more in garlic fed group. The predominant species of bacteria found in the intestines of freshwater fish are Aeromonas, Enterobacter, Flavobacterium, Pseudomonas and Acinetobacter (Sugita et al., 1992; Ringo et al., 1995) so also in this study. According to them, most microbes are transients in aquatic animals and may change rapidly with the intrusion of microbes coming from water and food.

None of the bacteriological parameters of gut in the garlic fed and control groups differed significantly (P>0.05) probably due to an acidic (pH 3 or lower) environment in the gastric cavity, which can irreversibly neutralize alliinase (Lawson and Hughes, 1992). Without that enzyme, no allicin can be formed in the stomach no matter how much alliin might be ingested. These results indicated that the garlic extract did not have any significant effect on fish gut bacterial flora in vivo when administered through feed. The results, however, contradict with that of in vitro study (Fig. 1), which demonstrated that garlic extract was capable of inhibiting Aeromonas spp. and Pseudomonas spp. Certainly, better information on the chemistry of gastric juices in fish would be useful. Another probable reason is that the length of the alimentary tract of fish is much shorter than in terrestrial animals (Hsu and Wu, 1979). Consequently, the time that the garlic supplemented feed remains in the alimentary tract of fish is much shorter than that in terrestrial animals. This explains that garlic remains in the alimentary tract for a short period, which is insufficient to allow it to interact with constantly changing intestinal bacterial flora, which fluctuates strongly on a daily basis (Spanggaard et al., 2000). The bacterial flora of the C. auratus gut would have behaved in a similar manner.

There are many factors that influence the sensitivity of animal to pathogens and the efficacy of chemical protection. According to Cortes-Jorge Jr (2001) garlic therapy can potentially fend off secondary infection, neutralize the chemicals used by the parasite to destroy host tissue, mask host tissue, making it difficult for the parasite to recognize it and deliver outright damage to the parasite. Further research on the supplementation of herbal products in fish feeds could contribute to more environmentally friendly production.
Acknowledgement

The Indian Council of Agricultural Research, Government of India, New Delhi under the AP Cess Fund Project financed this study for which the authors are thankful.

References


