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Dietary supplementation of garlic (Allium sativum) to prevent Acanthocephala infection in aquaculture

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Abstract
This study showed the uses of garlic (*Allium sativum*) supplemented feed on *Clarias batrachus* for the judgment of garlic potential against Acanthocephala. Garlic allicin content (an active component of garlic) worked for *C. batrachus* to inhibit infection by Acanthocephala. Four garlic supplemented diets (50, 100 150 and 200 ml/kg) of different concentrations and a non-supplemented control diet were fed to *C. batrachus* for three months. Long-term (90 days) supplementation with garlic significantly reduced infection success by up to 80% as compared to controls and did not negatively affect palatability of the feed, Specific Growth Rate (SGR) (3.60±0.13) and Food Conversion Ratio (FCR) (2.33±0.38). Infection success was not influenced by short-term (30 days) supplementation suggesting that a delayed host response must occur to improve resistance to infection [prevalence (71%, 35%) and intensity (0.8, 0.5)] and 60 days conditioning period showed reduction of parasite respectively [prevalence (75%, 25%) and intensity (1, 0.6). Incorporation of garlic into a pressure-extruded pellet was found to be an effective method of delivery as only minimal leaching of allicin from the diet occurred (< 3% of allicin detected) during the interval of water contact between delivery and consumption. This study demonstrates that garlic extract (200 mL/kg) administered as a dietary supplement is one of the most practical methods to prevent Acanthocephala infection in Aquaculture.

Keywords: Allium sativum; Acanthocephala; *Clarias batrachus;* Infection; supplementation and Inhibition.

1. Introduction
In Bangladesh parasitic study has been conducted in both freshwater as well as in marine environment where several protozoan, helminthes and crustacean parasites recorded in different fish species. As the *Clarias batrachus* is most popular fish in Jessore as well as throughout the country, their abundance is reducing due to over exploitation, environmental stress and the free occurrence of diseases. *Clarias batrachus* is an omnivorous fish and due to its food habit, it can act as an intermediate host for many helminth parasites. Parasite infection has harmful influence on fish health and thus inhibits the normal growth of the fishes and outbreaks results in high mortalities. Also, the parasitic infection of this experimental fish results in economic losses due to not only mortality, but also treatment costs, decreasing growth that reduces the expansion of aquaculture. As a consequence, parasitic infestation has been provoked in fisheries stock over the time.

At present, there are no methods to prevent monogenean infections (Whittington *et al.*, 2012) [1]. The treatments of fish must occur at regular intervals as parasite life stages often exist outside the treatment area and can be resilient to treatment (Militz *et al.*, 2013; Mueller *et al.*, 1992) [2, 3]. Furthermore, there are many treatments such as bathing treatments are labor-intensive, time consuming, weather dependent, environmentally deleterious and harmful to fish welfare (Wilkinson *et al.*, 2006) [4]. There is a preventative agent which is administered by feed is a practical alternative treatment on bathing applications and the treatment is delivered directly to the host rather than to the host's environment (Dunn *et al.*, 1990) [5]. Different antibiotics and drugs has been used to treat parasitic infection in fish which created problems. The residual effect after human consumption many such as carsinogenic effect of human body, solubility, palatability, toxicity, cost and delivery, especially in aquaculture. Garlic has been used for its health benefits for thousands of years. Modern research confirmed many of the healing Properties of garlic, including its antiparasitic activity.

The value of garlic, *Allium sativum*, extract for bacterial disease control and immunostimulation has previously been demonstrated for a number of cultured fishes (Aly and Mohamed, 2010; Nya and Austin, 2009) [6, 7]. The present work has been undertaken to investigate the different parasite communities, and to find out the prevalence and intensity of infestation to *Clarias batrachus* with different habitat of varying water quality in Jessore.

2. Materials and Methods

2.1. Collection of sample fish and their maintenance

Clarias batrachus is one of the common fresh water fish species studied. Fingerlings (N= 50 pics) of *Clarias batrachus* [weight (8±1.5) g, length (11±2) cm] were obtained from a private fish farm in an oxygenated polythene begs from Chanchra more, Jessore, Bangladesh to Fisheries Laboratory, Jessore University of Science & Technology (JUST). The fingerlings were carefully transferred to circular tank and left undisturbed overnight. Fish were fed with a commercial catfish feed twice

Table 1: Proximate composition of supplementary fish feed

Composition	%
Moisture (Max)	12
Protein (Min)	33
Lipid (Min)	03
Ash (Min)	08

(**Source:** Nourish Poultry Feed Ltd.)

Table 2: Proximate composition of prepared feed

Composition	%
Moisture(Max)	14
Protein (Min)	30
Lipid(Min)	06
Ash(Min)	10

2.2. Preparation of garlic extracts and diet formulation

Fresh garlic was bought from a local supermarket and a crude aqueous extract was prepared by grinding with distilled water in a domestic blender. The stock extract was stored at 4 °C in a sealed bottle and was used within one month. Garlic extract was incorporated into the diets directly to formulate four diets (50 ml kg^{-1}, 100 ml kg^{-1}, 150 ml kg^{-1} and 200 ml kg^{-1}) and a control diet without extract. The resulting diets were then formed into pellet by hand and the control diet pelleted first to ensure against the possibility of garlic contamination. Diets were dried under direct sunlight for 3 days. After drying the diets were stored in air tight containers.

2.3. Experimental fish and feeding trial

Clarias batrachus fingerlings were randomly divided into four treatment group (T_1, T_2, T_3 and T_4) and one control (T_0). Each group was having ten fingerlings in aquarium. Each aquarium was supplied with aeration by aquarium aerator. T_0 was fed with basal diet and treated as control. The treatment groups were fed with 50 ml garlic extract/kg of feed (group T_1), 100ml garlic extract/kg of feed (groupT_2), and 150 ml garlic extract/kg of feed (groupT_3), 200ml garlic extract/kg of feed (groupT_4) for three weeks. The fish were fed twice daily at morning and afternoon. Water exchange (50.0%) was done daily and water quality was monitored throughout the experimental days at weekly intervals. Temperature (28±1.5) ^{0}C, pH (6.5±1.5), dissolved oxygen concentration (5.2±0.3) mg/L. Specific growth rate (SGR) and feed conversion ratio (FCR) were estimated for both control and experimental groups.

The following formula was used to calculate the growth parameters.

$$SGR\ (\%/day) = 100 \times [\ln w1 - \ln w0)/t]$$

Where w0 and w1 are average initial and finial body weights, respectively and t is time (days).

$$FCR = \frac{Food\ consumed(g)}{Weight\ gain(g)}$$

2.4. Parasite investigation

At fifteen days intervals one fish was randomly selected from each group for external and internal experiment. Fish sample were taken in live condition, weighted and took the total length by scale of that live fish. After that fish was killed by hand and examined immediately for parasitological study by using photographic microscope (Axiocam ERc 5s with Axio vision driver Carl zeiss, Germany). A clean spatula was held to the body of each individual and it was drawn backwards towards the tail in a smooth movement and lifting off a small amount of mucous from the different sites of the body for investigating parasites from skin. Later on, for each sites, mucous scrapings was placed on a clean glass slides and examined under the 4x, 10x and 40x lenses of the photographic microscope for observing the presence of parasites. In gill biopsy, a fine pair of scissors was used to cut open the operculum from both sides to reveal the operculum cavity.

2.5. Internal observation

Gill filaments were taken out by cutting off the two ends of the gill arches, and kept on glass slide. Furthermore, small sample of gills was made by splitting up gill filaments using fine scissors and observed under microscope. Each fish was dissected with a fine scissors used to make an incision along the mid-ventral line of the body to find out the parasites from the internal organs. All internal organs like liver, kidney, stomach, spleen, gastrointestinal track and anal cavity were investigated through a microscope and separated by needle and forceps. The intestines were carefully opened out by an incision from the body and put into a glass slide. Sometimes larger acanthocephalan (spiny headed worm) were visible in naked-eyes lying in the body cavity with their heads buried in the intestines and were quickly isolated using forceps, fixed on glass slide with glycerin. All the organs are kept on glass slide and viewed under several microscopic lenses.

Table 3: Parasites found in different organ of *Clarias batrachus*

Sample no.	Weight (kg)	Std Length (cm)	Site of infection	Name of parasites
01	16.26	11.6	Stomach	Acanthocephala
02	14.9	12.5	Intestine	Nematode
03	16.12	13.9	Gill	Absent
04	12.20	12.5	Liver	Absent
05	13.54	13.3	Intestine	Platyhelminth
06	19.90	14.6	Stomach	Acanthocephala
07	15.50	15.5	Intestine	Acanthocephala
08	16.31	14.4	Stomach	Cestode
09	13.92	10.4	Liver	Absent
10	14.25	11.9	Intestine	Nematode

2.6. Parasite maintenance
Isolated parasites were preserved in a glass container. Parasites were preserved with 35% formalin solution and 10% alcohol that kept in glass container for experiment.

2.7. Effect of Garlic extract on the mass motility of whole Acanthocephala
To support the findings of in vitro studies, gross motility of whole Acanthocephala was recorded in the presence of various concentrations of garlic extract. Acanthocephala were kept in petridishes containing different concentrations (50, 100, 150 and 200 mL/kg) of garlic extract for 5 to 10 min in deep bath (25mL distilled water). One control was used without any garlic extract. The mass motility of the Acanthocephala was recorded visually during the bath treatment in vitro graded as 0, ±, +, ++ and +++ representing nil, feeble, poor, moderate and good motility, respectively. (Table 4)

Table 4: Mortality of acanthocephala in different concentration of garlic extract.

Concentration	Whole acanthocephala (no.)	Time	% of mortality
Control	10	5-10 min	0%
50mL/kg	10		20%
100mL/kg	10		50%
150mL/kg	10		60%
200mL/kg	10		80%

2.8. Host challenge
Hatchery records indicated that all experimental fish were of good health and disease free. Fish were fed with a daily ration of the commercial diet used in the formulation of the experimental diets equivalent to approximately 1–2% of their body weight for 7 days to acclimate them to experimental conditions. *Clarias batrachus* were randomly assigned to five experimental groups based on dietary treatment and period of conditioning, the four dietary treatments (50, 100, 150 and 200 ml/kg) and the control diet fed over 30, 60 and 90 days. Fish were fed to satiation twice daily, one pellet at a time. After 30 s the uneaten pellet was removed and the average weight of one pellet was omitted from the quantity of feed delivered. Temperature (26.7 ± 0.1 °C) of the system were recorded at each feeding for the duration of the trial. At the end of the conditioning period (30, 60 and 90 days) fish were challenged with 15 Acanthocephala. To facilitate infection, aeration and water flow to individual aquaria were suspended for 1 h (Hirazawa *et al*., 2010) [8]. Following challenge, the dietary trial resumed and continued for another 5 days to allow Acanthocephala the opportunity to attach to their host and commence development. After day six of post-challenge, fish were taken for freshwater bathing to remove parasites which was bear in gut of the fish as detailed by Militz *et al*., (2013) [2]. Infection success was expressed as the number of Acanthocephala introduced to each aquarium. Infection intensity and prevalence were determined as per (Bush *et al*., 1997) [9].

Table 5: Prevalence and intensity of Acanthocephala infecting *Clarias batrachus* fed diets of varying garlic extract concentrations for 30 days prior to challenge.

Diet	No. of fish	Prevalence (%)	x̄ intensity (range)
Control	10	100%	0.9 (1-10)
Garlic 50mL/kg	10	80%	0.88 (2-9)
Garlic 100mL/kg	10	76%	0.78 (1-7)
Garlic 150mL/kg	10	70%	0.71 (0-5)
Garlic 200mL/kg	10	65%	0.61 (1-5)

2.9. Statistical analyses
Data for growth (SGR and FCR). Values for each parameter measured were expressed as the arithmetic mean ± standard error (SE) by using Tukey statistical analysis by stats soft 2007. Effects of herbal diets on growth performance, hematological and immunological parameters were tested using one-way ANOVA and the mean values were compared by using Duncan's multiple range tests. Significance was accepted at $p<0.05$.

Table 6: Prevalence and intensity of Acanthocephala infecting *Clarias batrachus* fed five diets of varying garlic extract concentrations for 60 days prior to challenge.

Diet	No. of fish	Prevalence (%)	x̄ intensity (range)
Control	10	100%	0.9 (1-10)
Garlic 50mL/kg	12	75%	0.88 (2-10)
Garlic 100mL/kg	12	68%	0.85 (1-8)
Garlic 150mL/kg	12	50%	0.66 (0-4)
Garlic 200mL/kg	12	40%	0.62 (0-3)

Table 7: Prevalence and intensity of Acanthocephala infecting *Clarias batrachus* fed five diets of varying garlic extract concentrations for 90 days prior to challenge.

Diet	No. of fish	Prevalence (%)	x̄ intensity (range)
Control	10	100%	1 (0-10)
Garlic 50mL/kg	14	71%	0.74 (0-7)
Garlic 100mL/kg	14	55%	0.64 (1-6)
Garlic 150mL/kg	14	40%	0.53 (0-3)
Garlic 200mL/kg	14	20%	0.35 (0-1)

3. Results
3.1. Fish growth and Feed efficiency
The growth parameters of *C. batrachus* fingerlings fed with various dietary supplementation of garlic extract are presented in Table 8. The dietary inclusion of garlic extract had no significant ($P>0.05$) impact on weight gain, specific growth rate and feed conversion ratio of *C. batrachus* fingerlings when compared to garlic extract fed groups and control group. No mortalities were observed in all the treatment groups and control group during the feeding trial.

Table 8: Feeding efficience & growth observation during the experimental period.

Treatment	Initial weight (g)	Final weight (g)	Weight gain (g)	SGR (%/day)	FCR (%)
T_0 (control)	8.77±0.18	18.05±0.85	9.28±0.67	3.60±0.13	2.14±0.47
T_1 (50mL/kg)	8.71±0.30	17.67±0.49	8.95±0.52	3.53±0.16	2.33±0.38
T_2 (100mL/kg)	8.88±0.27	18.09±0.52	9.22±0.25	3.56±0.10	2.10±0.17
T_3 (150mL/kg)	8.79±0.01	17.99±0.76	9.20±0.75	3.57±0.20	1.75±0.55
T_4 (200mL/kg)	8.81±0.30	17.77±0.59	8.96±0.29	3.55±0.15	1.95±0.25

3.2. Infection success

After taking the dietary supplementation feed with garlic extract 90 days prior to challenge, it was shown that Acanthocephala infection success of *Clarias batrachus* significantly reduced (Fig.1; $p<0.05$). Inclusion of garlic extract in the diet (at 50, 100, 150 or 200 mL/kg) reduced Acanthocephala success. In comparison, Acanthocephala were successful in infecting *C. batrachus* fed with control diet which did not contain allicin (<3% of allicin detected) (Militz *et al.*, 2013). The prevalence of infection was 100% in control fish, while only 71%, 55%, 40% and 20% of fish in the 50, 100,150 and 200 ml/kg garlic supplemented treatments, respectively (Table 7). The mean intensity of infection was also greater among control fish (1) as compared to fish fed diets containing allicin (0.74, 0.64, 0.53 and 0.35; Table 7). Fish supplemented with garlic extract for 60 days showed prevalence 75%, 68%, 50% and 40% of fish in the 50, 100,150 and 200 mL/kg garlic extract. The 30 day conditioning period with garlic extract did not lead to a difference in Acanthocephala infection success of *C. batrachus* (Fig. 1; $p>0.05$). All four diets (50, 100, 150 and 200 ml/kg garlic extract) demonstrated comparable infection intensity (0.88, 0.78, 0.71 and 0.61 respectively) and prevalence (all ≥ 65%; Table 5). Acanthocephala infection success did not correlate with the total garlic extract quantity ingested for either the 30 day ($p>0.05$) or the 90 day ($p>0.05$) conditioning period trial.

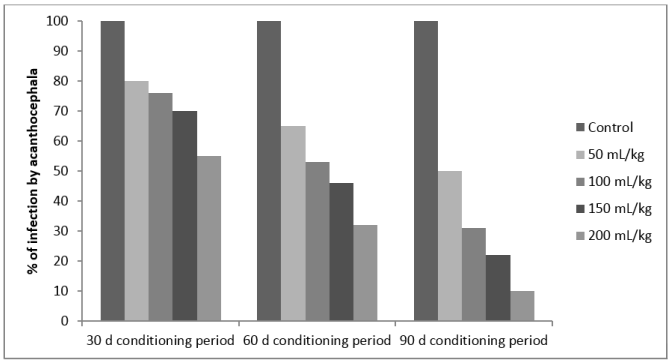

Fig 1: Mean (±SE) percent acanthocephala infection success of *Clarias batrachus* fed with control and garlic supplemented diets over a 30, 60 or 90 days conditioning period before parasite challenge.

5. Discussion

The results of this study reinforce the growing view that dietary supplementation of garlic extract is beneficial to fish health by conferring protection against pathogens. Feeding garlic extract to *C. batrachus* for a period of 3 months significantly reduced infection. All garlic diets conferred similar degrees of resistance to Acanthocephala and fall within the range of garlic concentrations (0.5–30 g kg^{-1}) was shown that garlic supplemented feed was effectively prevent bacteria associated mortalities in cultured fishes (Aly and Mohamed, 2010; Nya and Austin, 2009; Sahu *et al*., 2007; Talpur and Ikhwanuddin, 2012) [6, 7, 10, 11]. It has long been considered that garlic (*Allium sativum*) has several beneficial effects for human and animals, exhibiting antimicrobial, antioxidant, and antihypertensive properties (Konjufca *et al.*, 1997; Sivam G P.2001) [13, 12]. *Entamoeba histolytica*, the human intestinal protozoan parasite, is very sensitive to allicin, as only 30 g/ml of allicin totally inhibits the growth of amoeba cultures (Mirelman *et al.*, 1987; Ankni *et al.*, 1997) [14, 15]. Madsen reported that the raw and squeezed garlic (*Allium sativum*) at the concentration 200 mg/l with feed had potential effect on trichodiniasis in eel after the treatment (Madsen *et al.*, 2000) [16]. In this present study the garlic extract to *C. batrachus* for 90 days in supplementary feed showed highest prevalence and intensity (20% & 0.35). Chitmanat (2005) [17] was used crude extracts from of *Allium sativum* (800 mg/l) for making dietary feed and gave to in tilapia (*Oreochromis niloticus*) fingerlings for eliminating *Trichodina* sp. infection. Immunoregulatory responses to garlic, lysozyme activity, a component of teleost mucus, has specifically been associated with innate immunity to monogeneans (Jones 2001) [18]. The fact that individual *L. calcarifer* demonstrated a poor correlation between garlic extract consumption and infection success suggests that as with most chemical compounds (Sharp *et al*., 2004) [19], host response is not only dependent on concentration and/or exposure time but also on an individual's capacity to respond. Garlic extract used for juvenile *Clarias batrachus* to reduce infection of Acanthocephala which was determined took greater than 30 days. Sahu *et al*. (2007) [10] have demonstrated that when supplementing juvenile carp, *Labeo rohita*, for 20 and 40 days, several immunological indices continually increased (e.g. leucocyte density, superoxide anion production and antimicrobial activity of the serum) with longer periods of conditioning. Thus, it can be assumed that a similar graded response to garlic supplementation occurred with the *Clarias batrachus* in this study and that the degree of immunostimulation following 30 days supplementation with garlic was insufficient to reduce infection success of Acanthocephala. Thus, garlic possesses a range of attributes making it the ideal dietary supplement for controlling Acanthocephala infection in aquaculture pond.

Conclusion

Measures to prevent or reduce the intensity of Acanthocephala infections would substantially assist in on-farm disease management. This study clearly shows that garlic extract (200 ml/kg) administered as a dietary supplement is the most practical preventative method currently known for Acanthocephala infection that showed lowest prevalence 20% and intensity 0.35 in 90 days. Oral treatments are directly applicable in a diversity of aquaculture systems where chemical or freshwater bathing is impractical and garlic can be fed continuously in contrast to praziquantel treatments (Hirazawa *et al.*, 2004; Williams *et al.*, 2007) [20]. Confirming histological immunoregulatory changes at the host parasite interface following garlic extract supplementation would help to clarify garlic's mechanism of

action following ingestion and allow for further development of an already effective control measure for Acanthocephala and other potential pathogens and parasites.

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