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RESEARCH PAPER

Significant enhancement of survival, intestinal digestive enzymes, vitellogenin content, immune response, and antioxidant defense system of *Artemia urmiana* fed with garlic (*Allium sativum*) powder

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Abstract

Garlic is widely known for its antimicrobial, antioxidant, and immunostimulant properties. The present study aimed to assess the effects of garlic (*Allium sativum*) powder on the survival, growth, immunological response, antioxidant activities, and reproductive performance of *Artemia urmiana*. Three diets containing 5%, 10%, and 20% garlic powder feed, and a control diet (C) without garlic were used through a completely randomized design (treatments with triplicates). Twelve 60-L plastic tanks with a density of 20 nauplii per milliliter were used. *Artemia* were fed with Baker's yeast (1.25 mg yeast per one thousand nauplii) during the 30-day experiment. The study showed that 10% garlic treatment resulted in the highest survival rate ($P < 0.05$). The growth factor (dry weight) remained unchanged throughout the experiment. The activities of digestive enzymes (protease, amylase, lipase, and alkaline phosphatase), immune response (lysozyme and phenoloxidase activities), and antioxidants (superoxide dismutase and catalase) considerably increased in the treated groups ($P < 0.05$). Supplementation of *A. urmiana* diet with varying concentrations of garlic powder significantly increased the level of vitellogenin. However, no significant difference was found in nauplii and cyst production across all the groups. The study suggested that garlic, especially at 10% added feed, might effectively enhance the survival rate, immune response, digestive enzyme activity, antioxidant status, and vitellogenin content in *A. urmiana*.

Introduction

Various species of *Artemia* genus, also known as brine shrimp, are zooplankton of hypersaline habitats. They have been used as a model organism in ecological, physiological, ecotoxicological, and genetic studies since the early 1900s, and also in aquaculture (Léger *et al*., 1987; Nunes *et al*., 2006; Agh *et al*., 2007). Artemia is a kind of live feed that is used commonly in shrimp hatcheries due to its suitable size for larvae, high nutritional value and simplicity for digestion (Yuhana & Zairin Jr, 2017).

In recent years, there has been a significant deal of interest in the therapeutic applications of herbal products, particularly garlic, in the treatment of diseases, due to their lower impact on the environment and fish health compared to chemical therapies used in aquaculture (Phan Van *et al*., 2020). Schelkle *et al.* (2013) determined that garlic would be a good alternative for the control of *Gyrodactylus turnbulli* in *Poecilia reticulata*. Yavuzcan Yildiz *et al.* (2019) proved that the inhibitory effect of garlic juice on female *Lernanthropus kroyeri* depended on both the concentration and duration of exposure. In another study, Yavuzcan Yildiz & Bekcan (2020) exhibited that treatment of common carp (*Cyprinus carpio*) with garlic extract (GE) decreased the growth of *Gyrodactylus elegans* seen on the skin. Similarly, Sahandi *et al.* (2023) applied GE at a concentration of 0.2 g/L to guppy (*P. reticulata*) through bathing and found that it inhibited the growth of *Ichthyophthirius multifiliis* parasites.

Garlic has a strong antimicrobial effect due to its active ingredient allicin. Also, allicin increases growth and improves the function of the immune system (Shoaib Akhtar *et al*., 2001; Sh *et al*., 2019). This plant is widespread worldwide and contains amino acids, minerals, vitamins, flavonoids, trace elements (selenium and germanium), and volatile and nonvolatile compounds with medicinal value (Skidmore-Roth, 2009; Sh *et al*., 2019). In addition, garlic possesses antioxidant, antibacterial, antimicrobial, anti-protozoal, antifungal, antiviral, antiparasitic, and immunostimulatory effects (Harris *et al*., 2001; Singh *et al*., 2015). Furthermore, allicin and its derivatives, which are in the form of an organosulfur structure, have larvicidal and pesticidal effects (Rahmah *et al*., 2019; Hardiansyah *et al*., 2020). Roy *et al*. (2008) showed the efficiency of garlic lectin (LC50 value: 12.4 ± 1.918 lg/mL) on red spider mites of tea plants. Kalu *et al*. (2010) reported the larvicidal effectiveness of the ethanolic extract of *Allium sativum* (garlic bulb) against second, third, and fourth instar larvae of *Culex quinquefasciatus*. Ali Al-Shuraym (2022) explored the onion–garlic combination as a natural organic insecticide to repel palm aphids.

The oral administration of garlic has been reported to have various beneficial effects on shrimp, including growth improvement (Amoah *et al*., 2021; Kim *et al*., 2011; Kumar *et al*., 2019; Labrador *et al*., 2016; Pamanna *et al*., 2020; Pazir *et al*., 2018; Samadi *et al*., 2016; Poongodi, 2012; Malar & Charles, 2013), survival rate (Javadzadeh *et al*., 2012; Labrador *et al*., 2016; Pazir *et al*., 2018; Malar & Charles, 2013), immune enhancement (Amoah *et al*., 2021; Pazir *et al*., 2018; Samadi *et al*., 2016; Tanekhy & Fall, 2015; Malar & Charles, 2013), oxidative enzyme activity (Chirawithayaboon *et al*., 2020), digestive enzyme activity (Amoah *et al*., 2021), meat yield (Kim *et al*., 2011), antioxidant activity (Amoah *et al*., 2021), and disease resistance (Chirawithayaboon *et al*., 2020; Vaseeharan *et al*., 2011). Although the effect of garlic on growth and health performance has been well studied, no studies have explored the effects of garlic on the reproductive performance of shrimp or brine shrimp.

Earlier research has documented the effects of garlic on the reproductive performance of birds and mammals. Abdel-Wareth *et al*. (2019) reported that the supplementation of garlic oil at 700 mg kg^{-1} to the diets of male Californian rabbits increased the testosterone level and semen quality. In another study, Onyekwere *et al*. (2018) conducted an experiment using male and female rabbits.

They fed them with diets containing 0%, 5%, 10%, and 15% of garlic flour meal. They found that using a 15.0% dietary level of garlic flour meal enhanced the hormonal profile and levels of sex steroid hormones (testosterone and progesterone). Similarly, Ekuma *et al*. (2017) reported an increase in the semen characteristics (spermatozoa mass motility, spermatozoa proportion, sperm concentration, normal spermatozoa proportion, and total viable spermatozoa), testicular morphology (testis weight, testis volume, and paired testis volume), and levels of male reproductive hormones (testosterone and follicle-stimulating hormone) when rabbit bucks were fed with a diet supplemented with garlic. On the contrary, the crude extracts of garlic reduced the levels of serum testosterone and luteinizing hormone in male rats (Hammami *et al*., 2009). Qian *et al*. (1986) showed that garlic given to rats decreased sperm quality and functionality. Morakinyo (2008) reported that administering an aqueous extract of garlic decreased sperm count, motility, normal morphology, and epididymal volume in rats. Hammami *et al*. (2009) found that garlic intake led to male sexual dysfunction contrary to the previous finding that garlic intake had a negative impact on male reproductive function. The basic reasons for the discrepancy in results might be the lack of standardization among research models and the varying concentrations of garlic given to test subjects.

No studies investigated the effect of garlic on the growth, immunity, and reproduction of *Artemia*. Therefore, the present study was conducted to investigate the effects of different percentages (0%, 5%, 10%, and 20%) of garlic powder on *the* growth factor (dry weight), survival, digestive enzymes [protease, amylase, lipase, and alkaline phosphatase (ALP)], immune response [lysozyme and phenoloxidase (PO) activities], antioxidant activities [superoxide dismutase (SOD) and catalase (CAT)], and reproductive performance (vitellogenin, nauplii, and cyst production) of *A. urmiana*.

Materials and Methods

Hatching and *Artemia* rearing condition

Dried cysts of *A. urmiana*, originally from Lake Urmia, Iran, were purchased from an ornamental fish market (Topazland, Mashhad, Iran). The standard hatching and rearing conditions were maintained in this study as follows: salinity 35 ± 2 g L^{-1}, temperature 28°C ± 3°C, pH 9 ± 1, light 2000 lux, and photoperiod 12-h light/12-h dark (Motlagh *et al*., 2012). All the cysts (3 g per tank) were hatched in a single 20-L glass incubator. The nauplii were separated from the shells and unhatched cysts after 26 h and transferred to the experimental units with the initial stocking density of 20 nauplii per milliliter. No water exchange occurred until the tenth day of the experiment. Then, 10% of the water was discharged and replaced with reserve water every 3 days. A certain amount of fresh water was added to the tanks daily to maintain the salinity at the desired level. Gentle aeration was provided throughout the experiment.

Garlic and feed preparation

Fresh garlic bulbs were obtained from a local supplier and cut into small pieces after peeling and washing. They were completely powdered using an electric mill after drying. Nauplii were not fed for 24 h at the beginning of the trial, and then they were fed Baker's yeast at a rate of 1.25 mg per 1000 nauplii every day (Motlagh *et al*., 2017). The garlic powder required for each treatment was mixed with Baker's yeast and stored in a dry place at room temperature until further use.

Garlic proximate chemical composition and allicin content

Proximate chemical composition of garlic was determined following standard methods **(**AOAC, 1990) (Table 1). The allicin content was determined equal to 3.66±0.82 mg per grams of garlic powder, and it was determined spectrophotometrically by Miron *et al*. (2002) according to the protocol prepared. This method monitors the decrease in absorbance at 324 nm of 4-mercaptopyridine (4-MP) (10^{-4} M) in 50

mM NA-phosphate buffer, 2 mM EDTA, pH 7.2, incubated with growing concentrations of garlic powder.

Experimental design

Using a completely randomized design, four experimental diets with varying amounts of garlic powder (0, 5, 10, and 20%) were administered (four treatments with triplicates). Further, 60-L plastic cylindrical tanks were used for nauplii culture during the 30 days of trial.

Sampling, survival, and growth monitoring

Artemia in each tank were counted by random sampling (50 ml) from different parts of the tank, and individuals in all five samples were counted at the end of the feeding trial to assess the survival rate, and then length was measured with a digital caliper. Five samples in 10 ml of rearing water were taken to count released nauplii and cysts. Formalin (10%) was used to fix Artemia for survival, growth and nauplius production assays.

The survival rate, dry weight, and production of nauplius and cyst per Artemia were calculated using the following formula:

Survival rate= (Final surviving Artemia /Initial number nauplii) × 100

Dry weight = $10^{[-2.53 \times \log (\text{length}) + \log (\text{length})2]} \times 100$

Nauplius/cyst production per Artemia = Nauplius/cyst released/total number of Artemia

The sampling for biochemical evaluations was performed through filtering water, and then one gram of the biomass was sampled and stored at -20°C. Three samples were taken for each tank.

Vitellogenin

The egg yolk proteins were purified as previously described (Lui & O'Connor, 1976). After the *A. urmiana* ovaries were homogenized in a cold extraction buffer containing 0.5M NaCl, 5mM EDTA, and 0.5M Tris (pH 7.0), the resulting homogenate was centrifuged at 8000*g* for 5 min at 4°C. Vitellin was purified by HPLC for analyzing vitellogenin, and polyclonal antibodies were arranged by a previously established method (Arcos *et al*., 2003). Vitellogenin concentrations were determined using a quantitative enzyme-linked immunosorbent assay. The link between optical density and the amount of pure vitellin was determined by plotting a standard curve for the compound and calculating linear regression. All calibration curve and tissue sample measurements were performed in duplicate.

Digestive enzymes

At the end of the experiment, samples were collected to measure protease, lipase, and amylase activity. After rinsing the samples with cold fresh water, they were transported to 15-mL Falcon tubes and stored (at -80°C) until analysis (de Paiva-Maia *et al*., 2013). To homogenise the samples thawed in the laboratory for enzyme extraction, saline solution (0.9% NaCl) was added to a total amount of 1.6 mL per sample. The homogenised solutions were centrifuged at 5,000g for 5 minutes, and the supernatants were then utilised in enzymatic tests. The methodology established by de Paiva-Maia *et al*. (2013) was followed for the protease activity test, and the measurements were made using casein hydrolysis at pH 8. Starch was used as the substrate to determine amylase activity (Worthington, 1991). The lipase activity was measured using the method established by Worthington (1991). According to this method, the lipase activity in the samples was determined by titration at room temperature using olive oil emulsion substrate-gum arabic.

The activity of ALP was determined spectrophotometrically as previously described (Hadas & Pinkas, 1997). Briefly, 2.6 mL of 0.05 mol L^{-1} Tris buffer (pH 8.4), 0.03 mL of 0.1 mol L^{-1} $MgCl_2$, and 0.1 mL of 10.0 mmol L^{-1} pNPP were added to 1-g samples, and the samples were incubated at 37°C for 1 h. The readings were analyzed spectrophotometrically at 410 nm.

Antioxidant enzymatic activities

Artemia samples were homogenized at a ratio of 1:4 wet weight/buffer volume at 4°C in 100mM phosphate buffer (pH 7.4) containing 100mM KCl and 1mM EDTA. After the homogenates were centrifuged for 10 min at 10,000*g*, the supernatants were used as the enzyme source (Viciano *et al*., 2017).

As indicated in a previous work, The reduction in absorbance at 240 nm (extinction coefficient of 40 M cm^{-1}) owing to H_2O_2 consumption was used to measure CAT activity (Aebi, 1974). The volume and time required for the reaction were both 1 ml and 1 minute, respectively. Moreover, 50mM phosphate buffer, pH 6.5, and 50mM H_2O_2 constituted the reaction solution.

The SOD activity was measured as previously established (Fridovich, I. 1972). The suppression of cytochrome c reduction was utilized in this indirect technique. Following the reduction of cytochrome c, the absorbance at 550 nm increased for one minute. The reaction solution contains 50mM phosphate buffer, pH 7.8, 0.1mM EDTA, 50M hypoxanthine, 5.6 mU xanthine oxidase, and 10M cytochrome c in a 1 mL volume (Livingstone *et al*., 1990).

As detailed in a previous work (Smith & Soderhall, 1983), PO activity was evaluated spectrophotometrically by monitoring the production of dopachrome from L-dihydroxyphenylalanine (L-DOPA). Then, 50 µL of the samples was incubated with 50 µL of a convenient elicitor (1 mg mL^{-1}) for 30 min at 20°C. Each sample took 50 µL of the enzymatic substrate L-DOPA (3 mg/mL) for 5 min at 20°C, and 850 µL of distilled water was added to decelerate the reaction. The PO activity was determined spectrophotometrically by the degree of absorbance at 490 nm because of the formation of the red pigment DOPA-chrome. Spontaneous oxidation was monitored by incubating L-DOPA with 0.45 M NaCl. The PO activity was explained as the change in absorbance min^{-1} mg protein $^{-1}$ (Söderhäll & Hall, 1984).

The lysozyme activity was determined by the standard assay method on Petri dishes, as recommended in a previous study (Stabili *et al*., 1999). Briefly, 700 µL of 5 mg/mL peptidoglycan taken from *Micrococcus luteus* (Sigma, MO, USA) was suspended in 7 mL of 0.05M phosphate buffer-agarose (1.2%, pH 5.0) and then spread on Petri dishes. The wells with 6.3 mm diameter were sunk in the agarose gel, and each well was filled with 30 µL of the samples. Diameters of lysed areas were measured during an overnight incubation at 37°C and compared to those of standard hen egg-white lysozyme and reference samples (Merck, Darmstadt, Germany). Every sample was examined for how pH, ionic strength (I), and temperature influenced the outcome. The same I value was used to dissolve agarose in phosphate buffer. Using Petri dish assays and incubating the plates at 5 °C, 15 °C, 22 °C, and 37 °C, the temperature effect was determined. Petri dish tests with varying samples were used to generate a dose-response curve for lysozyme-like activity (20, 30, 40, 50, 60, or 100 µL of samples in each well in triplicate).

Statistical analysis

All percentage data were converted to Arcsin. Statistical analysis was performed through SPSS version 18. Once the two main conditions of the parametric tests of variance analysis were fulfilled homogeneity of variance and normality of data, one-way analysis of variance test was used to examine the main factors or the mutual influence between them. Also, to check the difference between means, the Duncan's multiple range test was performed at a significance level of 5%. Charts and tables were prepared using Excel version 2013.

Results

All groups were defined as follows: *A. urmiana* in the G5 group were fed with 5% garlic powder. The species in the G10 group were fed with 10% garlic powder. The species in the G20 group were fed with 20%

garlic powder. The species in the control group were not fed with garlic powder. Proximate chemical composition of garlic was determined (Table 1).

Tablo 1. Proximate composition of the garlic powder (*n* = 3)

Nutrient	Percent
Crude Protein	17.43±10.33
Crude Fat	3.24±0.99
Crude Fiber	2.76±0.47
Carbohydrate	65.2±12.24
Moisture	6.42±1.00
Ash	4.95±0.13

Growth performance

The dry weights of *A. urmiana* fed with different concentrations of garlic powder are shown in Figure 1. The dry weights in the control, 5%, and 10% groups, besides the 20% group, were statistically similar to the values recorded ($P > 0.05$). The survival rates of *A. urmiana* fed with different concentrations of garlic powder are presented in Figure 2. On the contrary, the 10% garlic powder group showed the highest survival rate ($P < 0.05$), and there are no survivors in the 20% garlic powder group

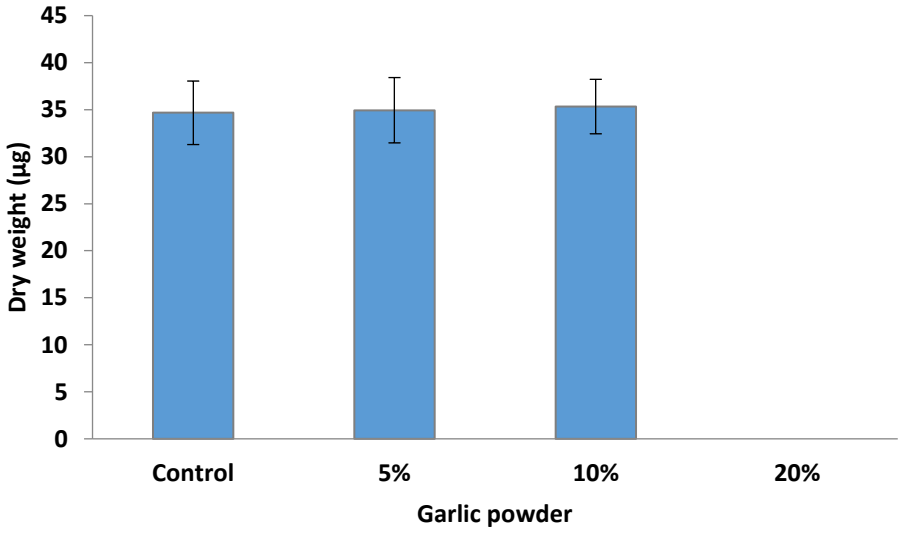

Figure 1. Mean (±SD) of the dry weight of *A. urmiana* species fed with different concentrations of garlic powder in the experiment (*n* = 3)

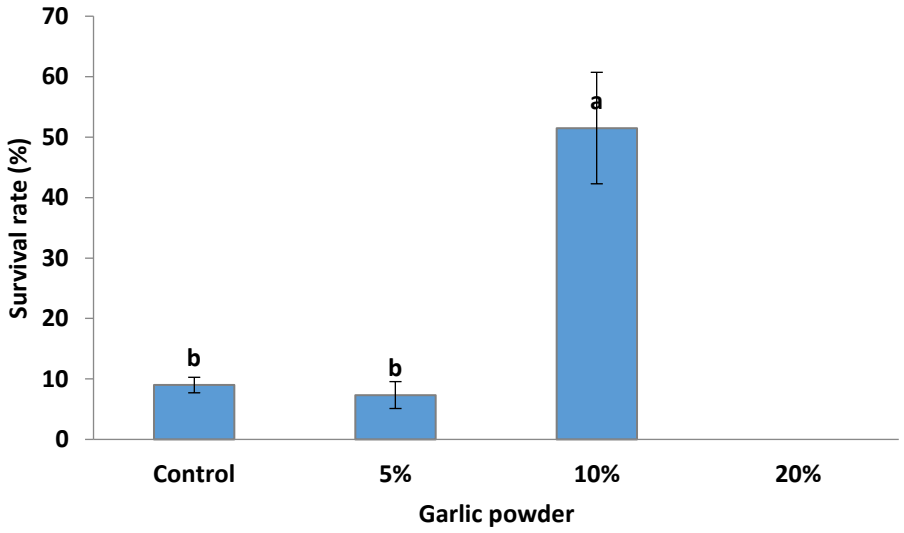

Figure 2. Mean (±SD) of survival rate of *A. urmiana* species fed with different concentrations of garlic powder in the experiment (n = 3)

The changes in nauplii production of *A. urmiana* are presented in Figure 3. No significant differences were found in the nauplii production values between the control, 5%, and 10% groups ($P > 0.05$). The variations in the cyst production of *A. urmiana* are shown in Figure 4. Similarly, no significant differences were observed in the cyst production values between the control, 5%, and 10% groups ($P > 0.05$). However, the significantly highest vitellogenin level was seen in the 5% group compared with all other experimental treatment groups ($P < 0.05$) and there are no survivors in the 20% group. (Figure 5).

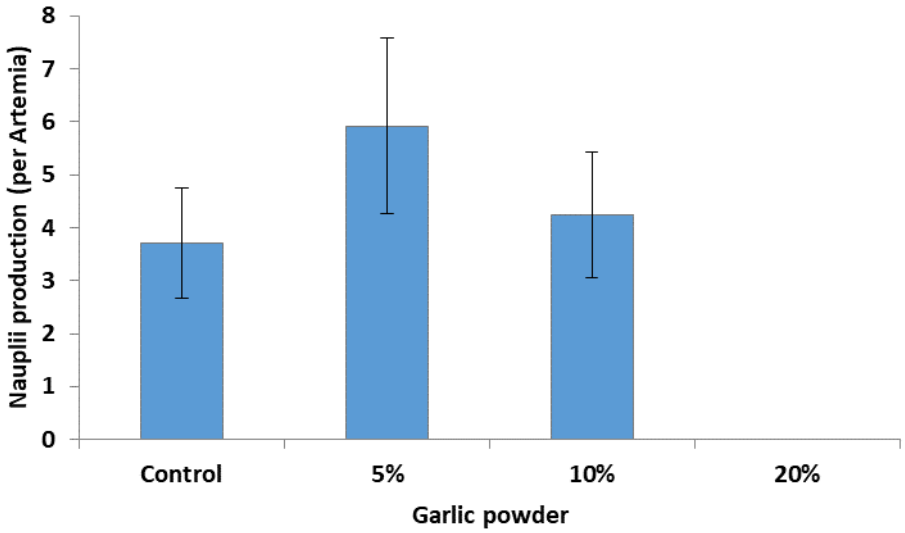

Figure 3. Mean (±SD) of nauplii production per *A. urmiana* species fed with different concentrations of garlic powder in the experiment (n = 3)

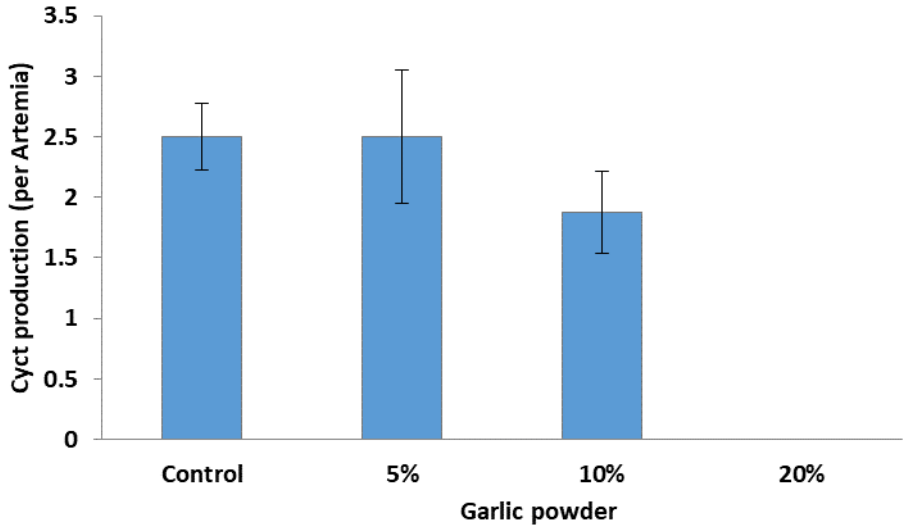

Figure 4. Mean (±SD) of cyst production per individual *A. urmiana* species fed with different concentrations of garlic powder in the experiment (n = 3)

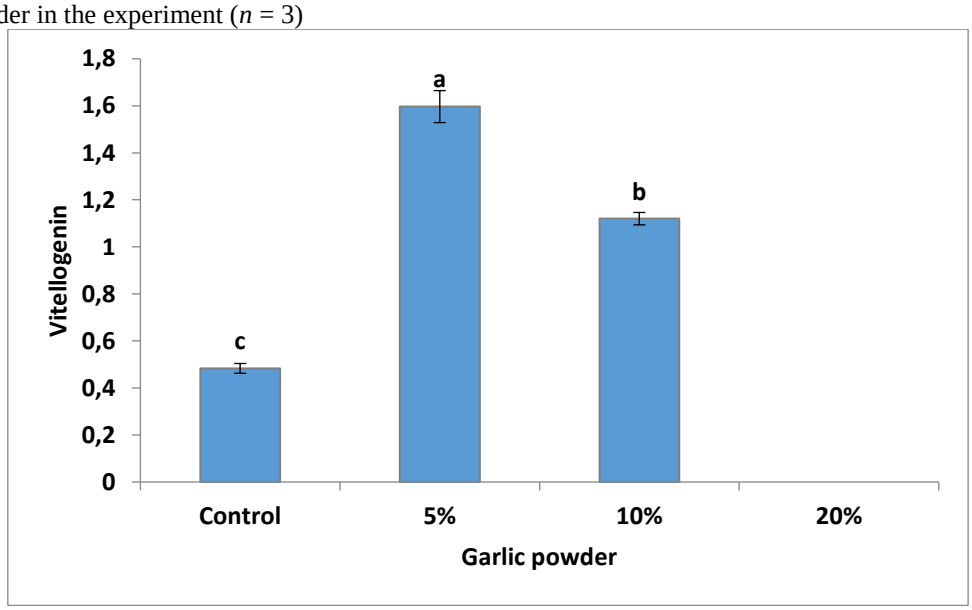

Figure 5. Mean (±SD) of vitellogenin content in *A. urmiana* species fed with different concentrations of garlic powder in the experiment (n = 3)

Digestive enzyme analysis

Table 2 shows the effects of different levels of dietary garlic powder supplementation on digestive enzyme activities (U mg protein min^{-1}) in *A. urmiana.* The highest protease, lipase, amylase, and ALP activities were recorded in the 10% group ($P < 0.05$). Meanwhile, there are no survivors in the 20% group.

Table 2: Effects of different concentrations of dietary garlic powder supplementation on digestive enzyme activities (U mg protein min^{-1}) in *A. urmiana* (n = 3)

	Dietary garlic powder content (%)			
	0	5	10	20
Digestive enzyme activity				
Protease (U mg protein min^{-1})	0.96 ± 0.05^{c}	1.72 ± 0.01^{b}	1.93 ± 0.04^{a}	
Lipase (U mg protein min^{-1})	0.23 ± 0.03^{c}	0.75 ± 0.01^{b}	1.09 ± 0.01^{a}	
Amylase (U mg protein min^{-1})	1.41 ± 0.04^{c}	2.17 ± 0.00^{b}	2.39 ± 0.04^{a}	
ALP (U mg protein min^{-1})	0.57 ± 0.05^{c}	0.96 ± 0.01^{a}	0.90 ± 0.04^{a}	

Treatments with different letters within a row are statistically different. ALP, Alkaline phosphatase.

Immuno-antioxidant parameters

The SOD, CAT, lysozyme, and PO levels, which are presented in Table 3, were the highest in the 10% group and there are no survivors in the 20% group.. Statistically significant differences were observed between the groups ($P < 0.05$).

Table 3: **Effects of different concentrations of dietary garlic powder supplementation on immuno-antioxidant parameters (U mg protein min^{-1}) in *A. urmiana* (n = 3)**

	Dietary garlic powder content (%)			
	0	5	10	20
SOD activity (U mg protein min^{-1})	0.54 ± 0.03^{c}	1.33 ± 0.04^{b}	1.62 ± 0.02^{a}	
CAT activity (U mg protein min^{-1})	0.65 ± 0.01^{c}	1.43 ± 0.03^{b}	1.68 ± 0.02^{a}	
PO (U mg protein min^{-1})	0.50 ± 0.02^{c}	1.07 ± 0.01^{a}	1.33 ± 0.01^{a}	
Lysozyme activity (U mg protein min^{-1})	1.34 ± 0.02^{c}	1.87 ± 0.05^{b}	3.10 ± 0.04^{a}	

Treatments with different letters within a row are statistically different. CAT, Catalase; PO, phenoloxidase; SOD, superoxide dismutase.

Discussion

In recent years, many studies were conducted on the use of garlic as an additive in fish and crustacean diets. Most of the results of these studies showed an improvement in the survival rate, growth, digestion, and resistance against pathogens in aquatic animals. However, using garlic as a food additive in commercial crustacean diets has not yet become a common practice.

Survival rate

In the present study, the survival rate (%) increased when *A. urmiana* were given garlic powder-supplemented diets, especially at concentrations of 5% and 10%. Consistent with this study, most studies also reported that the dietary supplementation of garlic improved the survival rate of Pacific whiteleg shrimp *Penaeus vannamei* (Chirawithayaboon *et al*., 2020; Javadzadeh *et al*., 2012; Pazir *et al*., 2018; Kumar *et al*., 2019; Labrador *et al*., 2016; Lokesh *et al*., 2020) and *Penaeus monodon* (Malar & Charles, 2013). The improved survival rate observed in the treatment groups was attributed to the constituents of garlic, such as immunostimulants, antistress factors, antioxidants, and antimicrobial factors (Kumar *et al*., 2019).

Besides antioxidant, antibacterial, antimicrobial, anti-protozoal, antifungal,

antiviral, antiparasitic, and immunostimulatory effects, garlic also has larvicide effects and is used as an organic pesticide to defeat invasive arthropods. The pesticidal effect of plants has been used against arthropods (Benelli, *et al*., 2015a; Benelli *et al*., 2015b; Karunamoorthi *et al*., 2009). Ramakrishnan *et al*. (1989) reported that GE was a highly active larvicide against *Culex* larvae, with 90% larval mortality after 8 h at a concentration of 50 ppm. Thomas & Callaghan (1999) found that GE had extremely high larvicide potential against the *C. pipiens* larvae, with 90% larval mortality after 90 h at concentrations of 0.16 and 0.32 g/L. Similary, GE was found to be effective against the second and third instar larvae of *Aedes aegypti* (Nasir *et al*., 2022). Farial *et al*. (2022) showed that the lethal concentrations of 21.91% and 37.34% of garlic led to 50% and 90% larval mortality, respectively, in *T. absoluta*.

Numerous studies were performed on the effects of garlic on fish arthropod parasites (Peña *et al*., 1988; Peyghan *et al*., 2008; Militz *et al*., 2013; Schelkle *et al*., 2013; Yavuzcan Yildiz *et al*., 2019; Yavuzcan Yildiz & Bekcan, 2020; Sahandi *et al*., 2023). In the present study, the larvicide potential and anti arthropod activity of garlic was considered to be the reason why the use of 20% garlic powder led to the death of *A. urmiana*.

Activities of digestive enzyme in the intestine

Crustaceans contain digestive enzymes directly related to the digestion of feed and absorption of nutrients. Proteases (trypsin, pepsin, carboxypeptidases A and B, leucine aminopeptidase, and arylamidase), lipases, esterases, and carbohydrases (amylases, maltase, chitinase, and cellulase) are examples of these digestive enzymes (Li *et al*., 2009; Qiu *et al*., 2018; Zhao *et al*., 2017). Lipase is generally considered an enzyme that catalyzes the hydrolysis of ester bonds in substrates such as cholesteryl esters, triglycerides (TGs), phospholipids, and vitamin esters. ALP is associated with the absorption and transport of lipids and carbohydrates across the intestinal cell wall (Fraisse *et al*., 1981). ALP is also an immune enzyme, catalyzes bacterial motifs (Balbaied & Moore, 2019). Garlic facilitates the secretion of bile acid to regulate digestion. It stimulates the digestive enzyme activity of lipase, trypsin, ALP, protease, and amylase, and also facilitates the digestion and absorption of proteins, carbohydrates, and lipids (Elkayam *et al*., 2003; Platel & Srinivasan, 2004).

Previous studies reported a significant influence on digestive enzyme activity after feeding a garlic-supplemented diet, for example, feeding trials in whiteleg shrimp *Penaeus vannamei* (Amoah *et al*., 2021) and narrow-clawed crayfish *P. leptodactylus* (Rezaei *et al*., 2022). In accordance with our observation, *Penaeus vannamei* fed with black garlic supplementation at 80 g kg^{-1} inclusion level showed higher amylase activity (Amoah *et al*., 2021). In addition, Rezaei *et al*. (2022) reported that feeding narrow-clawed crayfish a diet supplemented with 2% garlic powder for 90 days resulted in increased protease and amylase activities. However, no significant differences were observed in lipase values among the experimental groups; also, the activity of amylase was not significantly influenced by garlic powder. This discrepancy in results could be attributed to different fish/shrimp species and or different ingredients of diets or culture conditions. In conclusion, considering the effects on the overall performance of *A. urmiana*, garlic powder is a promising functional additive, and 10% dietary garlic powder was found to be a suitable dietary level for *A. urmiana.*

Immune responses and antioxidant enzymes

The immune responses and antioxidant activities (lysozyme, phenoloxidase, SOD, and CAT) play critical roles in combating infectious agents and provide information about the health status of the organism (Amoah *et al*., 2021). Similar to immunity-related enzymes that help boost immunity,

antioxidant enzymes also help repair the damage caused by free radicals. They have also been reported to increase immunity (Amoah *et al*., 2021; Martínez-Álvarez et al., 2005). The breakdown of bacterial cell walls activates lysozyme, a key biomolecule in nonspecific immunity that helps enable phagocytosis through the opsonization of invading pathogens. In addition, lysozyme has antibacterial properties and hydrolyzes and breaks glycosidic bonds in peptidoglycans (Oliver & Wells, 2015). Previous studies showed that lysozyme activity and subsequent immune capacity were improved by garlic-added feed (Oh *et al*., 2022). Initiating melanogenesis requires a series of enzymes, the last of which being phenoloxidase (Oh *et al*., 2022; Ratcliffe *et al*., 1985; Söderhäll & Cerenius, 1992). This enzyme regulates the number of bacteria in the hemolymph, making it an important marker of a crustacean's overall health (Söderhäll & Cerenius, 1992).

Garlic shows antioxidant activity due to the phenols and saponin compounds it contains (Oh *et al*., 2022). These chemicals can prevent the generation of free radicals, enhance the absorption of endogenous radicals, and enhance the antioxidant enzyme activities of SOD and CAT in cells (Chowdhury *et al*., 2021; Li *et al*., 2019; Nijveldt *et al*., 2001; Oh *et al*., 2022). SOD is the most important antioxidant defense system against ROS and superoxide anion radicals (Hoseini *et al*., 2021; Yousefi *et al*., 2021). SOD, present in both cytosol and mitochondria, specifically neutralizes superoxide radicals and protects cells from the harmful effects of superoxide radicals. It acts by catalyzing the conversion of superoxide free radical (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Hayyan *et al*., 2016; Kuramitsu *et al*., 2007). CAT reacts with hydrogen peroxide to form water and molecular oxygen and performs peroxidase activity by reacting with H donors (methanol, ethanol, formic acid, or phenols). In addition, it plays an important protective role, catalysing the disproportionation of toxic hydrogen peroxide into O_2 and H_2O. (Heuer *et al*., 2009). Evaluating the antioxidant and immune defense systems indicates good health conditions (Zhang *et al*., 2013).

In this study, the dietary supplementation of garlic powder in all the groups significantly enhanced the parameters of immuno-antioxidants (SOD, CAT, lysozyme, and PO) in *A. urmiana* compared with the nonsupplemented control group, except the 20% group. In agreement with our observation, Metwally (2009) indicated that SOD and CAT activities in blood serum and liver significantly increased in *O. niloticus* fed with garlic compared with those in the control group. Also, SOD activity showed a dose-dependent increase, whereas the serum CAT activity decreased in *O. mykiss* that received 10, 20, or 30 g kg^{-1} of dietary garlic compared with that in the control group (Mohebbi *et al*., 2012). Moreover, Yahyavi (2017) reported that the SOD activity increased in fish fed with diets supplemented with 1% of ginger or garlic. According to Adineh *et al*. (2020), SOD and CAT activities were enhanced in microencapsulated garlic-fed groups compared with the control group in *O. mykiss*. In female guppy (*P. reticulata*), diets supplemented with 0.15 mL kg^{-1} GE increased lysozyme activity in the skin mucus (Motlagh *et al*., 2020). Similar to the findings of our study, according to Chowdhury *et al*. (2021), SOD and CAT activities significantly increased in *Labeo rohita* fingerlings in groups fed with turmeric and garlic a herbal immunostimulator .In agreement with our results, dietary black garlic could significantly improve lysozyme, PO, SOD, and CAT activities in *Litopenaeus vannamei* (Amoah *et al*., 2021). Rezaei *et al*. (2022) showed that the narrow-clawed crayfish (*P. leptodactylus*) fed with garlic powder had significantly higher lysozyme, SOD, and PO activities than the control group. Besides, juvenile black rockfish (*Sebastes schlegelii*) fed with diets containing garlic juice processing waste revealed improved activities of SOD, CAT, and lysozyme (Oh *et al*., 2022). Jahanbakhshi

et al. (2022) indicated that SOD and CAT activities increased in giant freshwater prawn (*Macrobrachium rosenbergii*) fed with 5, 10, and 20 g/kg of GE. Adding garlic powder to the diet of crustaceans, such as Chinese mitten crabs (*Eriocheir sinensis*), increased the activities of the SOD and CAT (Zhou *et al*., 2022). This elevation in antioxidant performance might be caused by garlic inhibiting the production of free radicals, encouraging endogenous antioxidant enzyme mechanisms, and improving antioxidant enzyme activity. These different results might be associated with various factors such as experimental conditions, living species, and feeding duration.

Reproduction

The impact of garlic on reproductive metrics is little understood. This was the first study to report garlic powder's influence on vitellogenin in *A. arumina*. In this experiment, the highest level of vitellogenin was observed under 5% garlic powder treatment, and the lowest level was related to the control treatment. Motlagh *et al*. (2020) found that the dietary concentration of GE for guppy (*P. reticulata*) must be 1.64 ± 0.03 and 1.63 ± 1.01 mL kg^{-1} to achieve higher activities of sex steroids. The thiosuffinate component allicin is responsible for garlic's antibacterial properties (Chung *et al*., 2007). In this study, since garlic had an antibacterial effect, it was assumed that it restricted the growth of harmful bacteria and allowed the growth of beneficial bacteria. The beneficial bacteria participated in digestion, and better digestion led to higher vitellogenin production along with an increase in digestive enzymes. Hence, vitelligen can be considered as a reproduction indicator.

Conclusions

Administration of garlic in powder form, could markedly enhance the survival rate, immune response, digestive enzyme activity, and antioxidant status, while simultaneously improving the reproductive performance of *A. urmiana*. Here, the suggested level of garlic powder dietary supplementation was 10%. Based on the encouraging results of this study, researchers may want to look into the possibility of employing garlic powder to improve sustainable aquaculture in future studies.

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Ethical approval

The authors declare that experiments were done according to FUM animal ethics and Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Informed consent

Not available

Conflicts of interest

There is no conflict of interests for publishing of this study

Data availability statement

The datasets generated during current study are not publicly available due to the personal contract between the corresponding author and the ordering organization, but are available from the corresponding author on reasonable request.

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Author contribution

Hamidreza Ahmadniaye Motlagh: Conceptualization, Formal analysis, Project administration and Supervision. **Mehrdad Gheibdust** and **Ali Baghalian:** Data curation. **Ebru Yilmaz:** Writing - review & editing. **Omid Safari**, **Saeid Zahedi and Majid Taherpour:** Formal analysis

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