Fish and Shellfish Immunology 97 (2020) 96–99

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

Non-specific immunity promotion in response to garlic extract supplemented diets in female Guppy (*Poecilia reticulata*)

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ARTICLE INFO

Keywords:
Garlic (*Allium sativum*)
Guppy (*Poecilia reticulata*)
Non-specific immunity

ABSTRACT

In the present study, the effect of aqueous garlic (*Allium sativum*) extract on skin mucus immune parameters of *Poecilia reticulata* was evaluated. A total of 240 *P. reticulate* juveniles, weighing 0.013 ± 0.001 g, were randomly stocked in 12 experimental glass tanks at a density of 30 fish per tank. The fish were fed with diets supplemented with garlic extract at the concentrations of 0, 0.10, 0.15, and 0.20 ml kg^{-1} of diet three times a day at a ratio of 2.5% of their body weight for 80 days. Results of the study showed that, lysozyme activity increased significantly in the skin mucus of garlic-treated Guppy ($P < 0.05$). Values of ACH50 significantly increased ($P < 0.05$) in fish by increasing concentration of garlic extract from 0 to 0.15 ml kg^{-1} in diet. ACH50 also showed a significant declining trend ($P < 0.05$) by increasing concentration of garlic extract from 0.15 to 0.20 ml kg^{-1}. Total Ig content was enhanced significantly in 0.15 and 0.20 ml kg^{-1} treatments as well. ALP activity significantly increased in garlic-fed treatments compared to the control. Inclusion of garlic extract in diet was found to have no significant effect on final body weight and weight gain. Results revealed that, administration of 0.15 mL of garlic extract per kg feed is suggested to obtain optimal skin mucus immunity in *P. reticulata*.

1. Introduction

The Guppy (*Poecilia reticulata*) as a fish species native to the Caribbean, Central and South America is a highly favored ornamental fish because of its diverse coloration and comfortable conditions required for maintenance [1]. Value of ornamental fish trade has grown significantly over the past decades. Total trade in live marine ornamentals is estimated around US$44 million annually [2]. Yearly, 232,416 million ornamental fish are produced in Iran, creating a large turnover. This revenue-generating industry is continuously plagued by several problems, and there is a need to overcome culture system obstacles such as crowding stress and outbreeding of disease [3]. Today, farmers and large-scale producers are eager to apply feed additives having the least health and environmental concerns to attract consumer's attention. Among various candidates, herbal medicines seem to be a reliable choice because of their low price, affordability, and safe use [4].

Garlic (*Allium sativum*) belongs to the Alliaceae family. This family members are famous due to producing various organosulfur compounds, especially allicin [5]. Allicin is a principal bioactive agent found in this family like garlic and shallot. Many medicinal functions are described for allicin such as antioxidant [6], antibacterial [7], antiturmeric [8], and antidiabetic [9] effects. Garlic contains other compounds like prostaglandins, fructan, pectin, essential oil, carbohydrates, fats, proteins, minerals, and vitamins (A, B, C, and E) [10]. In aquaculture, garlic has been used to enhance growth [11,12], immune system [13–15], controlling pathogens [13,16,17], stimulating appetite [11,18],and improving flesh quality [19].

In case of Guppy, efficacy of garlic-based treatments against monogenean parasites has been proved [20]. However, there is no study investigated the effect of garlic on immune system, in relation to this valuable ornamental fish species. Therefore, the present study was conducted to investigate the effect of garlic extract on skin mucus immune parameters including lysozyme, alkaline phosphatase, complement activity, as well as total Immunoglobulin in *P. reticulata*.

2. Materials and methods

2.1. Preparation of garlic extract

Fresh garlic was purchased from a local producer in Birjand, Iran. Garlic bulbs were washed after removing their outer skin surfaces, and each bulb was chopped using a scalpel. Hundred grams of garlic pieces were blended with 200 ml of distilled water using a blender for 3 min.

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https://doi.org/10.1016/j.fsi.2019.12.007
Received 19 August 2019; Received in revised form 2 December 2019; Accepted 6 December 2019
Available online 10 December 2019
1050-4648/

Table 1
Comparison of skin mucus non-specific immune parameters and growth performance in *Guppy* fed with different concentrations of garlic extract for 80 days.

	Treatments			
	0.00	**0.10**	**0.15**	**0.20**
Skin mucus non-specific immune parameters				
Lysozyme (U mg^{-1} protein)	0.19 ± 0.01[a]	0.29 ± 0.02[b]	0.39 ± 0.02[c]	0.38 ± 0.01[c]
ACH50[1] (U ml^{-1})	0.35 ± 0.08[a]	0.51 ± 0.10[a]	0.87 ± 0.05[d]	0.77 ± 0.01[b]
Total Ig[2] (mg ml^{-1})	0.63 ± 0.11[a]	0.79 ± 0.10[a]	1.12 ± 0.18[b]	1.02 ± 0.15[b]
ALP[3] (U mg^{-1} protein)	0.83 ± 0.07[a]	1.24 ± 0.03[b]	1.45 ± 0.01[c]	1.34 ± 0.02[bc]
Growth performance and survival rate				
Initial weight (g)	0.014 ± 0.001	0.013 ± 0.001	0.012 ± 0.001	0.013 ± 0.001
Final weight (g)	0.33 ± 0.02	0.31 ± 0.06	0.35 ± 0.09	0.32 ± 0.06
Weight gain (g)	0.32 ± 0.03	0.29 ± 0.04	0.34 ± 0.05	0.31 ± 0.06

Values are mean ± SE of 3 fish; different superscript letters in a row indicate significant differences between groups ($P < 0.05$). 0.00 (control), 0.10, 0.15, and 0.20 ml kg^{-1} dietary garlic extract for 80 days; ACH50: Alternative complement pathway activity, Total Ig: Total Immunoglobulin.

To precipitate suspended garlic fractions, the homogenate was centrifuged at 10,000 rpm for 5 min at 4 °C. The supernatant was removed and was filtered through Whatman paper. Prepared extract was stored at −20 °C [21].

2.2. Experimental diets

Experimental diets were prepared by supplementing ornamental fish diet (Energy® dry matter 69.74 ± 2.25, nitrogen-free extract 19.23 ± 1.12, crude protein 40.92 ± 1.37, ash 2.72 ± 0.59, crude lipid 3.72 ± 0.70, crude fiber 3.15 ± 0.95) with garlic extract at levels of 0 (control), 0.10, 0.15, and 0.20 ml kg^{-1} of diet. Experimental diets were also coated with gelatin (4 g l^{-1}) to prevent garlic extract from leaching.

2.3. Experimental design

240 female Guppy (*P. reticulata*) juveniles of the same size (with average weight of 0.013 ± 0.001 g) were distributed randomly in 12 experimental glass tanks (50 l). Experiment was carried out in form of a completely randomized design. Feeding trial (2.5 mg/lb body weight, two times a day) lasted for 80 days, according to the Law of Animal Ethics presented in Ferdowsi University of Mashhad.

2.4. Mucus collection

Skin mucus samples were collected according to previous studies [22] with some modifications. To do this, fish were anesthetized with clove powder (5 mg L^{-1}) and placed in vicinity of 5 ml of 50 mM NaCl in individual plastic bags for 2 min. Then, mucus samples were centrifuged at 1500 × g for 10 min at 4 °C, and the supernatant was stored at −80 °C until use.

2.5. Mucus biochemical analyses and growth performance

Protein concentration was determined according to the method proposed by Lowry et al. (1951) [23]. Absorbance was read using a spectrophotometer at 750 nm. Skin mucus Alkaline Phosphatase (ALP) activity was estimated using Pars Azmoon (Pars Azmoon Co, Iran) commercial kit according to the manufacturer's protocol. Lysozyme activity was determined based on the lysis of the lysozyme-sensitive Gram-positive bacterium Micrococcus lysodeikticus according to the standard protocol [24]. Alternative complement activity was assayed using rabbit Red Blood Cells (RBC) [25], after preparation of Rabbit's red blood cells by buffer (pH = 7, 0.01 M), it was added to the samples, and the mixture was incubated at 20 °C for 90 min. Finally, 3.15 ml of 0.85% NaCl solution was added to each of the tubes; then the tubes were centrifuged at 1600 rpm for 10 min at 4 °C. The optical density of the supernatant was read at 414 nm. The volume of the serum or mucus, which causes 50% hemolysis, is the complement activity [26]. Based on previous studies [27],for assessment of total immunoglobulin, 0.1 mL of each plasma sample was mixed with polyethylene glycol (with a volume of 12%) and was incubated for 2 h, allowing immunoglobulin molecules to be deposited. Then, it was centrifuged (at 5000 rpm at 4 °C), and the supernatant was diluted 50 times with NaCl (0.85%). At the end, the fish were weighed on a digital scale (0.01 g) to evaluate final weight and weight gain. After determination of normality of data using Kolmogorov-Smirnov test and homogeneity of variance using Levene's test, the data were analyzed using one-way ANOVA. Tukey's test was used to compare means ($P < 0.05$).

3. Results

Results of this study demonstrated that activities of Alkaline Phosphatase (ALP), lysozyme, alternative complement (ACH50), as well as total immunoglobulin were significantly influenced by different levels of garlic extract. Lysozyme increased significantly in the skin mucus of garlic-treated Guppy ($P < 0.05$); the highest value was related to 0.15 ml kg^{-1} treatment. Values of ACH50 significantly increased ($P < 0.05$) in fish by increasing concentration of garlic extract from 0 to 0.15 ml kg^{-1} in the diet, it showed a significant decline ($P < 0.05$) by increasing concentration of garlic extract from 0.15 to 0.20 ml kg^{-1}. Total Ig also increased significantly in experimental treatments compared to the control. There was no significant increase in ALP between garlic-treated groups, even though this enzyme content was significantly lower in the controls compared to garlic-fed groups. Neither final weight nor weight gain represented a significant increase in treated animals; however, they showed the highest values in the group received 0.15 ml kg^{-1} treatment (see Table 1).

4. Discussion

Environmental stresses trigger immune system of aquatic vertebrates, as they can lower resistance to the pathogens [28]. Proliferation of mucus is among innate defense mechanisms, playing a critical role in host responses against pathogens and stressful environment. Since it causes complement, lysozymes, proteases, immunoglobulins and other immune components to reach mucosal surface of fish [29]. Efficacy of garlic extract as an immune stimulant has been reported to be mostly attributed to presence of allicin [30] as it is highly permeable through biological membranes, influencing its absorption, concentration, and bioavailability in target tissues [31].

In the current study, activities of skin mucus immune parameters increased by increasing garlic extract concentration in the diet of Guppy, and they were found to reach their peaks in the fish fed with 0.15 mL kg^{-1} garlic extract. Garlic has been reported to be useful for Guppy against a major aquarium pathogen, *Gyrodactylus turnbulli* [20,32]. Inclusion of garlic in diets of Rainbow Trout (*Oncorhynchus*

mykiss), Rohu (*Labeo rohita*), and Asian Seabass (*Lates calcarifer*) has been found to increase globulin and lysozyme activity [14,33]. An increment in the activity of skin mucus ALP has been reported in Roach (*Rutilus rutilus*) fries by increasing garlic content in their diets [34]. Although the ability of garlic to stimulate complement in fish has been demonstrated [35], to our knowledge, there is no study evaluated complement activity in fish fed with garlic. Therefore, our study may be the first one evaluating the effect of garlic on the complement activity in fish.

Prolonged treatment with garlic or its high doses may distract behavior and health of fish, justifying a drop in activities of mucus immune parameters in *P. reticulata* fed with 0.20 mL kg^{-1} garlic extract. Similar results have been obtained in previous studies [33], reporting activity of lysozyme at its peak in *L. rohita* fed with 5 g kg^{-1} garlic powder after 40 days of feeding. A declining trend has been reported if the fish is fed with 5 g kg^{-1} garlic powder for more than 40 days. In another study [36], European Seabass (*Dicentrarchus labrax*) juveniles fed with 0, 20, 40, and 60 g kg^{-1} garlic powder for 60 days. Blood globulin contents were found to decrease in juveniles fed with 40 g kg^{-1} garlic powder. These findings highlight the significance of verifying appropriate dosage requirement and exposure time of fish to the garlic [37].

Most of previous studies have reported garlic as a growth promoter in aquaculture [38,39]. Nonetheless in the present study, *P. reticulata* fed with diets containing different concentrations of garlic extract did not show any significant changes in their growth performance, which is in agreement with results obtained in previous researches [16,38,40]. Growth rate of Nile Tilapia (*Oreochromis niloticus*) fed with 10 and 20 ml kg^{-1} did not show any significant increments after 1 or 2 months, but increased significantly after eight months. Such a considerable variation in the efficacy of garlic could be attributed to the species and type of treatment preparation because of instability of allicin [37]. Also, since garlic interferes with fat metabolism [41], it is thought that garlic reduces fat accumulation in garlic-treated fish tissues.

5. Conclusion

Results of the present study demonstrated that the optimal dosage of garlic extract in the diet of *P. reticulata* fries was equal to 0.15 mL kg^{-1} in order to obtain optimal performance of mucus immune parameters for this species. Notwithstanding various advantages of herbal medicines for aquaculture, these materials have their limitation as well. For example, farmers got used to applying antibiotics for disease control, and they will not easily rely on herbs. The other problem would be exaggeration about some bioactive agents of plant like allicin. Many positive medicinal effects are described for allicin, but the truth, which is less considered is that, allicin is an unstable substance. It disappears quickly in garlic side products like essence, extracts, and powders; therefore other components should be responsible for garlic benefits besides allicin. Accordingly, more research is required to evaluate the effects of various garlic bioactive agents on Guppy under different environmental conditions, pathogenic elements, and exposure times.

Author Contribution

Hamidreza Ahmadniaye Motlagh; Project administration; Writing; Formal analysis, **Omid Safari**; Funding acquisition; Formal analysis;**Yahya Selahvarzi**; Investigation;**Ali Baghalian**; Data Curation; **Elham Kia**; Data Curation

Declaration of competing interest

None.

Acknowledgments

The authors appreciate the staff at the Faculty of Natural Resources and Environment, Ferdowsi University of Mashhad for their kindly support.

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