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Original Article

Effect of garlic extract supplementation on growth performance, nonspecific immunity, and antibacterial activity of skin mucus in goldfish, *Carassius auratus*

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Abstract: In the present study, the effects of dietary supplementation of garlic extract on growth performance, skin mucus immunological parameters and antibacterial activity of *Carassius auratus* were examined. Fish were stocked in 100 L glass tanks (6 fish per tank) in triplicate and fed diets containing different garlic extracts (0 (control), 5, 10, and 15 ml/kg] for eight weeks. At the end of feeding period, the fish skin mucus was collected for evaluating the components of non-specific immune system (including lysozyme, complement, total immunoglobulin, dissolved protein, and alkaline phosphatase). Additionally, antimicrobial activity of the skin mucus against *Aeromonas hydrophila*, *Yersinia ruckeri*, *Micrococcus luteus*, *Streptococcus faecium*, and *S. iniae* was assessed. After the feeding trial, the fish fed diets containing garlic extract showed no significant difference in growth parameters. Significantly higher skin mucus lysozyme, complement, alkaline phosphatase activities, and total immunoglobulin and dissolved protein concentration were observed in the fish fed garlic extract-supplemented diets (P<0.0001). The antimicrobial activity of the skin mucus increased along with the increase in the dietary garlic extract levels (P<0.0001). Moreover, garlic extract exhibited the antimicrobial activity against pathogenic bacterial species. The highest level of dietary garlic extract (15 ml/kg) led to significantly higher inhibition zones against pathogenic bacterial species compared to the other garlic extract levels (P<0.0001). The optimal administration of garlic extract at 15 ml/kg enhance skin mucus immune parameters and antimicrobial activity in goldfish.

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Introduction

Diseases are a notable restriction to the productivity of the aquaculture industry, imposing substantial losses. Antibiotics and chemical disinfectants have been routinely used to control diseases in aquatic animals (Li et al., 2019). Nowadays, their application has been limited or banned in many countries due to bacterial resistance and persistence in the fish tissues and aquatic environment (Fierro-Coronado et al., 2018). Moreover, antibiotics have negative effects on the host animal, including oxidative stress and immunosuppression, making the animal susceptible to non-bacterial infections (Hoseini and Yousefi, 2019; Zargar et al., 2020). Natural products such as prebiotics (Chen et al., 2016), probiotics (Daniels et al., 2013), medicinal plants (Harikrishnan, et al., 2011), phytochemicals (Hoseini et al., 2018) seaweeds (Tamadoni Jahromi et al., 2021), and acidifiers (Pourmozaffar et al., 2019) have been identified as promising alternatives to chemical drugs. In recent years, the use of medicinal plants in the aquaculture industry has increased and these herbal materials has been found to improve the immune system (Yin et al., 2014), antioxidant system (Hoseini et al., 2021), stress response (Paray et al., 2020; Yousefi et al., 2020a), growth performance (Yu et al., 2009), gut function (Ahmadniaye Motlagh et al., 2019), and resistance to pathogens (Yogeeswaran et al., 2012) of fish and crustaceans.

Garlic (*Allium sativum*) is used as alternative medicine for various illnesses in many different cultures (Tanekhy and Fall, 2015). It contains many

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valuable compounds such as vitamins (A, C, and B complex), iodine salts, linoleic acid (Labrador et al., 2016), organosulfur compounds (ajoene, allicin, diallyl disulfide, S-methylcysteine, S-allylcysteine, S-methylcysteine sulfoxide, and S-allylcysteine) (Lee et al., 2014) and flavonoid (Sharma et al., 2010). Allicin is the main biochemically and biologically active component of freshly crushed garlic (Guo et al., 2012). It improves protection against pathogenic agents through stimulation of immunological functions including antibody responses, lysozyme and complement activities, and phagocytic activity (Breyer et al., 2015). For example, the non-specific immune system of Nile tilapia (*Oreochromis niloticus*) has been improved, when garlic was incorporated into the diet (Aly and Mohamed, 2010). Nya et al. (2010) have reported that the addition of allicin to rainbow trout (*Oncorhynchus mykiss* diets enhances the red blood cells, serum lysozyme activity, phagocytic activity, and biochemical parameters such as total protein and globulin ratio. In another study, rainbow trout fed diets containing commercial garlic extract has shown a significant reduced mortality after challenge with *Aeromonas hydrophila* (Nya and Austin 2009). Several investigations have demonstrated the positive effects of dietary supplementation of garlic on the growth performance of aquatic animals such as Caspian roach (*Rutilus caspicus*) (Ghehdarijani et al., 2016), beluga (*Huso huso*) (Gholipour Kanani et al., 2014), grouper (*Epinephelus coioides*) (Guo et al., 2012), common carp (*Cyprinus carpio*) (Yousefi et al., 2020b), Caspian trout (*Salmo caspius*) (Zaefarian et al., 2017), and Asian sea bass (*Lates calcarifer*) (Talpur and Ikhwanuddin, 2012).

The skin mucus in fish contains many innate immune components such as lysozyme, alkaline phosphatase, lectins, proteolytic enzymes, and proteases, which play important roles against pathogens (Wang et al., 2020). Immune components in skin mucus are modulated by a number of abiotic and biotic factors (Lallès, 2019). According to earlier reports, diets enriched with myo-inositol (Wang et al., 2020), *Myrtus communis* (Mansouri Taee et al., 2017), *Aloysia citrodora* (Hoseinifar et al., 2020), and *Zingiber officinale* (Sukumaran et al., 2016) were found to improve skin mucus immunological parameters in various fish species. However, little information is available concerning the effects of diet supplementations with herbal additives on the skin mucus immunological parameters in goldfish, *Carassius auratus*.

Mimeault et al. (2005) reported that goldfish can be used as a model in nutritional and physiological studies. Due to the high stocking density and stressful conditions in rearing and transportation systems, goldfish will be susceptible to the invasion of opportunistic pathogens such as *A. hydrophila* (Harikrishnan et al., 2009) and *Yersinia ruckeri* (Wang et al., 2020). Therefore, the present study was designed to investigate the effect of different levels of dietary garlic extracts on growth performance, skin mucus immunological parameters and antibacterial activity of goldfish.

Materials and Methods

Fish: Goldfish with average weight (17.12±2.44 g) were obtained from a commercial fish farm (Topazland, Mashhad, Iran) and acclimated for 14 days into twelve 100 L glass tanks (6 fish/tank). During the acclimation period, the fish were fed a commercial feed (Energy® with a proximate composition of 40.92±1.37 crude protein, 3.72±0.70 crude lipid, 2.72±0.59 crude ash, 3.15±0.95 crude fiber, and 19.23±1.12 nitrogen free extract) three times a day at 8, 12, and 16, at a rate of 2.5% of their body weight. The water was exchanged daily at the rate of 30% for removing the uneaten food and feces. The water was maintained at a temperature of 29±2.5ºC, pH of 7.30±0.6, and dissolved oxygen of 7.90±0.14 ppm. All experiments were performed according to the ethical standards of working with laboratory animals issued by Ferdowsi University of Mashhad (number D/700/1028).

Preparation of garlic extract: Fresh garlics have been obtained from a local farm in Birjand, Iran. The garlic was extracted based on Ahmadniaye Motlagh et al. (2020). One hundred grams of fresh garlic pieces

were added to 200 ml of distilled water and blended for 3 min. The homogenate was centrifuged at 10,000 rpm for 5 min. The supernatant was removed and then the extract was filtered with Whatman filter paper. The prepared extract was stored at -20ºC until being used.

Experimental design: Goldfish (n= 72) were divided into four experimental groups in triplicate. Four experimental diets were prepared by supplementing the commercial diet with different levels (5, 10 and 15 ml/kg) of garlic extract. Garlic extract was sprayed over a basal diet. The gelatin powder (4 g/l) was used as a binder. Finally, the diets were dried for 3 h at room temperature. The fish were fed the experimental diets at 2.5% of whole body weight twice daily for 57 days.

Growth performance: After the feeding trial, all fish were fasted for 24 h and then the growth parameters were calculated according to the following equations:

Weight gain = $W_1 - W_0$

Specific growth rate (SGR) = $[(\text{Ln } W_1 - \text{Ln } W_0) \div (t)] \times 100$

Feed conversion ratio (FCR) = $[(FI) \div (W_1 - W_0)]$

Where, W_0 is initial weight; W_1 = final weight, t = experimental days and FI = feed intake.

Skin mucus biochemical analyses: At the end of the experiment, the fish were fasted for 24 h and three fish from each aquarium were randomly chosen. The method of skin mucus collection was performed according to earlier study (Mansouri Taee et al., 2017). Fish (3 fish per tank) were anesthetized with clove oil (0.2 ml/l). Individual fish were transferred into polyethylene bags containing 10 mL of NaCl (50 mmol) and shaken gently to extract the skin mucus in the NaCl buffer. After 2 min extraction, the mucus samples were transferred to sterile tubes and centrifuged at 1500 g for 10 min at 4°C. Finally, the supernatant was stored at −80°C. Protein concentrations were measured according to Lowry et al. (1951). Alkaline phosphatase (ALP) was assayed by detection kits (Co, Pars Azmon kit, Iran) (Tamadoni Jahromi et al., 2020). Each sample (0.1 ml) was mixed with 12% polyethylene glycol solution for assessment of total immunoglobulin (Ig). Ig molecules were precipitated down by centrifugation and total protein levels were re-determined. The difference in protein content was considered the total Ig content of skin mucus (Hoseinifar et al., 2016). Lysozyme activity was determined according to the method described by Zheng et al. (2009). Briefly, 100 µl of mucus sample was added to a 3 ml suspension of *Micrococcus lysodeikticus* (Sigma-Aldrich, Germany) was prepared in 0.05 M sodium phosphate buffer (pH= 6.2). The reaction was carried out at 25±1°C, and absorbance at 540 nm was measured after 0.5 and 4.5 min. Alternative hemolytic complement of skin mucus was determined using rabbit red blood cells. Rabbit's red blood cells were added to mucus samples and the solution was incubated for 90 min at room temperature. 3.15 ml of NaCl solution (0.85%) was added to the samples and the tubes were centrifuged for 10 min at 1600 rpm. The absorbance of the supernatants was measured at 412 nm. The values of maximum (100%) and minimum haemolysis were obtained by adding 100 µl of distilled water or buffer to 100 µl samples of RRBC, respectively. The volume of serum yielding 50% hemolysis was used to determine complement activity. The volume of the mucus, which causes 50% hemolysis, is the complement activity (Stolen et al., 1994).

Skin mucus antibacterial activity: The skin mucus antibacterial activity was assessed with the disc diffusion method. The following bacterial strains were used as substrates: Gram-negative bacteria, *A. hydrophila* ATCC 7966 and *Y. ruckeri* PTCC 1888. Gram-positive bacteria, *Micrococcus luteus* PTCC 1169, *Streptococcus faecium* ATCC 19434, and *Streptococcus iniae* PTCC 1887 (Magarinos et al., 1995). The bacterial species were grown in nutrient broth medium for 24 h at 37˚C, and then 0.1 ml of each broth culture medium (contain 1×10^5 CFU ml/1; OD 600) was cultured on nutrient agar. 6 mm diameter paper discs (four discs per plate) were impregnated with 150 µl of the mucus samples. The agar plates were incubated at 37°C for 24 h. The antibacterial activity of mucus was defined as the diameter of the clear inhibitory zone (mm) formed around the paper discs (Ahmadniaye Motlagh et al., 2019).

Table 1. Growth performance of goldfish (mean ± standard deviation) after 56 days feeding with diets containing 0-15 ml/kg garlic extract (n = 3).

Parameters	Control	5 ml/kg	10 ml/kg	15 ml/kg	P-Value
Initial weight (g)	17.10±0.11	17.09±0.11	17.18±0.16	17.13±0.21	P = 0.89
Final weight (g)	31.56±0.84	30.22±0.02	31.15±0.22	30.94±1.32	P = 0.29
weight gain (g)	14.46±0.86	13.13±0.08	13.98±0.07	13.81±1.39	P = 0.33
SGR	1.07±0.05	1±0.01	1.04±0.00	1.03±0.08	P = 0.38
FCR	1.83±0.09	1.96±.011	1.91±0.00	1.93±0.25	P = 0.65

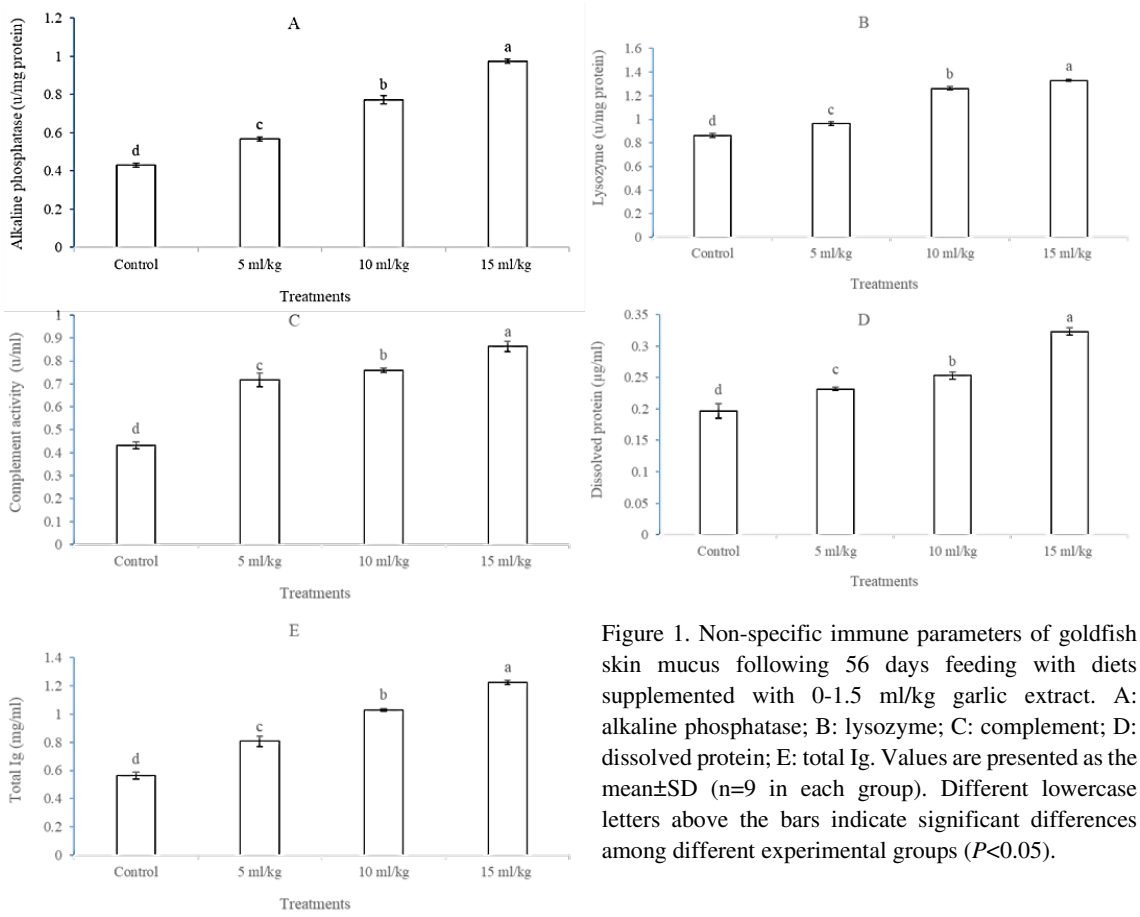

Figure 1. Non-specific immune parameters of goldfish skin mucus following 56 days feeding with diets supplemented with 0-1.5 ml/kg garlic extract. A: alkaline phosphatase; B: lysozyme; C: complement; D: dissolved protein; E: total Ig. Values are presented as the mean±SD (n=9 in each group). Different lowercase letters above the bars indicate significant differences among different experimental groups (P<0.05).

Statistical analysis: The data were expressed as average ± standard error. Statistical analysis involved one-way analysis of variance (ANOVA) followed by a post hoc least significant difference (LSD) test. Normality was tested using the Kolmogorov–Smirnov test. Data homoscedasticity was checked by Bartlett's test. A statistically significant difference was required at P<0.05. Statistical analyses were conducted using SPSS software version 17.0 (SPSS Inc., Chicago IL, USA).

Results

The growth performance of goldfish fed diets containing different levels of garlic extract are presented in Table 1. At the end of 57-day experimental periods, the final weight, weight gain, SGR, and FCR did not significantly differ among the treatments.

Non-specific immune parameters of goldfish fed dietary garlic extracts are shown in Figure 1A-E. ALP activity was significantly higher in the garlic extract-treated fish than the control fish (Fig 1A) (P<0.0001). Significantly lower lysozyme activity in goldfish mucus was observed in the control group than that in the others (Fig. 1B) (P<0.0001). Complement activity in the skin mucus increased significantly by 65, 75, and 100% for fish fed with 5, 10, and 15 ml/kg garlic extract supplemented diets, respectively (Fig. 1C)

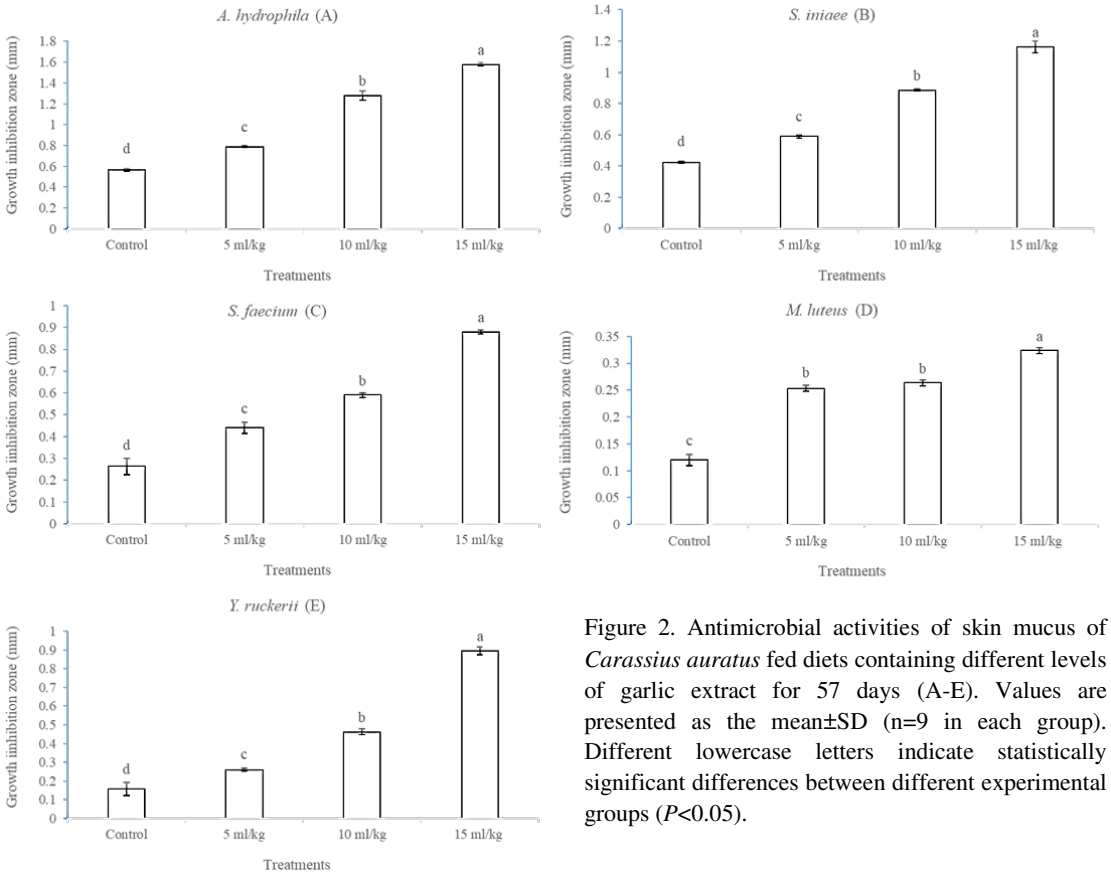

Figure 2. Antimicrobial activities of skin mucus of *Carassius auratus* fed diets containing different levels of garlic extract for 57 days (A-E). Values are presented as the mean±SD (n=9 in each group). Different lowercase letters indicate statistically significant differences between different experimental groups (P<0.05).

(P<0.0001). The total Ig was significantly increased when garlic extract was supplemented in the diet (Fig. 1D) (P<0.0001). Fish fed diets containing garlic extract showed significantly higher dissolved protein concentration than those fed with the control diet (Fig. 1E) (P<0.0001).

Experimental groups exhibited a different degree of inhibition against bacterial species (Fig. 2A-E). Anti-bacterial activity of the fish skin mucus significantly increased along with dietary garlic extract elevation. Fish fed with diet supplemented with 15 ml/kg garlic extract exhibited the highest antimicrobial activity against *A. hydrophila* (1.57±0.02 cm) (Fig. 2A), *S. iniaee* (1.16±0.04 cm) (Fig. 2B), *S. faecium* (0.88±0.01 cm) (Fig. 2C), *Y. ruckerii* (0.89±0.02) (Fig. 2E) and *M. luteus* (0.32±0.006 cm) (Fig. 2D) (P<0.0001).

Discussions

Based on the present results, the growth parameters of goldfish were not affected by the supplementation of garlic extract. Similarly, Mahmoud et al. (2019) found that Nile tilapia fed a diet containing 15 g/kg garlic powder for 60 days showed similar final weight. Similarly, Labrador et al. (2016) reported that diets enriched with garlic powder at 5, 10, and 15 g/kg did not have any significant effect on the growth performance of white leg shrimp, *Peneaus vannamei*. In another study, diets containing garlic extract at up to 30 g/kg inclusion had no notable effect on the final weight of Caspian trout (Zaefarian et al., 2017). Growth-boosting effects of herbal supplements are mediated by increased digestive enzymes' activity and/or improved nutrient absorption and utilization; therefore, garlic extract may have no such effects in goldfish (Hoseini et al., 2021) under the conditions of this experiment.

Fish mucus indicated valuable information for scientists to assess the fish health status (Talpur and Ikhwanuddin, 2012). The results showed the potentials of garlic extract in enhancing the skin mucus immune parameters ALP acts as antimicrobial enzyme against water pathogens (Lallès, 2019). In the present study, ALP activity was significantly higher in

the garlic fed groups. Ghehdarijani et al. (2016) found that Caspian roach fed with 5 and 10 g/kg garlic showed a significant increase in the skin mucus ALP. Change in skin mucus ALP due to the inclusion of plant components has been previously reported. Diets enriched with *M. communis* (0-15 g/kg), *Z. officinal* (0-10 g/kg), and *Phoenix dactylifera* water-soluble extracts (0-200 ml/kg) enhanced the skin mucus ALP activity in rainbow trout, Ruho carp (*Labeo rohita*), and common carp, respectively (Hoseinifar et al., 2015; Sukumaran et al., 2016; Mansouri Taee et al., 2017). The enhancement of ALP in goldfish might be due to the increased mucus secretion and mucosal immune response (Wang et al., 2020).

The goldfish fed diets enriched with garlic extract showed increased dissolved protein level, lysozyme, and complement activities in the skin mucus. Ahmadniaye Motlagh et al. (2019) found similar results in *Poecilia reticulate* fed with different levels of *A. sativum*. Rainbow trout and Ruho carp fed diets supplemented with different levels of garlic powder showed increased lysozyme activity and total protein in the plasma (Adineh et al., 2020; Sahu et al., 2007). Furthermore, diets enriched with garlic at 1-20 g/kg showed significantly enhanced plasma protein concentration in Asian sea bass, Caspian roach, and rainbow trout (Nya and Austin, 2009; Talpur and Ikhwanuddin, 2012 Ghehdarijani et al., 2016). Lectin is the most abundant protein in garlic, which involved in activation of the mannose-binding lectin. This protein is considered to bind to bacterial cells, trigger the complement cascade, and subsequently phagocytosis by macrophages (Nya and Austin, 2009). Igs play a vital role in the adaptive immune system through the production of antibodies against various antigens (Hoseinifar et al., 2020). In the present study, we found that dietary garlic extract significantly enhanced the total Ig level, which is in agreement with Ahmadniaye Motlagh et al. (2019) who reported increase in total Ig levels in *P. reticulate* fed diets containing garlic extract for 80 days. Meanwhile, the highest serum complement activity and skin mucus total Ig were observed in common carp fed 0.5 and 1.5 g/kg *Artemisia annua* extract, respectively (Sarhadi et al., 2020). It seems that sulfur compounds (such as allicin and S-allyl cysteine) in garlic contribute to activating the immune responses (Ndong and Fall, 2011). In addition, garlic can modulate cytokine production and activate immune response by stimulating antibody secretion and immune cells (Arreola et al., 2015).

In the present study, antibacterial activity against *A. hydrophila*, *S. iniaee*, *S. faecium*, *Y. ruckerii,* and *M. luteus* was observed in goldfish fed garlic extract. Wang et al. (2020) found similar results when different levels of myo-inositol were added to the diet of *Hucho taimen* fry. Additionally, the diets containing fermented *Saccharomyces cerevisiae* exhibited the strongest antimicrobial activity of rainbow trout skin mucus against *Y. ruckeri* (Sheikhzadeh et al., 2012). Antibacterial activity of garlic against *Vibrio parahemolyticus*, *V. harveyi* and *A. hydrophila* was also reported by Vuddhakul et al. (2007), Vaseeharan et al. (2011) and Natasya-Ain et al. (2018), respectively. Allicin is responsible for antimicrobial activity of garlic against pathogenic bacteria. When the garlic is crushed or cut, alliinase (C–S lyase enzymatic system) converts alliin (S (+)-allyl- L-cysteine sulphoxide) into allicin (Vaseeharan et al., 2011). In addition, allicin may react with sulfhydryl groups of the bacterial proteins. The ability of allicin to inhibit bacterial sulfhydryl enzymes such as urease, papain, amylase, and alcohol dehydrogenase reported by Bhatwalkar et al. (2021). The smallest inhibition was observed against *M. luteus* while the largest was against *A. hydrophila*. Ushimaru et al. (2007) reported that the efficacy of garlic against gram-positive bacteria is less than that of gram-negative bacteria. The enhancement of defense components with bactericidal properties such as lysozyme, complement, Ig, and lectin could improve the skin mucus antibacterial activity against pathogens (Wang et al., 2020).

In conclusion, the present study demonstrates positive effects of garlic extract on the skin mucus immune parameters. Dietary garlic supplementation at a dose of 15 ml/kg exhibits antibacterial properties against pathogenic bacteria.

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