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STUDIES ON THE GROWTH PERFORMANCE AND IMMUNE RESPONSE OF KOI CARP FINGERLINGS (*Cyprinus carpio* KOI) FED WITH AZOMITE SUPPLEMENTED DIET

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. Authors MSM, AJA and MJM designed the study, wrote the protocol and interpreted the data. Author MAJ anchored the field study, gathered the initial data and performed preliminary data analysis. Authors MSAK, VN and GT managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

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ABSTRACT

Effects of dietary azomite, a natural mineral of volcanic ash, on the growth performance and immune response of koi carp fingerlings (*Cyprinus carpio* koi) were investigated. Koi carp fingerlings were divided into four groups and each group was fed with azomite supplemented diet with three graded levels (2.0 g kg^{-1}, 4.0 g kg^{-1} and 6.0 g kg^{-1}) along with control group. After 8 weeks of feeding trial, the results indicate that the koi fed with diets supplemented with 4.0 g kg^{-1} azomite had the highest final weight, specific growth rate, total phagocytic activity, phagocytic index, NBT assay, lysozyme assay, total protein and immunoglobulin levels of koi carp fingerlings were significantly ($P < 0.05$) increased compared with control group. The results showed that the dietary level of 4.0 g kg^{-1} azomite can improve the growth performance and immune response of koi carp fingerlings.

Keywords: *Cyprinus carpio* koi; mineral supplement; total protein; serum lysozyme activity.

1. INTRODUCTION

The production and trade of ornamental fish is a profitable alternative in the aquaculture sector. Freshwater and marine species have been used successfully in the aquarium fish trade [1]. Despite the economical importance of this sector, the nutritional information for ornamental fish is scarce and often few or even no data of the nutritional requirements is available [1,2]. In natural conditions, fish can regulate and maintain their food intake and therefore their nutritional requirements, reducing the possibility of suffering nutritional deficiencies; however, this problem can be observed when the fish are subject to confinement conditions [3]. Most of the information is not specific to ornamental fish because

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it has been based on results from farm fish kept under different farming conditions, nutritional requirements and feeding habits. Therefore, the limited information about nutrient digestibility in ornamental fish increases the maintenance costs and the water pollution [4].

The most important minerals include calcium, phosphorus, copper, iodine, iron, magnesium, manganese, selenium and zinc within these, phosphorus is commonly supplemented since it is essential in growth, bone mineralization and lipid and carbohydrate metabolism [4] and because its concentration in water is low [3]. Some clinical manifestations of phosphorus deficiency in guppy include depressed appetite, scoliosis and lordosis has been observed [5].

Azomite is a naturally occurring mineral product. Its chemical name is hydrated calcium sodium aluminosilicate ($NaK_2Ca_5Al_3Si_{21}O_{70}6H_2O$). It is listed in the U.S. code of Federal Regulations (21 CFR 582.2729) as an anticaking agent for livestock feed and generally recognized as safe by the U.S. Food and Drug Administration. It falls within the guidelines for use in animal feed by the association of American Feed Control Officials (AAFCO) and recognized by Organic Materials Review Institute (OMRI) U.S.A. as kind of natural mineral that can be used to supplement livestock, aquatic diet and in organic agriculture. As a undersea volcanic sediment, Azomite was mixed with a large amount of plant and animal residues and minerals. It is especially rich in rare earth elements. Studies showed that adding 2.5, 5.0 g kg^{-1} Azomite in the diet improve the growth, pepsin activity and nutrient digestibility of tilapia (*Oreochromis niloticus* x *O. aureus*) [6].

In our previous study [7] the growth and immune response of freshwater fish *Oreochromis mossambicus* enhanced by the addition 4 and 6 g kg^{-1} of Azomite supplemented diet. Liu, et al. reported that the efficiency of feed utilization, activities of intestinal digestive enzymes and serum non-specific immune function of grass carp (*Ctenopharyngodon idellus*) were also improved by the addition of 2.0 g kg^{-1} Azomite [8]. Until now, no studies on the impact of Azomite on ornamental fish culture have been reported. Based on results from other animals, Azomite was expected to have the ability to promote growth and immune function in ornamental fish culture. So in this study, Koi carp fingerlings (*Cyprinus carpio* koi) was chosen as experimental animal to investigate effects of different dietary levels of Azomite on growth, serum non-specific immune indicators. If it is effective in improving these functions, it could be used in environment-friendly feeds to enhance ornamental fish culture. The aim of this article is to review the information available in the nutrition of freshwater ornamental fish with special emphasis in order to fulfill their optimum nutritional requirements, promote optimal growth, enhance the immune response, reduce the cost of feed and minimize the water pollution by using azomite supplemented feed.

2. MATERIALS AND METHODS

2.1 Experimental Fishes

Fingerlings of *C. carpio* koi used in this experiment were procured from a commercial ornamental fish farm, Kulathoor, Chennai. The animals were acclimatized for 1 week to the experimental conditions and diets. Before the experiment, the fishes were starved for 24 h and thereafter they were weighed. *C. carpio* koi (mean initial weight 2.90±0.50 g) were randomly selected and distributed into 4 rearing (with extra 8 tanks for triplicate) rectangular fiber tanks (capacity: 50 l) for the growth trial (12 fish/tank). Fishes were hand-fed to apparent satiation three times (08:30, 12:30 and 18:00) daily for 8 weeks. During the experiment, water temperature ranged from 26.8 to 29.3°C, pH 6.9–7.2, NH3–N 0.13±0.05 mg/l, dissolved oxygen content was approximately 6.5 mg/l, 12 h light/12 h dark cycle was used as photoperiod and the mortality was recorded.

2.2 Fish Feed

The basal diet (control) comprised of mackerel meal, dehulled soybean meal, and corn gluten meal as the protein sources; wheat flour, α-potato starch, and wheat gluten as carbohydrate and fish oil as lipid source in addition with vitamin and mineral premix (Table 1). The dietary Azomite (V5 Organic Biotech Private Limited, Chennai, India) was incorporated with the basal diet at doses of 2, 4, and 6 g kg-1 by evenly mixing with the basal diet thoroughly. The enriched feeds were dried in a vacuum freeze drier for 15 h and it was ground and extruded by passing through 5 mm mesh sieve. The prepared diets were stored at -20°C and it was used for the experiments. The proximate composition of the experimental diets were quantified following the AOAC method composed of different percentage of crude protein, crude lipid, crude ash, and crude carbohydrate.

2.3 Growth Performance

At the end of the experiment before harvesting, the fishes were starved for 24 h and total numbers were counted and the mean body weight of fishes were

Table 1. Ingredients and proximate composition (g) of experimental diets

Ingredients (g)	Control	2 g kg^{-1}	4 g kg^{-1}	6 g kg^{-1}
Mackerel meal	50	50	50	50
Dehulled soybean meal	10	10	10	10
Corn gluten meal	5	5	5	5
Wheat flour	17	17	17	17
α-potato starch	4	4	4	4
Wheat gluten	6	4	2	0
Fish oil	5	5	5	5
Vitamin premix[a]	2	2	2	2
Mineral premix[b]	1	1	1	1
Azomite	0	2	4	6
Proximate composition in dry matter (%)				
Moisture	7.12	7.11	7.34	7.02
Crude protein	54.3	50.7	50.2	51.8
Crude lipid	8.6	8.7	8.9	9.2
Crude carbohydrate	14.2	13.5	13.2	13.0
Crude ash	7.6	7.1	6.8	6.4

[a]Vitamin premix per kg: Vitamin A = 700000 IU; Vitamin D = 140000 IU; Vitamin E = 500 mg; Vitamin B_{12} = 1000 mcg; Folic Acid = 100 mg; Nicotinamide = 1000 mg, [b]Mineral premix per kg: Copper = 1200 mg; Cobalt = 150 mg; Iron = 1500 mg; Zinc = 3000 mg; Iodine = 325 mg; Selenium = 10 mg; Magnesium = 6000 mg; Manganese = 1500 mg; Potassium = 100 mg; Calcium = 270 gm; Phosphorus = 130 gm; Sulphur = 7.2 gm; Fluorine = 300 mg.

measured. Based on recording the weight of each fish and counting the number of koi, specific growth rate (SGR), feed conversion ratio (FCR) and survival rate were calculated using the following equations:

SGR = 100 x [*l*n final weight - *l*n initial weight] ÷ Total duration of the experiment

FCR = Feed given (dry weight) ÷ weight gain (wet gain)

Survival (%) = (Final number of koi ÷ Initial number of koi) x 100

2.4 Collection of Blood

The blood samples were drawn from the caudal artery of *C. carpio* koi by sterilized syringes. They were centrifuged at 2000g (10 min, at 4ºC). The serum was stored at-20ºC for immunological assay and another set of blood samples were collected by heparinised tubes for total protein and total immunoglobulin assay.

2.5 Phagocytosis Assays

Phagocytic cells were detected using *Staphylococcus aureus* (Sigma) as described by [9]. Blood sample (0.1 mL) was placed in a microtiter plate well, 0.1 mL of Staphylococcus aureus 1×10^7 cells suspended in phosphate buffered having saline pH of 7.2, was added and then mixed well. The bacterial blood solution was incubated for 20 min at room temperature. Five µL of this solution was taken on to a clean glass slide and a smear was prepared. The smear was air dried, then fixed with ethanol (95%) for 5 min and air dried. Then the smear was stained with Giemsa stain for 10 min. The two smears were made from each fish. The total of 100 neutrophils and monocytes from each smear were observed under the light microscope and the number of phagocytizing cells and the number of bacteria engulfed by the phagocyte were counted. Phagocytic activity and phagocytic index were calculated as follows: Phagocytic activity equals the number of phagocytizing cells divided by the total number of phagocytes counted. Phagocytic index is expressed as the total number of bacteria engulfed by the phagocytes, divided by the total number of phagocytes containing engulfed bacteria.

2.6 Nitroblue Tetrazolium Assay

Production of oxygen radicals from phagocytes in the blood was measured using nitroblue tetrazolium (NBT) dye as described by [9]. A sample (0.1 mL) of heparinized blood was placed in to a microtiter plate well and equal amount of 0.2% NBT (Sigma) was added, the NBT-blood cell suspension was incubated for 30 min at room temperature. A sample (0.05 mL) of the NBT-blood cell suspension was taken out and added to a glass tube containing 1.0 mL of N,N-dimethylformamide solution. Then the mixture was centrifuged for 5 min at 3000 g. The supernatant was

taken into a glass cuvette and absorbance was read at 540 nm using a spectrophotometer.

2.7 Lysozyme Assay

Lysozyme activity of blood serum was determined as described by [9] with some modifications. Blood serum was prepared by centrifuging the blood at 3000 g for 5 min. Serum (0.1 mL) was placed in test tubes and 0.9 mL of a 0.75 mg mL-1 Micrococcus lysodeikticus (Sigma) suspension in phosphate buffered saline, pH 6.2 was added and mixed well. The absorbance was measured at 450 nm by a spectrophotometer at 1 min intervals for 10 min after mixing with bacteria and rate of change of absorbance was calculated. Lysozyme activities were calculated using hen's egg white lysozyme (Sigma-Aldrich) as a standard.

2.8 Total Protein and Total Immunoglobulin in Plasma

Total protein content in blood plasma was determined using Peterson's modifications of the micro-Lowry method using a protein assay kit (Sigma). The protein concentrations were determined using a calibration curve prepared using bovine serum albumin as the standard (50 – 400 µg mL-1). For the determination of the immunoglobulin in the plasma, immunoglobulins were separated from the plasma by precipitation with polyethylene glycol as described by [9]. Plasma (0.1 mL) was placed in plastic serum vial and 0.1 mL of 12% polyethylene glycol was added and incubated at room temperature for 2 h under constant mixing. After incubation, the solutions were centrifuged at 7000 g for 10 min. The protein content in the supernatant was determined using protein assay kit. The total immunoglobulin content was determined by subtracting the protein content in the supernatant from the total protein content in the plasma.

2.9 Statistical Analysis

The data of each parameter were expressed as the mean±standard error of mean (SEM) and the effects of Azomite were tested using one-way analysis of variance (ANOVA) followed by Tukey's pair wise comparison test using SPSS (version 16 for windows). Differences were considered statistically significant when $p < 0.05$.

3. RESULTS

3.1 Growth Performance

The results of the growth performance levels in the control and azomite fed koi (*Cyprinus carpio*) fingerlings are shown in Table 2. Compared with the control group, final weight of 4.0 g kg^{-1} and 6.0 g kg^{-1} azomite fed groups were significantly increased by 66.4%, 58.6% ($P < 0.05$), feed conversion ratio significantly decreased (reduced value indicating higher efficiency) by 75.6% and 70.0% ($P < 0.05$). The survival rate of each group was higher than 98.0%, no significant difference was found among the experiment groups. There were no significant difference in the growth parameters among the control and 2.0 g kg^{-1} azomite fed groups.

3.2 Immunological Parameters

The immunological parameters total phagocytic activity, phagocytic index, NBT assay, lysozyme assay, total protein and immunoglobulin in the blood of both control and experimental groups are presented in Figs. 1 – 6.

The phagocytic activity in 4.0 g kg^{-1} azomite fed group significantly ($P < 0.05$) increased when compared with respective control group. Phagocytic index and NBT assay does not show any significant difference among the control and experimental fish groups. The activity of lysozyme in the serum was significantly ($P < 0.05$) higher in 4.0 g kg^{-1} azomite fed fish group when compared to the respective control group, while the total protein and immunoglobulin levels in the plasma does not showing significant increase in the experimental group, but 4.0 g kg^{-1} azomite fed group shows elevated levels when compared to control 2.0 g kg^{-1} and 6.0 g kg^{-1} groups.

Table 2. Effect of dietary azomite on the growth performance and feed utilization of Koi (*Cyprinus carpio*) fingerlings

Experiment	Initial weight (gm)	Final weight (gm)	SGR	FCR	Survival (%)
Control	2.90±0.15	3.72±0.23	1.36	0.197	98.0
2 g kg^{-1}	2.92±0.17	4.27±0.17*	2.25*	0.133*	98.6
4 g kg^{-1}	2.80±0.13	6.19±0.30**	5.65**	0.048**	100.0
6 g kg^{-1}	2.95±0.15	5.90±0.25*	4.91*	0.059*	99.0

** Significant difference at p<0.05, ** Significant difference at p<0.01*

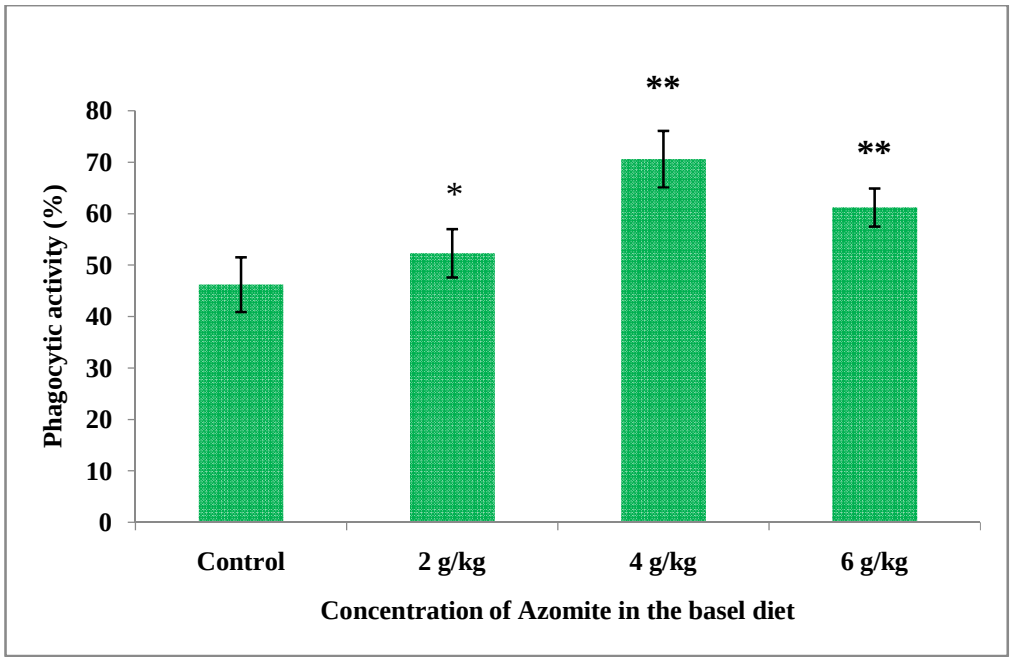

Fig. 1. Phagocytic activity (%) of koi carp fingerlings *(Cyprinus carpio* Koi*)* (mean ± SEM, n = 6) fed dietary supplementation diets with different concentration (2, 4, and 6 g kg^{-1}) of azomite
Significant different (p< 0.05 and p< 0.01) from the control are indicated by asterisks

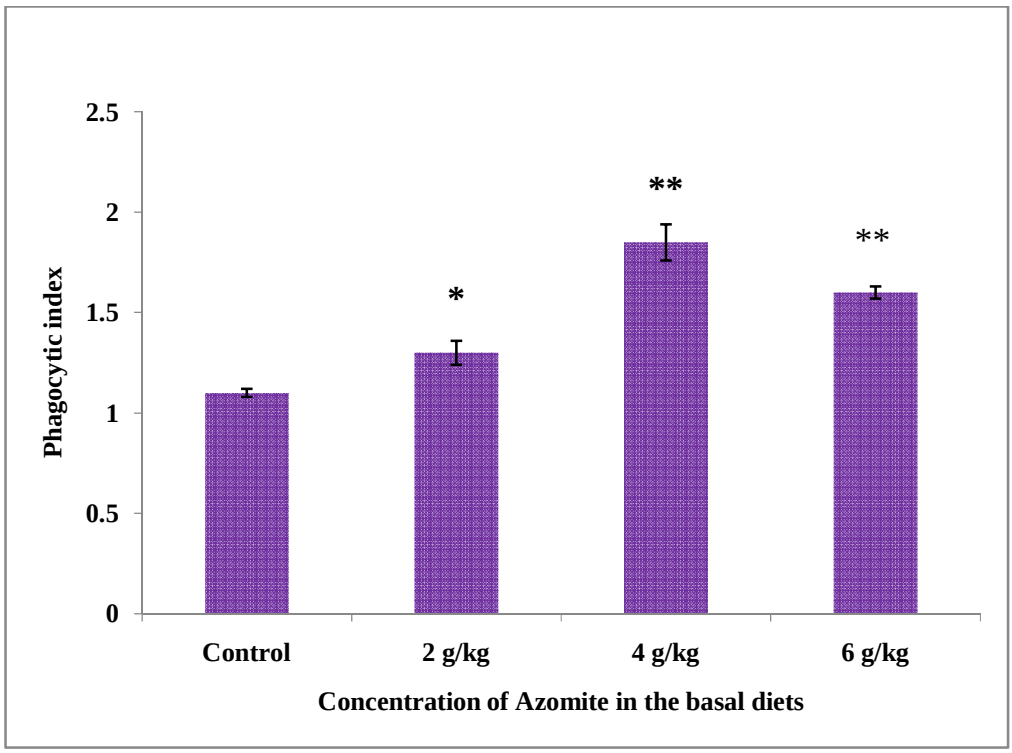

Fig. 2. Phagocytic indexof koi carp fingerlings *(Cyprinus carpio* koi*)* (mean ± SEM, n = 6) fed dietary supplementation diets with different concentration (2, 4, and 6 g kg^{-1}) of azomite
Significant different (p< 0.05 and p<0.01) from the control are indicated by asterisks

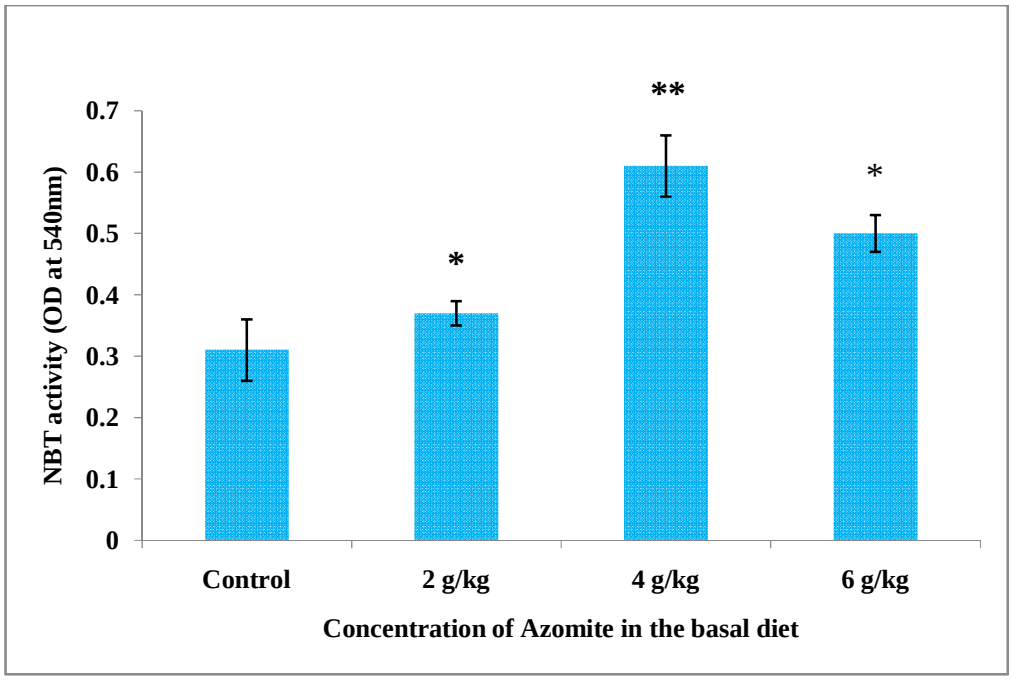

Fig. 3. NBT activity (OD at 540 nm) of koi carp fingerlings *(Cyprinus carpio* koi*)* (mean ± SEM, n = 6) fed dietary supplementation diets with different concentration (2, 4 and 6 g kg^{-1}) of azomite
Significant different (p< 0.05 and p<0.01) from the control are indicated by asterisks

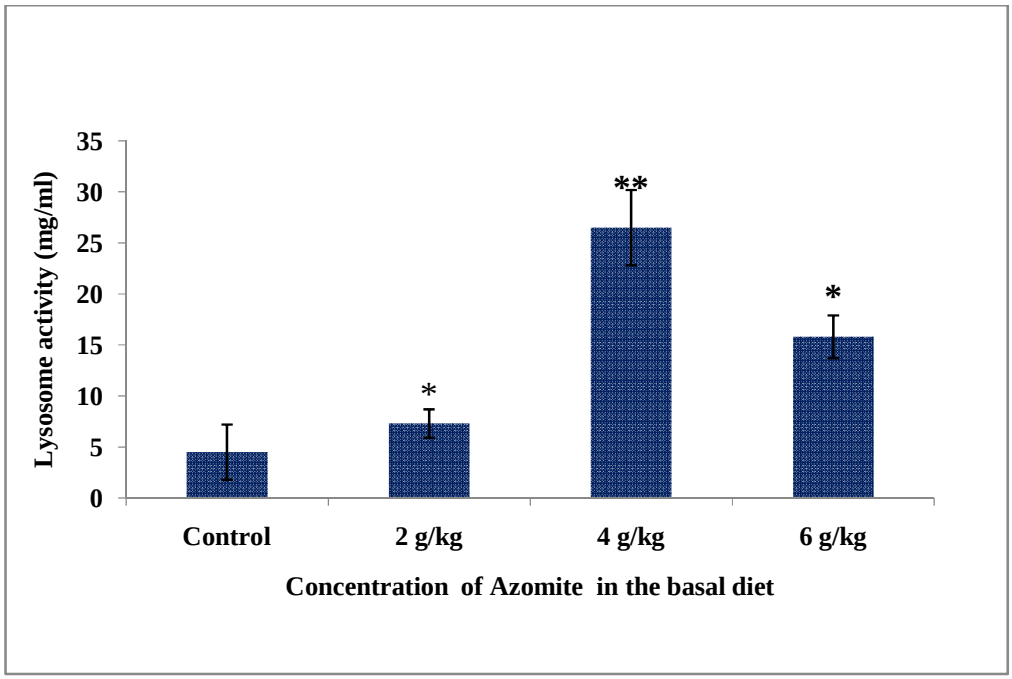

Fig. 4. NBT activity (OD at 540 nm) of koi carp fingerlings *(Cyprinus carpio* koi*)* (mean ± SEM, n = 6) fed dietary supplementation diets with different concentration (2, 4 and 6 g kg^{-1}) of azomite
Significant different (p< 0.05 and p<0.01) from the control are indicated by asterisks

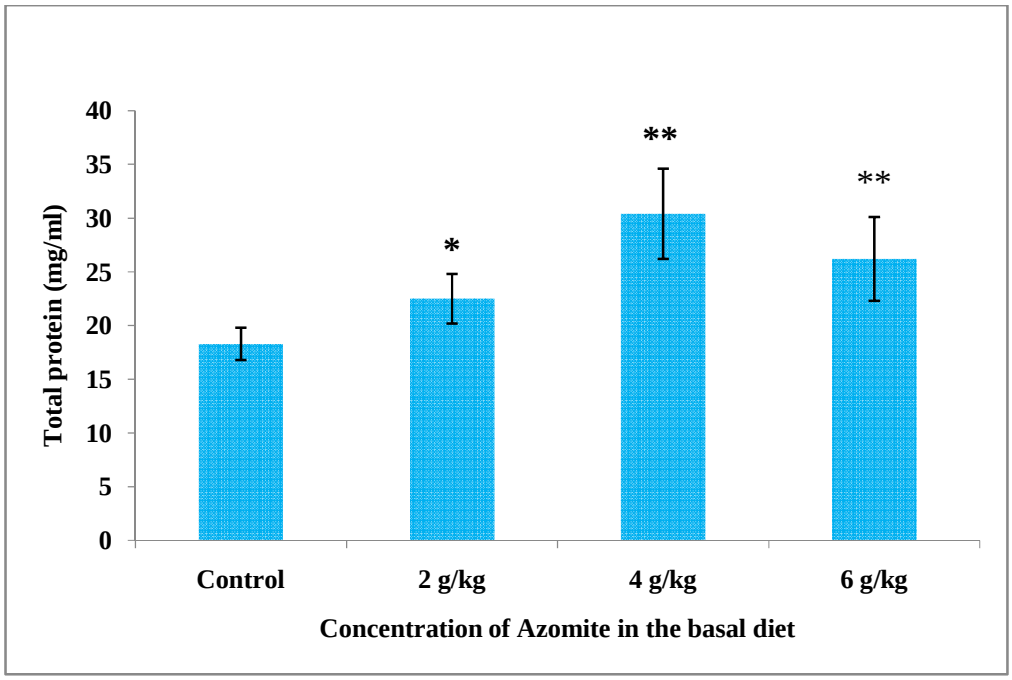

Fig. 5. Total protein (mg/ml) of Koi carp fingerlings (*Cyprinus carpio* koi) (mean ± SEM, n = 6) fed dietary supplementation diets with different concentration (2, 4, and 6 g kg^{-1}) of Azomite
Significant different (p< 0.05 and p<0.01) from the control are indicated by asterisks

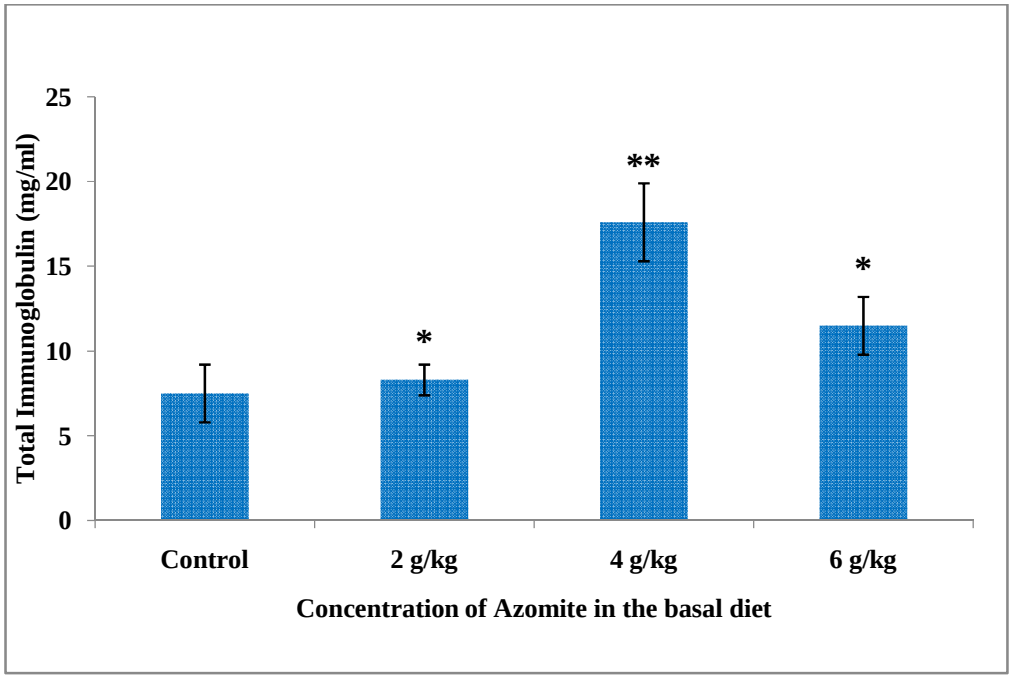

Fig. 6. Total Immunoglobulin (mg/ml) of koi carp fingerlings (*Cyprinus carpio* koi) (mean ± SEM, n = 6) fed dietary supplementation diets with different concentration (2, 4, and 6 g kg^{-1}) of azomite
Significant different (p< 0.05 and p<0.01) from the control are indicated by asterisks

4. DISCUSSION

In aquaculture, application of traditional medicine is more advantageous to overcome the drawbacks in traditional chemotherapy; hence application of natural mineral supplements or herbals with multifunctional active principles can be ideal alternatives. A number of studies demonstrated that rare earth minerals possess certain antibacterial properties [10-12] the effects are dose-dependent. For example, Cerium ions inhibit the growth of several bacteria including *E. coli*, *Bacillus pyocyaneus*, *Staphyloccous aureus*, *Leuconostoc*, and *Streptococcus faecalis* at concentrations ranging from 10^{-3} mol/l to 10^{-2} mol/l [13,14]. Dietary supplementations of rare earth elements cause bacterial flocculation by changing the structure and altering the surface charge of bacterial membranes [15].

A study by Liu et al. [6]; showed that weight gain of tilapia *Oreochromis niloticus* x *O. aureus* can be increased by 12.7% and 9.9% by adding 2.5 and 5.0 g kg^{-1} Azomite in diet ($P < 0.05$). Grass carp fed with a diet containing 2.0 g kg^{-1} Azomite had higher weight gain and lower feed conversion ratio ($P < 0.05$) than carp of the control group [8]. Azomite has been used in animal husbandry and crop production [16,17]. Apart from aquaculture practices, dietary azomite improves broiler breast meat by feeding 3.0–5.0 g kg^{-1} [17]. Studies on Azomite showed that adding 2.5 g kg^{-1} Azomite in the diet improves serum SOD activity of tilapia *Oreochromis niloticus* x *O. aureus* ($P < 0.05$) [6], and adding 2.0 and 4.0 g kg^{-1} Azomite has improved serum AKP activity of grass carp ($P < 0.05$) [8].

Azomite® is a natural mined, hydrated sodium calcium aluminosilicate (HSCAS) product from an ancient mineral deposit in Utah (USA) that comprises a broad spectrum of over 70 minerals and trace elements, including calcium, phosphorus, copper, iodine, iron, magnesium, manganese, selenium, zinc, etc. It plays a pivotal role in the physiology of organisms. For example zinc directly increases the production and function of leucocytes and indirectly affects the immune system by acting as a cofactor of enzymes and improving the organ functions in man [18]. In fish, the role of dietary zinc, immune response and disease resistance has been reviewed by [19]. Selenium is another important trace element for fish because it is a constituent of selenoproteins and has structural and enzymatic roles similar to glutathione peroxidase which is an antioxidant enzyme. Selenium modulates the immune functions such as inflammation and virulence development [20]. In channel catfish selenoyeast and selenomethionine are better selenium sources compared to sodium selenite, in increasing the antibody titer [21]. Administration of 0.4 mg kg^{-1} from the organic sources improved the macrophage chemotactic response offering protection against *E. ictaluri* in channel catfish. Poultry, shrimp, and tilapia farmer use in the feed in conjunction with their regular trace mineral mix add Azomite for many years for improvements to improve the weight gain, feed conversion, and survival [22].

The nonspecific immune response depends on the function of macrophage activity such as phagocytosis and chemotaxis. Phagocytic cells are the most important cellular components of the innate immune system of fish [23] their phagocytic activity constitutes a primitive defense mechanism [24] which is an important characteristic of the nonspecific immune system [25]. In the present study, koi (*Cyprinus carpio*) fingerlings fed with 4 g kg^{-1} Azomite supplementation diets were able to significantly enhance the phagocytic activity.

Lysozyme is an important defence molecule of the innate immune system, playing a role in mediating protection against microbial invasion. It is a mucolytic enzyme produced by leucocytes, especially monocytes, and neutrophils. Fish lysozyme possesses lytic activity against bacteria and can activate complement and phagocytes. Lysozyme is found in a wide range of vertebrates including fish and is one of the defensive factors against invasion by microorganisms. In the present study, serum lysozyme levels of koi (*Cyprinus carpio*) fingerlings fed with 4.0 g kg^{-1} azomite incorporated diet significantly elevated when compared to the control group. Immunoglobulins are a major humoral component of the specific immune system. The total immunoglobulin and total protein levels in the plasma of koi (*Cyprinus carpio*) fingerlings fed with 4.0 g kg^{-1} azomite was increased when compared to control fish group.

5. CONCLUSION

As far as our knowledge is concerned, this is the first study focusing on the impact of Azomite supplemented diet on the growth and immune response in koi (*Cyprinus carpio*) fingerlings. The present study reporting positively enhanced immunological parameters in koi (*Cyprinus carpio*) fed with 4.0 g kg^{-1} azomite incorporated diet. Incorporation of azomite into feed holds a potential for immune enhancement and growth performance in koi (*Cyprinus carpio*) fingerlings thereby increasing the resistance of the fish to diseases and stress which may reduce fish mortality rates and offer economic benefits. The optimum level of azomite for ornamental fish culture is suggested to be 4.0 g kg^{-1} in the basal diet.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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