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*Full Length Research Paper*

# The effect of garlic (*Allium sativum*) on growth and immune responses of hybrid tilapia (*Oreochromis niloticus x Oreochromis aureus)*

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**Garlic (*Allium sativum)* was incorporated into diets (0 (control group), 0.5 and 1 g/kg (test groups)) of juvenile hybrid tilapia, *Oreochromis niloticus* x *O. aureus*. The fish initial weight was 25.5 ± 1.0 g (mean ± SD) with no significant size difference among the treatments. Innate immune response responses were evaluated for 2 to 4 weeks. Total leucocyte count, respiratory burst, phagocytic activity, phagocytic index and lysozyme activity were enhanced in garlic 0.5 g/kg treated groups compared to the control group after 4 weeks. Fish fed with garlic supplemented diets at 0.5 g/kg showed decreased on weight gain at 2 and 4 weeks as compared to those fed with control diet.**

**Key words:** Hybrid tilapia, *Oreochromis niloticus* x *O. aureus*, juvenile, garlic, *Allium sativum*, innate immune response.

## INTRODUCTION

Tilapia is the third most commonly farmed fish after carp and salmon with global production of 1.49 million metric tonnes (mmt) in 2002, and is expected to grow to 2.0 mmt in 2010 (Fitzimmons, 2003). However, the outbreak of diseases is a limiting factor in tilapia culture production. At many tilapia farms and hatcheries several antibiotics, vaccines, and chemotherapeutic agents as well as some immunostimulants have been used to prevent viral, bacterial, parasitic, and fungal diseases. Fish as well as human rely on both specific and innate immune mechanisms to protect themselves against invading pathogens. Phagocytosis is one of the main mediators of innate immunity to pathogens including bacteria, viruses, and parasites in fish. The most important cells involved in this defence are the phagocytes. Several reactive oxygen species (ROS) are produced by fish phagocytes during the respiratory burst. Once bacteria or fungi are engulfed by leucocytes, the host's NADPH-oxidase is activated, which in turn increases oxygen consumption and subsequently produces ROS such as superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), hydroxyl radical (·OH), singlet oxygen ($^1O_2$) (Roch, 1999). The release of superoxide anion is known as the respiratory burst, and together its derivates are bactericidal (Secombes and Fletcher, 1992). Since $O_2^-$ is the first product to be released from respiratory burst, the measurement of $O_2^-$ has been accepted as direct and accurate way of measuring respiratory burst activity (Secombes, 1990; Secombes and Olivier, 1997; Roch, 1999). These reactive oxygen species are supported by several soluble factors, such as lysozyme (Dalmo et al., 1997; Verlhac and Gabaudan, 1999; Yano, 1996). Lysozyme found in cutaneous mucus, peripheral blood and certain tissue rich in leucocytes, is an enzyme which catalyses the hydrolysis of N-acetyl muramic acid and N-acetyl glucosamine of peptidoglycan in the bacterial cell walls (Jollès and Jollès, 1984). This protein functions as a crucial role in the defense immune system. It is also well known that the innate immune system in fish can be triggered by many immunostimulants, such as levamisole

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**Table 1.** Composition of the experimental diets.

| Ingredients | 0 g garlic /kg diet | 0.5 g garlic/kg diet | 1 g garlic/kg diet |
|---|---|---|---|
| Fish meal | 24 | 16 | 12 |
| Garlic seed meal | 0 | 8 | 12 |
| Vitamin mix [a] | 2 | 2 | 2 |
| Mineral mix [b] | 4 | 4 | 4 |
| Dextrin | 10 | 10 | 10 |
| Wheat flour | 25 | 25 | 25 |
| α-starch | 10 | 10 | 10 |
| Cellulose | 20 | 20 | 20 |
| Oil (Cod liver oil/Corn oil) [c] | 5 | 5 | 5 |
| Energy (kcal/100g) | 281 | 281 | 281 |

[a]Calcium carbonate 2.1%, calcium phosphate dibasic 73.5%, citric acid 0.227%, cupric citrate 0.046%, ferric citrate (16to 17% Fe) 0.558%, magnesium oxide 2.5%, magnesium citrate 0.835%, potassium sulfate 6.8%, sodium chloride 3.06%, sodium phosphate 2.14%, zinc citrate 0.1335, potassium iodine 0.001%, potassium phosphate dibasic 8.1%.[b]Thiamin HCl 0.5%, riboflavin 0.8%, niacinamide 2.6%, D-biotin 0.1%, Ca-pantothenate 1.5%, pyridoxine HCl 0.3%, folic acid 0.5%, inositol 18.1%, ascorbic acid 12.1%, para-aminobenzoic acid 3%, cyanobalmin 0.1%, BHT 0.1%, α-cellulose 60.3%. [c]Cod liver oil / corn oil = 2:1.

(Siwicki, 1987, 1989; Siwicki et al., 1990; Jeney and Anderson, 1993), glucan (Engstad et al., 1992; Jorgensen and Robertsen, 1995; Chen and Ainsworth, 1992; Ainsworth, 1994; Jeney et al., 1997), glucan plus vitamin C (Verlhac et al., 1996), yeast RNA (Sakai et al., 2001), lipopolisaccharide (Dalmo and Seljelid, 1995; Solem et al., 1995) and kitosan (Siwicki et al., 1994). However, some of the immunostimulants cannot be used because of various disadvantages such as high cost and limited effectiveness. Besides, a large number of plants have been used in traditional medicine for the treatment and control of several diseases (Duke, 1987). Garlic has shown antimicrobial (Kumar and Berwal, 1998), antihypertensive (Suetsuna, 1998), hepatoprotective (Wang et al., 1998) and insecticidal (Wang et al., 1998) properties. Garlic extract has also been shown to reduce serum cholesterol levels (Bordia et al., 1975; Augusti, 1977) and increase blood coagulation time (Bordia et al., 1975). An antifungal activity of garlic bulbs (Fromthing and Bulmer, 1978) is also on record. S-allyl cysteine present in crushed garlic was found to inhibit tumor metabolism and enhance immune response (Sumiyoshi, 1997). Allium species of garlic also have immune enhancing activities that include promotion of lymphocyte synthesis, cytokine release, phagocytosis and natural killer cell activity (Kyo et al., 1998). To date, most previous studies on fish were carried out on antioxidant and antimicrobial properties of garlic and its derivatives such as essential oil and oleoresin (Akgül, 1993; Zaika, 1988). To our knowledge no work has been reported on growth and immune response of juvenile hybrid tilapia fed with garlic supplemented diets. Therefore, this study was aimed at determining the effect of garlic on growth and immune parameters of hybrid tilapia.

## MATERIALS AND METHODS

### Animal

Two hundred Juvenile hybrid tilapia were obtained from Freshwater Aquaculture Research Center (FARC, Taiwan) and held in 1000 l fiberglass tanks supplied with a filter and an aeration system at 27 ± 1°C. During acclimatization, fish were fed daily with a commercial diet (Grobset, Taiwan).

### Preparation of diets

To evaluate the effects of garlic on growth and immune responses of juvenile hybrid tilapia reared under freshwater, three diets containing 0 g garlic /kg diet as control, 0.5 g garlic /kg diet and 1 g garlic /kg diet respectively, were formulated. Fresh garlic bulbs were purchased from a local market (Keelung, Taiwan). The main protein sources (fish meal: crude protein 66%, crude lipid 6.7% and garlic meal: crude protein 17.3%, crude lipid 0.34% (Haciseferogullari et al., 2005)) already ground into meal were passed as particles through an N° 40 (425 µm) mesh sieve. Mineral and vitamin mix were prepared into the laboratory according to Sheen and Wu (1999). After all the ingredients were mixed thoroughly, adequate quantity of water (30% for 100 g of mixed ingredients) and oil (cod liver oil and corn oil in the ratio 2:1) were added. Then, the dough was passed through an extruder to make spaghetti, and dried at 35°C for 8 h. The dried diet was packaged into plastic bag and stored frozen at -20°C until use (Table 1).

The experimental diets were analyzed for proximate composition (Table 2) based on AOAC International (1984) methods. Crude protein was determined with a Kjeltec system 1002 (Tecator). Crude lipid was determined by chloroform-methanol (2:1, v/v) extraction

**Table 2.** Proximate analysis of the experimental diets.

| | 0 g garlic/kg diet | 0.5 g garlic/kg diet | 1 g garlic/kg diet |
|---|---|---|---|
| Moisture | 9.57 | 10.64 | 10.67 |
| Crude protein* | 13.20 | 11.9 | 10.00 |
| Crude lipid* | 6.34 | 6.02 | 5.70 |
| Crude fiber* | 10.36 | 11.87 | 11.72 |
| Ash* | 12.72 | 13.54 | 13.32 |

* Presented as percentage of dry weight.

method (Folch et al., 1957). Crude fiber was determined by the Fibertec system M 1020 hot extractor (FOSS Tecator). Gross energy was obtained by IKA calorimeter system C 2000 basic. Ash and moisture were determined by conventional methods using muffle furnace at 505°C and a 105°C oven.

**Growth study**

To determine the effects of garlic on the growth of hybrid tilapia, the experiment was carried out in three replicates test and control groups consisting of 10 fish in 60 l glass tank containing 40 l of water. Fish were fed to satiation with different diets containing garlic at concentrations 0 g/kg (control), 0.5 and 1 g/kg (test groups) two times a day for 4 weeks. The fish initial weight was 25.5 ± 1.0 g (mean ± SD) with no significant size difference among the treatments. During experiments, temperature was maintained at 27 ± 1°C. Growth performance of fish was determined in terms of final individual fish weight:

Weight gain (%) = 100 (Final Body Weight – Initial Body Weight)/ Initial Body Weight.

**Formalin-Killed *Escherichia coli***

An *Escherichia coli* (DH5α) culture grown overnight in 100 ml tryptic soy broth (TSB) at 37°C. Formaldehyde (37%) was added to give 2% final concentration and the culture was shaken at 22°C overnight. Stock cultures were centrifuged at 700 x g for 10 min at 4°C. The supernatant fluid was removed and the bacterial pellet washed twice with 50 ml PBS (NaCl, 8.0 g $l^{-1}$; $KH_2P0_4$, 200 mg $l^{-1}$; $Na_2HPO_4$, 1.15 g $l^{-1}$; KCl, 200 mg $l^{-1}$; $CaCl_2.2H_20$, 133 mg $l^{-1}$; $MgCl_2.6H_20$, 100 mg $l^{-1}$) and re-suspended in 50 ml PBS and kept at 4°C for phagocytosis test.

**Zymosan**

A suspension of 50 mg zymosan (Sigma) in 5 ml PBS was prepared in capped glass culture tube, and the tubes was placed in a boiling water bath for 30 min with frequent shaking. The solution was centrifuged at 600 x g for 5 min. The pellet was re-suspended in 10 ml chicken serum (Sigma), and incubated for 30 min at 30°C. Then, it was centrifuged at 600 x g for 5 min. The supernatant fluid was removed and the bacterial pellet was washed twice with 10 ml PBS, re-suspended in 50 ml PBS to give 1 mg $ml^{-1}$ and stored at 4°C for respiratory burst assay.

**Effect of garlic on the immune parameters of hybrid tilapia**

For the studies of immune parameters, tests were carried out in three replicates test and control groups consisting of 10 fish in 60 l glass tank. Three fish were randomly sampled per replicate at the beginning, after 2 and 4 weeks of treatment. A total of 90 fish (3 x 3 x 10) were used for the study. Blood (1.0 to 1.5 ml) was sampled individually from the caudal vein using a heparinized syringe (25 g) fitted with a needle at the beginning of the test, at 2 and 4 weeks. Total leucocyte count was measured using an automated hematology analyzer (KX-21, Sysmex, Japan). The remainder of blood was used for the subsequent tests.

**Separation of leucocyte**

The separation of leucocytes was followed the methods of (Law et al., 2001). Briefly, blood (500 µl) was mixed with 500 µl of AL medium (AIM-V medium and Leibovitz`s L 15 medium, GIBCO BRL, Gaithersburg, MD, USA), streptomycin (100 µl) and penicillin (100 µl). Percoll (55%, Sigma) was added to the mixed blood solution, and then centrifuged at 400 x g (Model 5403, eppendorf, Hamburg, Germany) for 15 min at 10°C. The leucocytes were obtained from the interface and washed with AL medium by centrifugation at 600 x g for 10 min at 10°C. After centrifugation, the leucocytes were suspended in AL medium with 5.5 mM glucose. The number of cell viability was analyzed by trypan blue (0.1%) with a haematocytometer.

**Measurement of innate cellular response**

The respiratory burst of the leucocytes (intracellular superoxide anion production ratio) was quantified using the reduction NBT (Nitro Blue Tetrazolium) to formazan as a measure of superoxide anion ($O_2^-$) production (Chung and Secombes, 1998). The absorbance at 630 nm was measured spectrometrically in triplicates with a microplate reader (Model VERSAmax, Molecular Devices, Sunnyvale, CA, USA) using DMSO/KOH alone as a blank. Respiratory burst was expressed as NBT-reduction in 100 µl of leucocyte suspension (about 1.5 x $10^6$ leukocytes). The phagocytosis was measured based on the method of Mathews et al., 1990. Briefly 300 µl leucocyte suspension (about 4.5 x $10^6$ leukocytes) in L-15 medium were added in triplicate tubes.

Three hundred microlitre of formalin-killed *E. coli* in PBS was added to each tube and incubated for 1 h at 17°C. Then, 900 µl cold PBS was added, and the tubes were centrifuged at 300 x g for 5 min. The supernatants were poured out and the pellets were

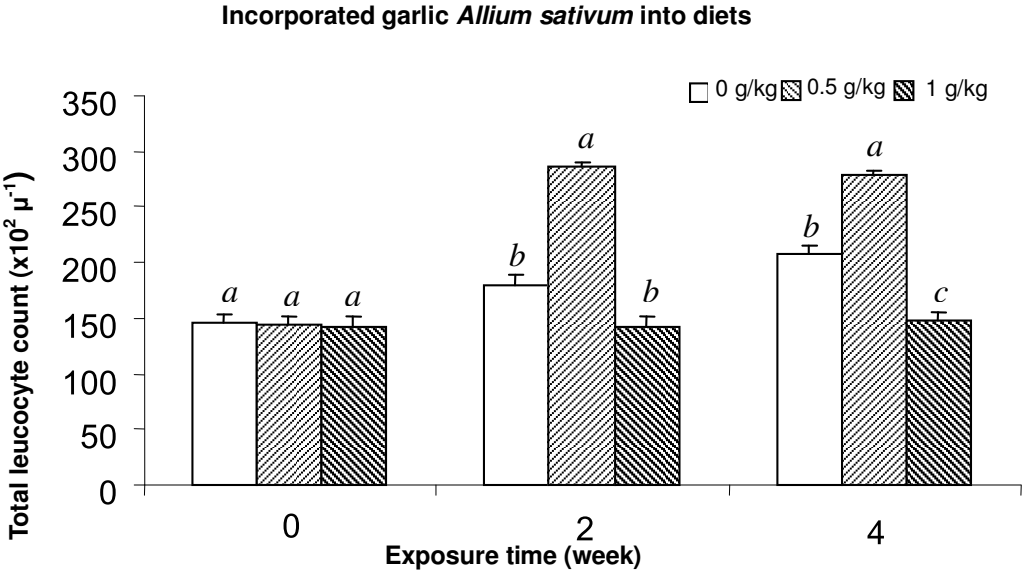


**Figure 1.** Mean (± S.E.) total leucocyte count of hybrid tilapia fed diets supplemented with garlic at concentrations 0 g/kg (control), 0.5 and 1 g/kg at the beginning, and after 2 and 4 weeks. Each bar represents the mean value from nine fish with standard error. Data (mean ± S.E.) in the same exposure time with different letters are significantly different ($p$< 0.05) among different diets.

taken up and smeared on slides. The slides were air-dried, then stained with GIEMSA solution (Sigma, St. Louis, MO, USA). Lastly, slides were examined using a microscope (Leica DMIL, Leica Microsystem. GMSH, Wetzlar, Germany) to determine the proportion of macrophage cells capable of phagocytosis (cells containing more than 5 bacteria were considered capable). The phagocytic activity was expressed as the number of phagocytic cells per 100 adherent cells. The phagocytic index (PI) was expressed as the average number of *E. coli* ingested by each phagocytic cell.

#### Measurement of innate humoral response

Lysozyme activity was measured based on the turbidimetric assay (Ellis, 1990). Briefly, a standard suspension (0.2 mg $ml^{-1}$) of *Micrococcus lysodeikticus* (Sigma) was prepared in 0.05 M sodium phosphate buffer (pH 6.2). Test plasma (10 µl) was added to 200 µl of the bacterial suspension in a 96-well microlitre plate, and the decrease in absorbance at 520 nm was recorded after 1 and 4 min at 22°C. Standard solution containing 0, 10, 20, 30, 50 and 100 units $µl^{-1}$ of hen egg white lysozyme (L6876 Sigma) was used to construct a standard curve. A unit of lysozyme activity was defined as the amount of plasma causing a reduction in absorbance of 0.001 $min^{-1}$.

#### Statistical analysis

Turkey's multiple range tests was used to determine the significant differences among treatments groups using SAS computer software (SAS Institute, 1990). Differences between means were considered statistically significant when $p < 0.05$.

## RESULTS

### Effect of garlic on the growth of hybrid tilapia

Fish fed with garlic supplemented diets at 0.5 g/kg showed a decrease about 20% on weight gain at 2 and 4 weeks as compared to those fed with control diet (Figure 4).

### Effect of garlic on the immune parameters of hybrid tilapia

### Total leucocytes count (TLC)

TLC increased significantly by 23.62 and 43.67% for the fish fed with 1% garlic supplemented diet after 2 and 4 weeks, respectively. TLC increased significantly by 98.72 and 93.87% for the fish fed with 0.5 g/kg garlic supplemented diet after 2 and 4 weeks, respectively. TLC of fish fed with diet (0.5 g/kg garlic) was significantly higher than those of that fed with diets (1 and 0 g/kg garlic) (Figure 1).

### Effect of garlic on the innate cellular response

Respiratory burst increased significantly by 8.73 and 11.83% for the fish fed respectively with diets supplemented with 1 and 0.5 g/kg garlic after 4 weeks.

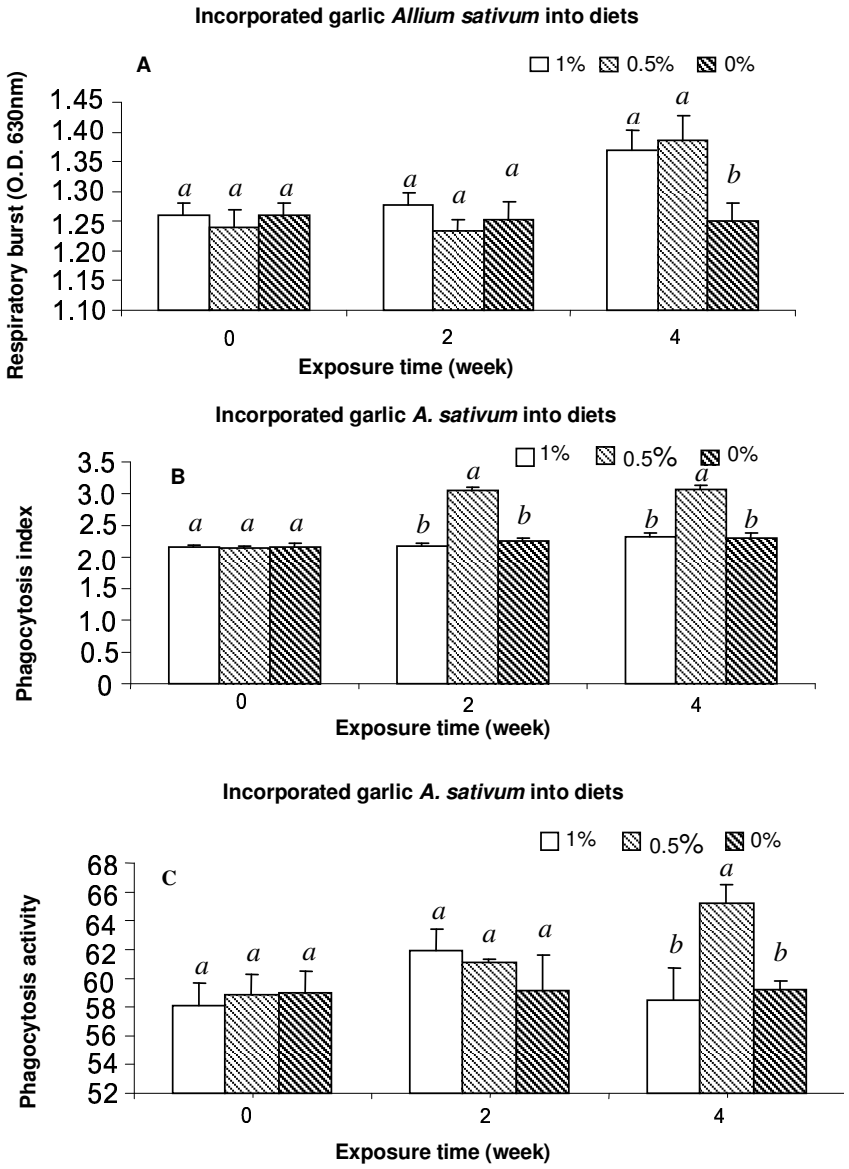


**Figure 2.** Mean (± S.E.) respiratory burst (A), phagocytic activity (B) and phagocytic index (C) of hybrid tilapia fed diets supplemented with garlic at concentrations 0 g/kg (control), 0.5 and 1 g/kg at the beginning, and after 2 and 4 weeks. Each bar represents the mean value from nine fish with standard error. Data (mean ± S.E.) in the same exposure time with different letters are significantly different ($p< 0.05$) among different diets.

No significant different of the respiratory burst activity among treatment was found after 2 weeks (Figure 2A). Phagocytic activity (PA) increased significantly by 10.92% for the fish fed with diet supplemented with 0.5 g/kg garlic after 4 weeks as compared to those fed with control diet (0 g/kg garlic). However, no significant difference was found after 2 weeks among treatments. The phagocytic index (PI) of fish fed with control diet did not significantly differ from those fed with 1 g/kg garlic supplemented diet after 2 and 4 weeks, respectively. Phagocytic index increased significantly by 43 and 43.46% for fish fed with 0.5 g/kg garlic supplemented diet respectively after 2 and 4 weeks as compared to those fed with control diet (Figures 2C and B).

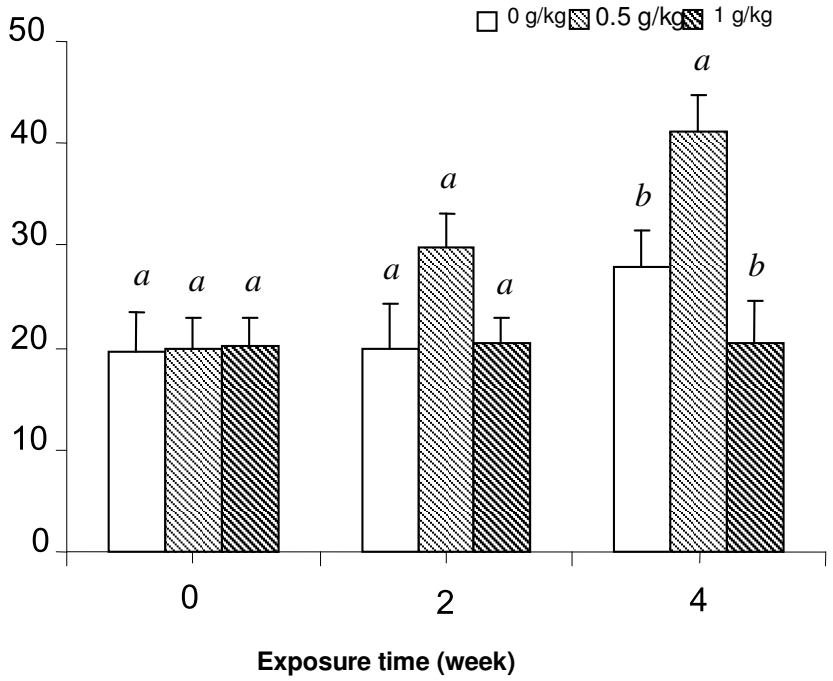


**Figure 3.** Mean (± S.E.) lysozyme activity in the plasma of hybrid tilapia fed diets supplemented with garlic at concentrations 0 g/kg (control), 0.5 g/kg and 1 g/kg at the beginning, and after 2 and 4 weeks. Each bar represents the mean value from nine fish with standard error. Data (mean ± S.E.) in the same exposure time with different letters are significantly different ($p< 0.05$) among different diets.

### Effect of garlic on the innate humoral response

Lysozyme activity increased significantly by 49.31 and 106.6% for the fish fed with 0.5 g/kg supplemented diet after 2 and 4 weeks, respectively. However, no significant difference was observed in lysozyme activity between control and fish fed with garlic supplemented diet at 1 g/kg over 4 weeks (Figure 3).

## DISCUSSION

Qureshi et al. (1983) reported no differences in final body weight of pullets fed diets with various garlic products at levels equal to about 50 kg/t of added garlic bulb. Body weight gain in broiler chickens, that received a diet supplemented with a commercial garlic product at concentrations up to 45 kg/t, were not affected (Horton et al., 1991b; Konjufca et al., 1997; Freitas et al., 2001). The body weight gain of broiler chickens fed low concentrations of commercial garlic products was improved (Lewis et al., 2003; Demir et al., 2003). In addition, lambs slaughtered did not differ in cold carcass weight and carcass yield due to the garlic bulb and garlic husk inclusion level (Bampidis et al., 2005). However, in the present study, body weight gain was decreased in juvenile hybrid tilapia fed diets supplemented with 0.5 g/kg garlic over 4 weeks. Respiratory burst has been found to increase in *Labeo rohita* fingerlings as a result of incorporated garlic into diets (Sahu et al., 2006).

In the present study, respiratory burst increased significantly for the hybrid tilapia fingerlings when fed garlic diets at concentrations 0.5 and 1 g/kg after 4 weeks. Therefore, incorporated garlic into the diets for hybrid tilapia juvenile cause increase in respiratory burst leading to enhancing immune ability. Garlic quickens macrophage phagocytosis, a process by which microorganisms and cellular debris are engulfed and destroyed (Lau et al., 1991). Germanium, a therapeutic factors contained in garlic, has been shown to enhance natural kill cell activity and macrophage activity in experimental animals (Aso, 1985). The present study indicated that both phagocytic activity and phagocytic index of blood leucocytes increased significantly in juvenile hybrid tilapia fed with

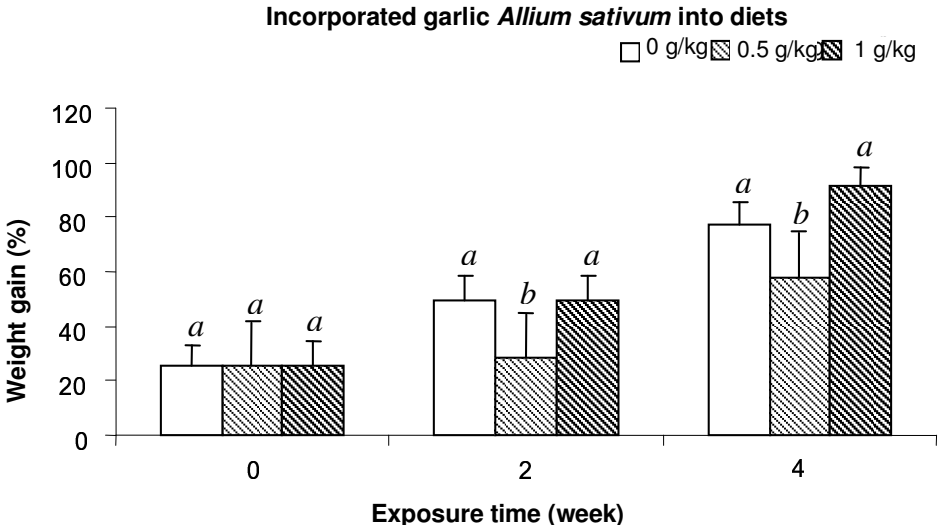


**Figure 4.** Mean (± S.E.) Weight Gain of hybrid tilapia fed diets supplemented with garlic at concentrations 0 g/kg (control), 0.5 g/kg and 1 g/kg at the beginning, and after 2 and 4 weeks. Each bar represents the mean value from nine fish with standard error. Data (mean ± S.E.) in the same exposure time with different letters are significantly different ($p< 0.05$) among different diets.

garlic at concentration 0.5 g/kg after 4 weeks. However, phagocytic activity and phagocytic index of juvenile hybrid tilapia fed with garlic at concentration 1 g/kg did not differ from those fed with control diet. In this study, the increase of phagocytosis (phagocytic index and phagocytic activity) as well as respiratory burst was well correlated with the increase of total leucocyte count in juvenile hybrid tilapia fed with 0.5 g/kg garlic incorporated into diet. This fact suggests that the presence of garlic in diet (at concentration of 0.5 g/kg) stimulates juvenile hybrid tilapia immunity. In so doing, garlic incorporated in diet can increase resistance to stress that has been shown to compromise the immune function of *O. mossambicus* (Ndong et al., 2006).

Humoral innate factors like lysozyme has been observed to be higher in garlic treated fish groups compared with the control fish group (Sahu et al., 2006). Similar result in lysozyme activity was obtained in juvenile hybrid tilapia when fed of garlic at concentration of 0.5 g/kg over 2 to 4 weeks. However, diet supplemented with garlic at concentration 1 g/kg had no effect in lysozyme activity of juvenile hybrid tilapia compared to unsupplemented control over 0 to 4 weeks. The present result suggests that garlic supplemented diet at 0.5 g/kg improves lysozyme activity in juvenile hybrid tilapia and therefore enhance its immune ability. As shown in results, higher concentration of 1 g/kg of garlic does not significantly influence the lysozyme activity. The increase of lysozyme activity was well correlated with the increase of the phagocytosis in hybrid tilapia. Our results are in line with the observation that humoral factors may enhance phagocytosis in fish (Chung and Secombes, 1987).

In conclusion, the present study documented that 0.5 g/kg supplementation of garlic had significantly improved leucocyte count, respiratory burst, phagocytic activity, phagocytic index and lysozyme activity, indicating the immunostimulant properties of garlic in juvenile hybrid tilapia. Juvenile hybrid tilapia fed of garlic 1 g/kg showed no improvement in lysozyme activity, phagocytic activity and phagocytic index which indicate that the immunostimulant properties of garlic seem to disappear at high concentration. Supplementation of garlic had no effect in growth performance of juvenile hybrid tilapia. In light of the enormous pressure which fish immune system sustain, supplemented nutrients like garlic are clearly needed.

This work provides a new perspective for use of medicinal plants as adjuvant therapy added to fish food to prevent diseases. Further studies including determination of required doses and the mechanism of action of garlic needed to be focused.

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