MONITORING THE EFFECT OF GARLIC ON THE IMMUNE RESPONSE OF NILE TILAPIA

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ABSTRACT

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The objective of this experiment to study the immunomodulation response of garlic as a feed additive for Nile tilapia. Therefore, to investigate the influence of this additive on the immune hematological parameters. Non-specific immune response of Nile tilapia and diseases resistance abilities will be evaluated. The current study demonstrated that garlic, *Allium Sativum* can be used to modulate the immune system of Nile tilapia to the favorer of resistance to diseases. Two diet regimes basic, (control), and 3% garlic were formulated and used to feed fish for 60 successive days. Fifty fish were used to investigate some of the immune parameters as serum globulins, total and differential white blood cell counts, phagocytic activities and phagocytic indices. The other fifty fish was subjected to infection challenge with *Aeromonashydrophila* to investigate the disease resistance ability of the fish received the feed additive. Results showed that the white blood cell counts, serum globulins, phagocytic activities and phagocytic indices have significantly increased in fish fed on 3% garlic supplemented diet than the control one. The mortality rate of fish fed on 3% garlic supplemented diet and challenged with *Aeromonashydrophila* were significantly lower than those that received basal diet. Obtained results concluded that garlic has an immunostimulative effect and garlic powder could be recommended to be used for farmed fish to decrease mortalities caused by pathogenic microorganisms.

Keywords: *Nile tilapia-fish immunomodulation-phagocytic assay-garlic.*

INTRODUCTION

Fish culture is an important industry in which the production of fish worldwide increases every year. Disease outbreaks were recently identified as a major constraint to aquaculture production and trade, with a consequent effect on the industry's economic development (Yunxia *et al.*, 2001). The use of antibiotics and chemotherapy was the method of choice as the disease control strategy, unfortunately, antibiotics treatment is not successful and sustainable due to increase antibiotic-resistant in bacteria, negative effects on the indigenous microflora of juveniles or adult fish (Misra *et al.*, 2006). Antimicrobials can generate cross-resistance against human antimicrobials, which could pose a hazard (Witte *et al.*, 1999).

Immunostimulants, also known as immunostimulators, are substances comprising of drugs and nutrients that activate the immune system by increasing activity of any of its components (Jadhav *et al.*, 2006). immunostimulants these are the products derived from natural sources or synthetically made with different chemical characteristics and varied modes and mechanism of action (Petrunov *et al.*, 2007). An immunostimulant is a chemical, drug, or stressor that enhances the innate (non-specific) immune response by interacting directly with cells of the system activating them. The use of immunostimulants is being introduced into fish farming routine procedures as a prophylactic measure. These substances haven't any negative side effects that live vaccines and antibiotics may have on consumers and on the environment, and are generally classified as biological response modifiers (Anderson, 1992 and Secombes, 1994). Immunostimulants can activate fish immune functions, even in immunosuppressive conditions caused by any form of stress or toxic situations. (Sakai, 1999; Sahoo and Mukherjee, 2002, 2003).

Garlic (*Allium sativum*), a member of the Liliaceae family, can help in the control of bacteria and fungi,

and increase the welfare of fish (Corzo-Martinez *et al.,* 2007). Garlic *(Allium sativum)* has proved to be hypolipidemic (Sumiyoshi, 1997), antimicrobial (Kumar and Berwal, 1998), antioxidant (Sivam, 2001), antifungal (Fromthing and Bulmer, 1978), antihypertensive (Suetsuna, 1998). The *Allium* species showed immune enhancing activities that included promotion of lymphocyte-synthesis, cytokine release, phagocytosis and natural killer cell activity (Kyo *et al.,* 2001). Previous research suggested that these functions are mainly attributed to the bioactive components of garlic (*Alliumsativum)*, including Sulphur containing compounds, such as allin, diallylsulphides and allicin (Amagase and Milner, 1993). Allicin (diallythiosulfinate) is the most abundant compound (70% of allthiosulfinates) present, or formed in crushed garlic *(Allium sativum)*(Block, 1992).Studies conducted by several researchers, mainly on Nile Tilapia (Oreochromisniloticus), reported that after administering garlic *(Allium sativum)*, an increased final weight and an improved growth rate compared to the control group were noticed (Shalaby *et al.,* 2006 and Metwally, 2009). Also, Ndong and Fall (2011) studied the effect of garlic on growth and immune response of hybrid tilapia (*Oreochromisniloticus* x Oreochromisaureus). Their study documented that 0.5 g/kg supplementation of garlic*(Allium sativum)* had significantly improved leukocyte count, respiratory burst, phagocytic activity, phagocytic index and lysozyme activity, indicating the immunostimulant properties of garlic in juvenile hybrid tilapia (Ndong and Fall, cited by Gabor *et al.,* 2011).

The objective of this experiment to study the immunomodulatory response of garlic as a feed additive for Nile tilapia, and to investigate the influence of this additive on the immune hematological parameters.Non-specific immune response of Nile tilapia and their diseases resistance abilities will be evaluated.

MATERIALS and METHODS

Fish:
A total of 100 alive Nile tilapia, Oreochromisniloticus, with an average body weight 100-150 g each, with total length of 14-17 cm were obtained from a private fish farm at Assiut governorate. Fish were transported immediately to the Aquatic Animals Wet Lab., Veterinary Hospital Clinic, Faculty of Veterinary Medicine, Assiut University. The fish were maintained in 400- L water recirculated tanks for adaptation for two week (Ellsaesserand Clem 1986). Then, the healthy fish were divided into two groups (one is control and the other is experimental group) of 50 fish each.

Diets and feed additives
Two different diets with or without additives in the form of dry pellets, representing two diet variants, were formulated to be used for feeding fish. The experimental diet was prepared using a hand palletizer and pellets left for 24 h to air dry, and stored in a refrigerator (4°C) for daily use. A basic control diet was formulated from grounded yellow corn (34.9%), soya bean meal (28.6%), fish meal (17.0%), wheat bran (9.3%), vegetable oils (6.5%), ground lime stone (0.70%), bone meal (0.30%), mineral mixture (1.7%) and vitamin mixture (1.0%). The other experimental diet was formulated as basal diet supplemented with 3% garlic powder (*Allium sativum*). Fish were fed twice daily at a rate of 3% of fish body weight per day for 60 days.

Blood sampling
Fifteen fish from each treatment were randomly collected at the end of the feeding experiment (2 month) and anaesthetized with MS-222. Blood samples were collected from the caudal vein, and were divided into two portions. One part was collected with 3% sodium citrate and used for total leucocytic count and differential leucocytic count, and other mixed with Candida albicans culture to assess phagocytic activity and phagocytic index. The second portion of the blood sample was allowed to clot overnight at 4°C, centrifuged at 3000 x g for 15 min and non-hemolysed serum was collected and stored at -20°C until use.

Total leucocytic count:
White blood cells (WBCs) were counted using the Neubauerhaemocytometer (Boeckel Co., Hamburg, Germany), in Natt-Herrick diluents according to the method of Stoskoph (1993).

Differential leucocytic count:
According to (Lucky, 1977) and (Belo *et al.,* 2008), the percentage value for each type of cells were calculated.

Determination of phagocytic activity and phagocytic index:
Phagocytic activity was determined according to (Kawahara *et al.,* 1991). 200 µl of each blood sample collected from fish were mixed with 10 mg of C. albicans culture in eppendorf, shaken and incubated 25°C for 5 hours. After incubation, smears of the blood samples were made, fixed, and stained with Giemsa stain. Phagocytic activities were estimated by calculating the proportions of macrophages which contained intracellular yeast cells by random count of 300 macrophages and expressed as percentages of phagocytic activity. The phagocytic indices (PI) were estimated by calculating the average number of yeast cells per one macrophage.

biochemical analysis:

Determination of serum total protein, albumin and globulin:

Serum total protein and albumin values were measured using spectrophotometry and "Total Protein" and "Albumin" kits (Spectrum, Egyptian Company for Biotechnology, ObourCity, Cairo, Egypt). Globulin values were determined by direct subtracting the values of the albumin from those of the total protein according to (Khalil, 2000).

Experimental challenge: Bacterial strain:

An *A. hydrophila* strain was isolated from a clinical case of infected fish during a mass mortality in a local farm of Nile tilapia showing signs of septicemia. The strain was identified by Gram stain, motility test, and various biochemical characters. The biochemical properties of the isolate confirmed the identity (Austin and Austin, 2007). Bacterial strains were kept in BHI broth with 15% glycerol (El-Gomhurrhia, Cairo, Egypt) at -20ºC. *A. hydrophila* strain was passed three times in Nile tilapia through intraperitoneal injection before using for experimental challenge.

Bacterial challenge suspension and counts.

Colony forming units (cfu) counts in bacterial suspensions were determined using spectrophometery optical density values at wavelength of 600 nm and standard-plate-count method with ten-fold serial dilution (Elkamel and Thune, 2003).

Experimental infection:

After the feeding trial 30 fish of each treatment were divided into two equal groups, the first group was intraperitoneal injection with 0.5ml of a bacterial suspension 1 X 107 cfu/ml pathogenic *A. hydrophila* .The second group was IP injected by 0.5ml of sterile saline as control. All groups were under observation for 14 days to record any abnormal clinical signs and the daily mortality rate. Re-isolation and identification of bacteria was done from freshly dead fish as mentioned above. The relative level of protection (RLP), among the challenged fish was determined according to Ruangroupan *et al.* (1986) using the following equation:

RLP = 100 - percentage of immunized mortality ÷ percentage of control mortality x100.

Statistical analysis:

Data were analyzed using the packaged SPSS program for windows version 10.01 (SPSS, 2000 Inc., Chicago, IL). Data were presented as mean ±standard error (SE). Differences between groups were determined by the one way analysis of variance (ANOVA). Significance level was set at P≤ 0.01 and P≤ 0.05.

RESULTS

Results of the current study showed that the WBCs of Nile tilapia fed on 3% garlic supplemented diet were significantly higher than those of fish fed on the control diet (Table 1). The lymphocyte showed significant increase at (p≤ 0.05) and monocyte also showed significant increase at (p ≤ 0.01) in garlic supplemented group than those of fish in the control group (Table 1). Phagocytic activity of Nile tilapia received the 3% garlic added diet was significantly higher than the control group, and also the phagocytic index of Nile tilapia received the 3% garlic supplemented diet was significantly higher than the control one (Table 1). Serum globulins of Nile tilapia fed on garlic supplemented diet was significantly higher than the control group (Table 2). Mortalities of Nile tilapia challenged with *A. hydrophila* was significantly less in fish supplemented group than those of fish fed in the control group (Table 3).

Table 1: Effect of garlic supplementation on immune response measurements of Nile Tilapia (Mean± S.E).

Group	Total lecocytic	Lymph.	Mono.	Neutro.	Baso.	Eso.	Phagocytic activity	Phagocytic index
control	35.3×10³ ±0.88	63.3±1.6	5.8±1.0	22.8± 1.9	0.66± 0.3	1.3±0.4	22.33± 0.86	1.39± 0.16
3% garlic	36.8×10³ ±0.89*	66.1±1.9*	8.0± 1.7**	22.2± 1.6	0.37± 0.1	1.0±0.2	27.94± 0.97**	2.16± 0.17**

*significant increase (p≤ 0.05) and, ** significant increase (p ≤ 0.01)

Table 2: Effect of garlic supplementation on protein status of Nile Tilapia (Mean± S.E).

Group	Total proteins	Albumin	Globulin
control	3.71±0.1	2.13±0.02	1.66±0.1
3% garlic	4.41±0.23*	2.39±0.06 **	2.16±0.14*

* significant increase (p ≤ 0.05) and ** significant increase (p≤0.01)

Table 3: Mortality percent and relative level of protection (RLP) after challenge with pathogenic bacteria (*Aeromonashydrophila*).

group	No.	Mortality		RLP	
		No.	%	No.	%
Control	15	8	53.33	7	0
3%garlic	15	2	13.33	13	75

DISCUSSION

The present study was designed to investigate the effect of dietary supplementation of garlic *(Allium sativum)* on the immune response and diseases resistance of cultured Nile tilapia. One of the most effective methods of controlling diseases in aquaculture is strengthening the defense mechanisms of fish through prophylactic administration of immunostimulants (Robertsen, 1999). Garlic *(Allium sativum)*, an important medicinal plant, has a wide spectrum of actions; not only antiviral, antibacterial, antiprotozoal, and antifungal but also has beneficial effects on the immune systems (Harris *et al.*, 2001).

The innate immune system of fish is the first line and primitive of defense against invading pathogens. The major components of the immune system are macrophages, monocytes, granulocytes and humoral elements, such as lysozomes, immunoglobulins and the complement system (Secombes and Fletcher 1992 and Magnadottir, 2006).

White blood cells (WBCs) of fish play a crucial role in the cellular immunity and resistance to infectious diseases (Whyte, 2007). There were significant increases in WBCs counts, lymphocytic count and monocyte count in fish fed with the basic diet with 3% garlic *(Allium sativum*) compared with the control one. This indicates a role of garlic *(Allium sativum)* onhaematogenesis, (Kumar *et al.,* 2006). Allium species of garlic *(Allium sativum)* also have immune enhancing activities that include promotion of lymphocyte synthesis, cytokine release, phagocytosis and natural killer cell activity (Kyo *et al.*, 1998).

These results are in accordance with results obtained by Fazlolahzadeh *et al.* (2011) who stated that Lymphocyte count increased significantly in fish (rainbow trout) fed on diets containing all garlic doses compared with the control group. Ndong and Fall (2011) reported that 0.5 % supplementation of garlic had significantly improved respiratory burst, leukocyte count, phagocytic index, and phagocytic activity, when compared with the control one.

The feed additives improved fish immunity and decrease mortality of fish through improvements of the differential leucocytic count (Lymphocyte, monocytes, basophils, eosinophils and neutrophils) in fish. Ali *et al.* (2004) and Ren *et al.* (2007) reported that, the beneficial effects of feed additives on various immunological parameters have been reported, that enhanced serum bactericidal activity, phagocytic activity, antibody levels, serum complement activity and lysozyme activity, and also, increased the total protein level in the blood (Dügenci *et al.*, 2003).

Phagocytosis is a primary non –specific defense mechanism against invasion of pathogenic organisms of hosts (Olivier *et al.*, 1988). The present study indicated that both phagocytic activity and phagocytic index of blood leucocytes increased significantly in Nile tilapia fed with garlic at concentration 3 g/kg after 8 weeks. The increase of phagocytosis (phagocytic index and phagocytic activity) was well correlated with the increase of total leucocyte count in Nile tilapia. This fact suggests that the presence of garlic in diet (at concentration of 3 g/kg) stimulates Nile tilapia immunity. Previous studies demonstrated that Nile tilapia fed garlic had significantly increased phagocytic activity and index (Ndong *et al.*, 2006).

Serum total protein and globulin are considered as good indicators of humeral defense system of fish (Siwicki *et al.*, 1994) and increases especially in the fish fed with plant extracts. Current results indicated a significant increase in total protein and globulin in fish group fed on garlic in comparison with the control one, this increase in serum antibodies provides immediate and broad protection against bacterial and viral pathogens (white, 2007). Total immune-globulin levels were significantly higher in Nile tilapia, Oncorhynchus mykiss, fed on garlic supplemented diet than the control group (Metwally, 2009). Total serum protein and globulin content were markedly increased after oral administration of garlic compared to the control (Dorucu *et al.*, 2009). High serum protein levels have been reported due to improve liver and other organs functions which synthesized plasma protein. In additions the increase in serum protein content might be in part due to an increase in the WBC, which is a major source of serum protein production such as lysozyme, complement factors and bactericidal peptides (Misra *et al.*, 2006).

The percentage of protection, after challenge was significantly greater ingarlic treated group when compared with the control group at the end of the

experiment. This indicates that these substances produced more resistance to challenge infection when fed for a long period. These results partially agree with those mentioned by Aly and Mohamed (2010). Garlic has traditional applications as an anti-infective agent against many bacteria (Ress *et al.,* 1993), fungi (Adetumbi *et al.,* 1986) and viruses (Weber *et al.,* 1992). The antibacterial properties of garlic clove homogenates are attributed to allicin (as one of the major essential oils of garlic, have a significant antibacterial activity. More obviously, Mohamed *et al*. (2013) showed that allicin in vitro inhibited the growth of the tested bacterial aeromonads. Moreover, extracts of fresh garlic enhanced the cellular oxidative enzymes, superoxide dismutase, catalase and glutathione peroxidase (Borek, 2001). Moreover, extracts of fresh garlic can increase theproduction of some chemical messengers (interferon, interleukins and complement proteins) that stimulate other arms of the immune system and increase the activity of T and B lymphocytes (Raa *et al.,* 1992).

Conclusion: Obtained results concluded that garlic has an immunostimulative effect and garlic powder could be recommended to be used for farmed fish to decrease mortalities caused by pathogenic microorganisms.

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رصد تأثير الثوم على الاستجابه المناعيه فى اسماك البلطى النيلى

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استهدفت هذة التجربه دراسة مدى تاثير الثوم كمادة مضافة للعليقة على الاستجابة المناعية لاسماك البلطى النيلى. تم تجهيز عليقتين الاولى هى العليقة الاساسية (الضابطة) والثانية مضاف اليها بودرة ثوم ٣ جم/كجم عليقة وغذيت الاسماك على العليقتين لمدة ٦٠ يوم تتالى. استخدمت نصف اعداد الاسماك من المجموعتين لدراسة بعض القياسات المناعية مثل العدد الكلى لخلايا الدم البيضاء، خلايا الدم البيضاء بمختلف أنواعها، نشاط الخلايا الأكولة والتحليل اللونى لمصل الأسماك. بينما تعرضت المجموعة الاخرى من الاسماك الى عدوى تجريبية باستخدام ميكروب الايروموناس هيدروفيلا فى نهاية فترة التجربة. وقد أظهرت النتائج وجود زيادة معنوية فى كلا من العدد الكلى لكرات الدم البيضاء ونشاط الخلايا الأكولة والجلوبيولين فى المجموعة التى غذيت على عليقة تحتوى على ٣% ثوم مقارنة بالمجموعة الضابطة. وجد أن معدل الوفيات فى مجموعة الاسماك التى غذيت على عليقة تحتوى على بودرة الثوم أقل من مجموعة الأسماك التى غذيت على العليقة الضابطة خلال فترة التجربة (وكان مستوى الحماية النسبى ضد العدوى التجريبية أعلى فى المجموعة التى غذيت على عليقة تحتوى على بودرة ثوم عن المجموعة الضابطة) خلصت هذة التجربة الى أن الثوم لة تاثير فعال فى تنشيط الجهاز المناعى للاسماك وكذلك مقاومتها للامراض.