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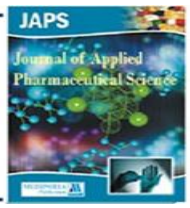

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Immunostimulant effects of dietary *Spirulina platensis* on tilapia *Oreochromis niloticus*

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ABSTRACT

Immuno-stimulant effects of the dietary Spirulina (*Spirulina Platensis*) were studied in tilapia, *Oreochromis niloticus*. For this purpose, fish were fed with Spirulina and the parameters of non-specific defense mechanisms, including serum bacterial activity, phagocytosis and lysozyme activity were performed 1. 2. 3 and 4 weeks after Spirulina administration. The efficacy also determined by bacterial challenge and monitoring specific antibody levels (Micro-agglutination). The results indicated that Spirulina enhanced responses of bactericidal, phagocytic activity and lysozyme activity. Determined by micro-agglutination, antiserum of experimental fish displayed high antibody titers. The fish fed with Spirulina and vaccinated fish conferred protection against Aeromonas hydrophila challenge 80% and 70% relative percentage survival (RPS) respectively. These findings suggest that dietary Spirulina has immunostimulatory effects on the immune system of tilapia (*O. niloticus*).

Keywords: *Spirulina platensis*, *Oreochromis niloticus*, immune assay, Phagocytic assay.

INTRODUCTION

Immunostimulants enhance the innate immune system, thereby preventing infectious diseases. In fish, several immunostimulants such as Chitin (Sakai *et al.*,, 1992 and Esteban *et al.*,, 2001), Lactoferrin (Sakai *et al.*,, 1993), dimerized lysozyme (Siwicki *et al.*,, 1998), CPG oligodeoxy nuclleotides (Tassakka and Sakai, 2002& 2003), Nisin (Villamil *et al.*,, 2004) have been reported and these substances play a promising role in aquaculture by enhancing the resistance of cultured fish against diseases. Spirulina (*Spirulina platensis*) is a marine blue-green filamentous alga that grows in carbonate-rich lakes. This cyanobacterium has been commercially produced for more than 10 years as a human food supplement because it contains high-quality protein and other nutritional components such as vitamins, minerals, essential fatty acids and B-Carotene(Hayashi *et al.*,, 1998) Recently, Spirulina has been speculated to be associated with modulation of the host immune system (Hironobu *et al.*,, 2006).

Spirulina Platensis was also found to improve the immune system by suppressing cancer development and viral infection in man (Hirahashi *et al.*,, 2002). In mice, Spirulina enhanced IL - 1 antibody production (Hayashi *et al.*,, 1994 & 1998). Hirahashi *et al.*,, (2002) also found that, administration of hot water extract of Spirulina orally to human activates the innate immune system by augmenting the production of interferon and cytolysis in human NK cells Few data are available on the immune-stimulatory effects of Spirulina in fish. Duncan and Klesius (1996) have demonstrated that Spirulina enhanced nonspecific immunity of channel catfish (*Ictalurus Punctatus*) while Hironobu *et al.*,, (2006) suggested that dietary Spirulina has immune-stimulatory effects on the innate immune system of carp (*Cyprirus carpio*). The current study aims to establish the immune-potentiating function of Spirulina (*Spirulina Platensis*) in tilapia fish (*Oreochromis niloticus*).

MATERIAL AND METHODS

Fish and experimental design

A total number of 240 *Oreochromis niloticus* with a mean weight of 50 g were stocked in 12 experimental units of 60 litter capacity containing dechlorinated filtered tap water at a temperature of 25.2 - 27.3 ^{o}C, PH 7.1 - 7.5, dissolved oxygen 4.2 - 5.6 mg / L, salinities 1.6 - 1.8%, unionized ammonia (NH3) 0.06 - 0.09 mg / L and nitrite (NO2) 0.02 - 0.23 mg / L. Each experimental unit was provided with aeration and fish were maintained in fresh water and fed commercial diets (32% crude protein) twice daily.

The fish were divided into 4 equal groups and each group was replicated three times (a total of 180 fish in each). The replicates of the first and second groups were administered orally with 1 mg and 10 mg of suspended Spirulina in sterilized physiological saline (0.85% Nacl) respectively, while the third group was vaccinated (intraperitoneally injection, IP) by 0.2 ml of inactivated bacterine of *Aeromonas hydrophila* by. Fish in the 4th group were kept as control received orally an equal amount of phosphate buffer saline. The experiment lasted for 4 weeks.

Preparation of *Spirulina Platensis*

S. Platensis, harvested from the culture in outdoor open tanks and dried after washing by direct sunlight, the strain was obtained from Agent Chemical Laboratories, Redmond, WA, USA. The dried Spirulina were suspended in sterilized physiological saline (0.85% Nacl) and 0.1 ml of this suspension was administered orally to fish for 4 weeks (Hironobu *et al.*,, 2006).

Blood samples

At the end of 1st, 2nd, 3rd and 4th week, blood samples were collected from replicates of different groups via the caudal vessels using disposable syring. After each time interval, 12 fish were sampled from replicates of each group and the serum of their blood was separated and used for specific and non-specific immune assays.

Non-specific immune assays

Serum bactericidal activity

Serum bactericidal activity to *Aeromonas hydrophila* strain (Standard strain ATCC obtained from Dept. Avian and aquatic Animal Med., Fac. Med. Alexandria University) was determined according to Rainger and Rowley (1993). Briefly, a 300 ul of *A. hydrophila* suspension (1.5 x 10^3 cells / ml) and 300 ml of fresh serum were mixed in sterile ependorf tubes. A blank consisted of 300 ul of bacterial suspension and 300 ul of sterile PBS. The tubes were incubated at 28 ^{o}C. A 50 ul sample was removed at 0, 1, 2, 3, 4h, and different dilutions were plated on nutrient agar for 24 h at 28 ^{o}C, and colony forming units (CFU) were counted. The results were recorded as survival index (SI) (WordLow & Unlles, 1978).

Value, Calculated as follows: SI = CFU at end / CFU at start x 100

Phagocytic assay

Phagocytic activity was determined according to fan *et al.*,, (1996). Anticoagulant - treated blood was mixed (1:1) with Staphylococcus albus (1.0 x 10^5 cells / ml) in PBS (PH 7.2) and incubated for 30 min at 37 ^{o}C. A drop of mixture was transferred to a microscope slide and flattened. After drying, the cells were fixed with methanol for 30 min. They were then stained with Levowitz - Weber for 1 - 2 min and washed three times with distilled water. The results were read via oil immersion light microscope. Phagocytic cells and engulfed bacteria were counted, and the percentage of phagocytic cells and phagocytic index were calculated as follows.

Phagocytic activity (PA) = Percentage of phagocytic cells containing bacterial cells.

$$\text{Phagocytic index(PI)} = \frac{\textit{Number of bacterial cells phagocytosed}}{\textit{Number of phagocytic cells}}$$

Lysozyme activity

Serum lysozyme activity was determined through the turbidimetry described by Hultmark *et al.*,, (1983) by using lyophilized Micrococcus lysodekticus ($OD_{570\ nm}$ = 0.3) as the substrate in phosphate buffer (0.1 M, PH 6.4). Fifty microlitres of fish serum was added to 3 ml of bacterial suspension. The 570 nm absorbance-was-measured-after-mixture (Ao) and incubation for 30 min at 37^{o}C (A). The result was expressed by the formula:

Lysozyme activity = (Ao - A) / A

Assay of specific antibodies by micro-agglutination test (MA) and challenge Detection of immunostimulation of Spirulina was evaluated against to *A. hydrophila* by micro-agglutination (MA) test. Agglutination titers were expressed as log2 of highest serum dilution still giving a clear agglutination according to (Eurell *et al.*,, 1979). The initial immunization with formalin killed *A. hydrophila* vaccine was followed by a challenge with the same living virulent organism at the end of the experiment in all treatments according to (Ellis, 1998).

Challenge Test

A virulent strain of *A. hydrophila* was inactivated by formalin according to (Sakia *et al.*,, 1984). The inactivated *A. hydrophila* was tested for safety and sterility according to (Andreson *etal.*, 1970). The inactivated bacterin was mixed with an equal volume of sterile saline(Badran, 1990). The Bacterial number was adjusted at MacCforland's No. 2 (6 x 10^8 cells / ml). 0.2 ml of inactivated bacterial suspension was injected interapertioneally (IP) into fish. Seven days post-injection with inactivated bacteria and on weekly intervals through out 6 weeks, 5 fish were taken from each group for blood collection from caudal vessels and serum was then separated. Fifty days after primary vaccination, the vaccinated fish (Group 3) received a second vaccination with the same dose and route of vaccination. The immune response to *A. hydrophila* was detected by MA test after preparation of stained antigen according to (Eurell *et al.*,, 1979 and Collins *et al.*,, 1976). After antibody titration the survival fish were i.p. challenged with 0.1 ml / fish containing 2.0 x 10^7 cells / ml of the virulent *A. hydrophila* after 28 days from second vaccination. Mortality each group was recorded for 20 days after challenge. All dead fish were examined to determine the aetidogical agent. Protection was calculated as the relative percentage survival (RSP) (Croy & Amend 1977) using the formula:

$$RSP = \frac{1 - \%\, vaccinated\, mortality}{\%\, non - vaccinated\, mortality} \times 100$$

Statistical analysis

The t-test ($P < 0.05$) was applied to evaluate differences of bactericidal activity, phagocytic assay, lysozyme activity and antibody titers in serum. The X^2 - test was used to assess the differences in mortality between groups.

RESULTS

1- Non-specific immune responses

Bactericidal activity of serum

There were significantly higher levels of bactericidal activities against A. hydrophila in Spirulina treated tilapia (10 mg / fish) and Spirulina treated tilapia (1 mg / fish) compared with the control (blank) ($P < 0.05$, Table 1). It was noted that the sampling time had a strong effect on the levels bactericidal activity against A. hydrophila in Spirulina – treated- tilapia and vaccinated fish ($P < 0.05$) After 1st week, 2nd week and 3rd week from Spirulina treated tilapia (1 mg / fish) was non-significantly different compared with vaccinated with non- Spirulina - treated tilapia, but only at 4th week the difference observed. On the other hand, all over the period of experiment (4 weeks) the Spirulina - treated tilapia (10 mg / fish) was significantly different compared with the Spirulina - treated tilapia (1 mg / fish), vaccinated tilapia and control group ($P < 0.05$).

Phagocytic assay (activity and index)

phagocytic assay indicated that tilapia phagocytes were phagocytic and Spirulina - treatment or vaccination enhanced the phagocytic activity of phagocytes ($P < 0.05$, Table1). Significant differences were observed in phagocytic activity and index between the different groups from the 1st week to end of the experiment. The significantly higher level of both phagocytic activity and index at 4th week of the experiment specially in Spirulina – treated tilapia (10 mg / fish) than Spirulina – treated tilapia (1 mg / fish) and vaccinated group. There was no significant difference between control groups at any sampling time.

Lysozyme activity in serum

Lysozyme activity in four groups are presented in table 1. lysozyme activities in the Spirulina - treated groups and the vaccinated group were stable. In a short, Spirulina supplementation and vaccination enhanced the lysozyme activities. No differences were observed in lysozyme activities between groups at 1st week of experiment, but after that and during course of the experiment the Spirulina - treated tilapia (10 mg / fish) recorded the highest levels compared with Spirulina - treated tilapia (1 mg / fish), vaccinated and control groups. Worthy to be noted that there was no significantly difference between Spirulina - treated tilapia (1 mg / fish) and vaccinated group during different sampling of experiment ($P < 0.05$).

II. Antibody titers

There were significant higher levels of specific antibodies against A. hydrophila in both Spirulina - treated tilapia (10 mg / fish) and vaccinated fish compared with the control non-vaccinated fish ($P < 0.001$, table 2). At day 28 after the Spirulina supplemented and first immunization, the antibody titers reached 4.5 ± 0.0, 6.5 ± 0.5, 5.0 ± 0.5 and 3.5 ± 0.5 in Spirulina - treated tilapia 1 mg & 10 mg / fish, vaccinated and control groups respectively. from then on, the antibody titers increased gradually. After the booster vaccination at 6th week, antibody titers recorded the highest level in Spirulina - treated tilapia (10 mg / fish) followed by vaccinated group and finally Spirulina – treated tilapia (1 mg / fish) where reached to 8.0 ± 0.0, 7.0 ± 0.0 and 6.0 ± 0.0 respectively, compared to control group 4.5 ± 0.5 ($P < 0.05$).

Challenge test

Significant protection was shown in the Spirulina - treated tilapia (10 mg / fish) and vaccinated groups after challenge with *A. hydrophila* (Table 3). In the control group challenged with A. hydrophila, severe congestion and hemorrhages were seen at the base of the fins, the belly region, dorsal musculature, around the anus and finally ulcer formation at the caudal peduncle area from day 5 were soon also when the infected fish were dissected large amounts of fluids tinged with blood were observed in the peritoneal cavity.

Control fish started to die on day 7 post- Challenge, and 90% mortality was reached on day 10. However, a lower mortality was observed in the Spirulina - treated tilapia (10 mg / fish) and vaccinated groups and resulted in RPS of 80% and 70% respectively. The results of RPS of Spirulina - treated tilapia (1 mg/ fish) and control group was 50% and 10% respectively.

Table. (1) : Showing the effect of dietary ***spirulina platensis*** on some parameters of non- specific immune response of ***Oreochromis niloticus*** (means ± S.E.).

parameters	Bactericidal activity survive index (SI)				Phagocytic activity (PH) %				Phagocytic index (PI)				Lysozyme activity in serum (units/ml)			
Treatments / Periods	1 mg	10 mg*	Vaccin-ated**	Control	1 mg	10 mg	Vaccin-ated	Control	1 mg	10 mg	Vaccin-ated	Control	1 mg	10 mg	Vaccin-ated	Control
Zero day	Da 40.00 ± 1.68	Da 45.00 ± 2.35	Da 47.00 ± 2.85	Aa 44.00 ± 2.18	Da 16.33 ± 1.45	Ea 18.33 ± 1.53	Db 14.33 ± 1.15	Aa 16.33 ± 1.35	Aa 3.91 ± 0.42	Aa 3.20 ± 0.37	Aa 4.10 ± 0.32	Aa 3.66 ± 0.41	Ca 0.06 ± 0.01	Da 0.04 ± 0.01	Ca 0.05 ± 0.01	Aa 0.06 ± 0.01
1st week	Cb 47.15 ± 1.33	Ca 53.67 ± 1.20	Db 46.00 ± 1.15	Ac 45.00 ± 1.86	Cb 20.33 ± 1.53	Da 23.67 ± 1.15	Cb 18.33 ± 1.76	Ac 15.67 ± 0.67	Aa 4.21 ± 0.34	Aa 4.73 ± 0.13	Aa 4.27 ± 0.48	Ba 3.75 ± 0.32	Ca 0.08 ± 0.01	Ca 0.1 ± 0.02	Ba 0.08 ± 0.01	Ab 0.05 ± 1.01
2nd week	Bb 53.20 ± 2.17	Ba 62.71 ± 2.33	Cb 51.54 ± 2.31	Ac 43.15 ± 0.44	Bb 24.33 ± 1.20	Ca 29.33 ± 1.73	Cc 21.33 ± 1.33	Ad 17.67 ± 0.88	Ba 6.27 ± 0.18	Aa 7.17 ± 0.30	Bb 5.52 ± 0.44	Cc 3.55 ± 0.19	Bb 0.12 ± 0.02	Ba 0.18 ± 0.02	Bb 0.1 ± 0.02	Ac 0.06 ± 0.01
3rd week	Ab 57.31 ± 2.34	Aa 69.33 ± 2.75	Bb 59.34 ± 2.08	Ac 42.02 ± 0.76	Ab 28.67 ± 1.45	Ba 33.33 ± 1.73	Bc 24.67 ± 1.20	Ad 16.67 ± 0.67	Bb 6.87 ± 0.41	Aa 8.45 ± 0.26	Bb 6.76 ± 0.19	Cc 3.37 ± 0.35	Bb 0.14 ± 0.02	Ba 0.22 ± 0.02	Ab 0.16 ± 0.01	Ac 0.06 ± 0.01
4th week	Ac 61.88 ± 2.27	Aa 82.30 ± 2.14	Ab 68.00 ± 2.19	Ad 46.54 ± 1.03	Ab 30.33 ± 1.53	Aa 38.33 ± 1.15	Ab 29.33 ± 1.53	Ac 16.33 ± 0.67	Bb 8.35 ± 0.62	Aa 10.71 ± 0.12	Bb 8.67 ± 0.47	Cc 3.68 ± 0.09	Ab 0.18 ± 0.02	Aa 0.32 ± 0.02	Ab 0.2 ± 0.02	Ac 0.06 ± 0.01

Means with different letters in the same column different significant (P< 0.005). * dose of ***Spirulina platensis*** . ** vaccinated with ***A. hydrophila*** vaccine.

Table. (2) : Showing antibody titers ($\log_{10}$) in different treated groups.

Groups	Week post vaccination					
	1	2	3	4	5	6
1 mg	Bc 3.0 ± 0.5	Cc 3.0 ± 0.5	Bb 4.0 ± 0.5	Cb 4.5 ± 0.0	Ba 5.0 ± 0.5	Ca 6.0 ± 0.0
10 mg	Ac 4.5 ± 0.0	Ac 5.0 ± 0.0	Ac 5.5 ± 0.0	Ab 6.5 ± 0.5	Ab 6.5 ± 0.0	Ac 8.0 ± 0.0
Vaccinated	Cc 2.5 ± 0.0	Bb 4.0 ± 0.5	Bb 4.5 ± 0.0	Aa 5.0 ± 0.5	Aa 6.0 ± 0.5	Ba 7.0 ± 0.0
Control	Cc 2.5 ± 0.0	Cc 3.0 ± 0.0	Bb 3.0 ± 0.5	Cb 3.5 ± 0.5	Cb 3.5 ± 0.0	Da 4.5 ± 0.5

Control non- vaccinated serum samples were antibody negative

Table. (3) : Showing effect of dietary feeding ***Spirulina platensis*** on protection of ***Oreochromis niloticus*** against a virulent strain of ***Aeromonas hydrophila*** after vaccination by imtraperitoneally injection of ***A-hydrophila*** bacterin (n = 30)

Treatments	dead	Survival	Mortality %	Relative Percentage survival (RPS)
1 mg	15	15	50	50
10 mg	6	24	20	80
Vaccinated	9	21	30	70
Control	27	3	90	10

DISCUSSION

A closed Ecological Recirculating Aquaculture System (CERAS) is being used to produce tilapia efficiently with limited space, energy and labor (Takeuchi *et al.,,* 1997). In order to overcome the diseases incidence of tilapia- Spirulina Supplementation is being studied (Hironabu *et al.,,* 2006). In the present study, treatment with Spirulina at dose of 10 mg/ fish increased the bactericidal activities of serum than that of Spirulina at the dose of 1 mg / fish and control group from day 14 after intubation. Moreorer, it was demonstrated that the bactericidal activities of serum of the vaccinated group with A. hydrophila bacterin were higher than Spirulina- treated tilapia (1 mg / fish) and control group and these results may be attributed to the significant rise in antibody – mediated complement – killing ability of immune serum when compared with non–immune serum (Bricknell *et al.,,* 2000 and Jian *et al.,,* 2005)

Granulocytes and mononuclear phagocytes or macrophages play a central role in the cellular part of the non-specific defense of fish (Dalmo *et al.,,* 1996). Some reports have described the effect of immunostimulants on the phagocytic activity of fish (khalil *et al.,,* 2001). Chen and Adams (1996) reported that the i.p. injection of extra cellular products of Mycobacterium spp. into rainbow trout, Oncoryhnchus mykiss (Walbaum) appeared to enhance the phagocytic activity of phagocytes in blood. In this study, the phagocytic activity and phagocytic index increased significantly in both spirulina – treated tilapia (10mg/ fish) and vaccinated groups compared to Spirulina – treated tilapia (1mg / fish) and control non – vaccinated groups. These results attributed to that Spirulina augmented the expression of cytokine genes in the leucocytes of tilapia. Cytokine genes are simple poly peptides or glycoproteins that act as signaling molecules within the immune system (Thomson, 1994).

Lysozyme, detected in the blood, mucus and organs of various fish, plays on important bactericidal role in the non - specific defense against pathogens primarily through lytic actions on the pathogen cell wall. High lysozyme activity may be desirable in cultured fish because it may aid against infection when fish are kept at high densities and consequently are exposed to high bacterial loads (Grinde *et al.,,* 1988). Jian *et al.,* (2005) observed the higher lysozyme activity in serum of Japanese flounder, Paralichthys olivaceus vaccinated with adjuvanted Vibrio anguillarum. The same results was found in the present study; Lysozyme activities of the Spriulina - treated tilapia (10 mg / fish) and vaccinated groups were significantly higher than Spriulina - treated tilapia (1 mg/fish) and control groups.

Antibody response is known to be an important competent of the fish immune system and the ability to monitor such a response is essential to understanding adaptive immunity. Passive immunization studies have shown that injection of high titer anti-pathogen serum into fish before or soon after pathogen challenge can confer significant protection (Marquis and Lallier 1989; La Patra *et al.,,* 1994; Akhlaghi *et al.,,* 1996; Shelby *et al.,,* 2002 and Jian *et al.,,* 2006). In this study, we examined the disease resistance in Spirulina - treated tilapia using the tilapia pathogen *A. hydrophila.* Challenge experiments confirmed that antibody titers were correlated with protection from *A. hydrophila* challenge, where Spirulina - treated tilapia (10 mg / fish) and vaccinated groups conferred good protection against *A. hydrophila* in the challenge test. Spirulina- treated tilapia (10 mg / fish) gave the best result (80% RPS), vaccinated group with adjuvanted 2.0 X 10^7 cells / ml was better (70% RPS) and Spirulina – treated tilapia (1 mg / fish) and control group recorded the lowest protection of 50% and 10% RPS respectively. These results suggest that antibody produced from immuno-stimulatory effects of Spirulina and vaccine plays a role in conferring significant protection. Thus this results show the increased resistance to *A. hydrophila* infection on tilapia treated with Spirulina, Similar results obtained by Hironobu *et al.,* (2006).

In this study, the immunostimulatory effects of spirulina are seen in fish. However, the mechanism of immunostimulation in fish is still not clear. In human, oral administration of hot water extract of spirulina significantly increased the production of interferon - γ in Nk cells (Hirahashi *et al.,,* 2002).

In conclusion, the present results indicated that oral administration of *Spirulina platensis* at a dose of 10 mg / fish for 4 weeks to tilapia (O.niloticus) leads to (a) enhanced bactericidal activities of serum, phagocytic activity and lysozyme activities (b) increased the specific antibodies against different antigens and (c) increased the relative percentage survival against *A. hydrophila* infection.

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