

Full Length Research Paper

Trials to improve the response of *Oreochromis niloticus* to *Aeromonas hydrophila* vaccine using immunostimulants (garlic, Echinacea) and probiotics (Organic Green™ and Vet-Yeast™)

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This work aimed to investigate the role of some immunostimulants and probiotics in improving the response of overwintered tilapia to *Aeromonas hydrophila* vaccine. In this study, 15000 Nile tilapia fry (*Oreochromis niloticus*) were collected and divided into five groups. Group 1 was the control, groups 2 to 5 were fed diet supplemented with garlic, Echinacea, Organic Green™ and Vet-Yeast™ respectively for 5 months. Vaccination with *A. hydrophila* bacteria was done by the end of the feeding experiment. The antibody titer of the vaccinated overwintered tilapia of all groups showed no significant changes during the same sampling time. A significant high value in the antibody titer was recognized in vaccinated overwintered tilapia at the end of 6th-8th week post-vaccination (PV) in the control group and between the 4th – 8th week PV in the immunostimulant supplemented groups (Groups 2-3), and between the 2nd – 10th week PV in probiotic supplemented groups (Groups 4 and 5). The challenge infection of the vaccinated tilapia showed the highest mortality in Group 1 while the lowest mortality was seen in Group 5. However, maximum protection after challenge was seen at 6th week PV in other treated groups. The immunostimulants and probiotics under test proved efficient in improving the immune response to vaccination which will improve the resistance of tilapia fry against infection during the winter. The overall results are promising to implement overwintering fry culture program to economically maximize and efficiently use the available aquaculture facilities throughout the year.

Key words: Tilapia, overwintering, immunostimulants, probiotics, vaccines, pathogens.

INTRODUCTION

Aquaculture is a promising sector of fish industry in the world with about 80 million tones being produced annually

(Kolkovski and Kolkovski, 2011). The development of aquaculture faced several constraints; among these are

diseases constituting the most limiting factors. Bacterial infections, pose one of the most significant threat to successful fish production throughout the world (Rahman et al., 2009). In Egypt, bacterial diseases induce heavy mortality in farm fish (Aly, 2013). Moreover, winter season in Egypt stress tilapia causing low survival and render hatcheries unable to produce fry to stock in the ponds during winter which limit the length of the production season until June with a huge economic loss for the inability to use the fish farm during that period. Recently, the control of bacterial infection in fish is managed through the use of vaccines or via biological control using safe microorganisms (Aly et al., 2015; Aly and Mohamed, 2010).

Overwintering of tilapia fry may provide sufficient fingerlings for the following season and is carried out using heated facilities, underground warm water and in green houses (Cruz and Ridha, 1994; Jiazhao, 1991). But these resources are generally not available in Egypt. Overwintering late-season fingerlings in deep hapas (3.0 m) suspended in deep ponds (3.5 m) has also been constructed (Nguyen and Little, 2000).

Aeromonas hydrophila is one of the most common bacterial pathogens in the Egyptian aquaculture. It causes septicemia with mortalities among tilapia and other fish species reared under the hatchery and farm environment in Egypt (Aly, 2013; Aly et al., 1998). The intestinal microbiota of fry plays a role in the defense against opportunistic pathogens. The application of immunostimulants and probiotics in improving fish health was tested previously (Aly et al., 2007, 2008a, b). Garlic and Echinacea improved the body gain, survival and resistance to infection in Nile tilapia (Aly and Mohamed, 2010). Garlic plays a role in the control of bacteria and fungi (Corzo-Martinez et al., 2007). The allium enhances the immune activities through promotion of lymphocyte-synthesis, cytokine release, phagocytosis and activation of natural killer cell (Kyo et al., 2001). Few data on the use of garlic extract as immune stimulant in fish in Egypt are available (Aly et al., 2008). Echinacea, also, seems to activate the macrophages and other immunological functions in laboratory animals and humans. The evidences on the role of the polysaccharidic fraction of Echinacea on immunity are reported (Bauer, 1999). On the other hand, *Bacillus* spp., lactic acid bacteria (LAB) and other Gram-negative bacteria have been tested as fish probiotics (Irianto and Austin, 2002; Vał zquez et al., 2005). Moreover, the usage of *Saccharomyces cerevisiae* in fish is a good enhancer of fish immune system, it also improves the survival and growth rate of supplemented fish (Abd El-Tawab et al., 2008). Previous study showed the effect of some immunostimulants and probiotics on

the survival rate and final weight of overwintered tilapia fry where no significant differences was recognized in the growth while significant improvement in the survival was seen in diets containing immunostimulents or probiotics (Aly et al., 2010). The current work was designed to investigate the role of selected immunostimulants (garlic and Echinacea) and probiotics (Organic Green™ and Vet-Yeast™) on improving the response of overwintered tilapia fry to vaccination with *A. hydrophila* vaccine.

MATERIALS AND METHODS

Fish

Nile tilapia fry, *Oreochromis niloticus* (Fifteen thousand, initial weight 0.02 g) were divided into five equal groups, each of three equal replicates that are reared in 15 cement ponds (5 x 2.5 x 1 m). Ponds were filled with tap water and partially renewed during the experiment. The water quality was optimal throughout the period of experiment, except temperature which was lower than optimal range for tilapia culture because of winter season (November-March). The water was exchanged 20% daily to maintain its quality within an acceptable range for the survival of tilapia (NO₃ (0.20 mg/L), total ammonia nitrogen (0.2 mg/L), Chl a (42.27 mg/L), and orthophosphate (0.02 mg/L)). Water temperatures during the experiment ranged from 10 to 20°C.

Preparation of diets

Dietary ingredients of 35% protein were prepared in the World Fish Center as pellets [21]. Ingredients were prepared by grinding the corn to granules (0.5 mm mesh size) (Thomes-Willey Laboratory Mill Model 4, Swedesboro, NJ 08085 U.S.A). Ingredients were mixed by mixer (Hobart model D300T, U.S.A.) at a low speed for 30 min. Oil (vegetable and cod liver) was added gradually to ensure the homogeneity of the ingredients.

The immunostimulants {Garlic (*Allium sativum* L), Echinacea (*Echinacea purpurea*)} and probiotics {Organic Green™ and Vet-Yeast™} were procured from the local market of Egypt. Echinacea (*E. purpurea*) extract contains 1.5% chicoric acid grade. Organic Green™ contains 1×10^{11} bacterial cells each from *Lactobacillus acidophilus*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Aspergillus oryzae*. One gram (1 g) of Vet-Yeast™ product contains 1×10^9 *Saccharomyces cerevisiae* dried cells according to the manufacturers.

The tested immunostimulants and probiotics prepared and mixed with formulated balanced diet where garlic (*A. sativum* L) used at a rate of 40 g of garlic kg⁻¹ feed, Echinacea (*E. purpurea*) extract at 4 g of Echinacea kg⁻¹ feed, Organic Green™ at 4 g Organic Green™ kg⁻¹ feed while Vet-Yeast™ at 4 g Vet-Yeast™ kg⁻¹ feed. The feed were prepared each two weeks and the pellets were left for 24 h to air dry, and stored in a refrigerator (4°C).

Bacterial pathogen

A pathogenic *A. hydrophila* was obtained as a reference strain from

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Table 1. Design of the vaccination experiment of overwintering tilapia and challenge following vaccination.

Groups	Treatment	Total number of fish	Number of tilapia (I/P injection)		Challenge infection in weeks		
			Saline	<i>Aeromonas</i> vaccine	6 th	8 th	10 th
1	a	Control negative	150	0	30	30	30
	b	Control Positive	150	150	30	30	30
2		Garlic	150	150	30	30	30
3		Echinacea	150	150	30	30	30
4		Organic green	150	150	30	30	30
5		Vet-Yeast	150	150	30	30	30

Fish Health Management Division of the WorldFish Center, which was isolated from infected tilapia species and was identified by polymerase chain reaction (PCR). The isolate was used in the vaccination trial to test response of the overwintered vaccinated-fry.

Vaccine preparation

Formalin-killed *A. hydrophila* bacterin was prepared by addition of formalin (0.3%) to the bacterial culture, which had been previously incubated at 35°C for 48 h (Baba et al., 1988). The formalized bacterial culture was held at room temperature overnight, and then subjected to sterility and safety tests according to earlier study (Cardella et al., 1990). The sterility test was performed by culturing washed bacterin on TS agar. Plates were incubated at 37°C for 24 h and examined for bacterial growth. The safety test was performed by the intraperitoneal (IP) inoculation of 20 susceptible tilapia (20 ± 4 g) with the prepared bacterin cells (0.1 ml). The fish were reared for 2 weeks post-injection and then morbid fish were subjected to necropsy for re-isolation of *A. hydrophila* using RS media. The prepared and tested vaccine was stored in the refrigerator at 4°C. Immediately before use, formalin killed bacterial cells were washed twice with sterile saline solution and prepared to a concentration of 3 mg wet-weight/ml saline.

Experiment

Fish of group 1 (control) was fed a balanced ration without other additives. Groups 2-5 were fed on a balanced diet supplemented with 4% garlic (group 2), 4 ppt Echinacea (group 3), 4 ppt Organic Green™ culture (group 4) or 4 ppt Vet-Yeast™ (group 5); respectively. Fish were fed three times daily in a plastic feeder for five months (November – March). At the end of feeding experiment, the overwintered *O. niloticus* (n = 900) were collected; 300 fish from the control group 1 (150 for control negative and 150 for control positive subgroups a and b) and 150 fish each from the 4 treated overwintered tilapia groups were also collected. Fish were collected equally from the three replicates of the 1st stage experiment in a random way and subdivided into 3 equal replicates groups (each of 50 fish). Fish were vaccinated following the schedule shown in Table 1. Fish were anaesthetized with 100 mg/L MS₂₂₂ (Tricain methane sulfonate; Argent Chemical Laboratories, Fisheries Division). The negative control fish of group 1 were intraperitoneally (IP) injected by 0.1 ml sterile saline solution. Fish of other treated groups (2-5) and control positive subgroup were inoculated with 0.1 ml formalin-killed *A. hydrophila* diluted in 0.1 ml sterile saline (Badran et al., 1993).

Laboratory evaluation

Antibody determination

By the end of 1st, 2nd, 3rd, 4th, 6th, 8th and 10th week post-vaccination (PV), fish were anesthetized by immersion in water containing 0.1 ppm MS-222 and blood samples were collected. Whole blood (0.5 ml) was obtained from caudal vein of fish of experiment 2, (n = 30/group, 10/replicate), using syringes (1-ml) and 27-gauge needles and transferred to blood Eppendorf tubes without anticoagulant. The blood samples were centrifuged at 3000 × g for 15 min and the supernatant serum was collected and stored at -20°C in screw-capped glass vials until used. Specific antibody titers, in collected sera, were determined using the bacterial agglutination test (Baba et al., 1988).

Challenge after vaccination

Fish (n = 90), with average weight of 6 ± 0.3 g, were randomly obtained from the three replicates of each treatment of experiment 2, and were used for challenge test at the end of the 6th, 8th and 10th weeks post-vaccination (30 fish/challenge group). Fish were splitted into 3 equal groups (each of 3 equal replicate (10 fish) being reared in a separate glass aquarium (50 x 60 x 70 cm). Fish was IP inoculated with 0.5 ml (10⁸ bacteria cells ml⁻¹) of culture suspension of the reference pathogenic strains of *A. hydrophila* (Aly et al., 2008a). Mortality was recorded and the dead fish was subjected to necropsy for bacterial re-isolation. The relative level of protection (RLP) among the vaccinated and challenged fish was determined using the following equation (Ruangroupan et al., 1986):

$$\text{RLP}\% = 100 - (\text{percent stimulated mortality} \div \text{percent mortality in the control group}) \times 100$$

Statistical analysis

One-way and two-way analyses of variance (ANOVA) were carried. Also, Duncan's multiple range test (Duncan, 1955) was used to determine differences among treatments (mean at significance level of P<0.05). Standard errors were also estimated. Analysis was carried out using the SAS package (Duncan, 1955).

RESULTS

The antibody titer was not different among all the

Table 2. Antibody titer of overwintered tilapia from the 1st till the end of the 10th week PV (Mean \pm SE).

Group	Antibody titer/ week					
	1 st	2 nd	4 th	6 th	8 th	10 th
(1) Control	4 ^{Ac} \pm 0.32	4.8 ^{Abc} \pm 0.37	5 ^{Abc} \pm 0.32	6.6 ^{Aa} \pm 0.51	6.2 ^{Aa} \pm 0.37	5.8 ^{Ab} \pm 0.37
(2) Garlic (4%)	4.6 ^{Ac} \pm 0.51	5.6 ^{Ac} \pm 0.40	5.8 ^{Ab} \pm 0.37	7 ^{Aa} \pm 0.45	6.6 ^{Aa} \pm 0.24	6.4 ^{Ab} \pm 0.24
(3) Echinacea (4 g/kg feed)	4.6 ^{Ab} \pm 0.40	5.6 ^{Ab} \pm 0.58	5.8 ^{Aa} \pm 0.51	6.8 ^{Aa} \pm 0.37	6.4 ^{Aa} \pm 0.40	5.8 ^{Ab} \pm 0.58
(4) Organic green (4 g/kg feed)	4.4 ^{Ab} \pm 0.51	5.2 ^{Aa} \pm 0.49	5.2 ^{Aa} \pm 0.37	6.6 ^{Aa} \pm 0.51	6.2 ^{Aa} \pm 0.37	6 ^{Aa} \pm 0.32
(5) Vet-yeast (4 g/kg feed)	4.8 ^{Ab} \pm 0.37	5.8 ^{Aa} \pm 0.55	6 ^{Aa} \pm 0.37	7 ^{Aa} \pm 0.32	6.6 ^{Aa} \pm 24	6.2 ^{Aa} \pm 0.37

Capital letters compare between columns and small letters compare between rows. Columns with the same letter are not significant different.

Table 3. Relative level of protection of overwintered vaccinated tilapia after challenge infection of all the groups at the end of the 6th, 8th and 10th week PV.

Group	RLP (%)		
	6 th	8 th	10 th
Negative control	0	0	0
(1) Positive Control	35.5 ^{Ca} \pm 0.61	35.3 ^{Ca} \pm 0.36	35.2 ^{Ca} \pm 0.55
(2) Garlic (4%)	45.1 ^{Ba} \pm 1.67	40.4 ^{Bb} \pm 1.16	40.7 ^{Bb} \pm 1.35
(3) Echinacea (4 g/kg feed)	50.7 ^{Aa} \pm 1.77	45.4 ^{Ab} \pm 1.22	40.7 ^{Ac} \pm 1.67
(4) Organic green (4 g/kg feed)	50.8 ^{Aa} \pm 1.67	40.5 ^{Ab} \pm 2.11	40.1 ^{Ab} \pm 2.07
(5) Vet-yeast (4 g/kg feed)	50.9 ^{Aa} \pm 0.67	50.4 ^{Aa} \pm 0.55	45.8 ^{Ab} \pm 1.49

Capital letters compare between columns and small letters compare between rows. Columns with the same letter are not significant different.

treatment groups (Table 2). The antibody titer of the vaccinated overwintered tilapia of the control group initially did not significantly differ but later started to increase at the 6th - 8th week PV. In the garlic and Echinacea supplemented group, a statistically significant increase began during the 4th - 8th week PV, while in the organic green and Vet-yeast supplemented groups, a significant increase was seen earlier than in the other groups (2nd - 10th week PV). *A. hydrophila* challenge infection of the overwintered-vaccinated tilapia induced the highest mortality in the non-vaccinated control group, followed by the vaccinated control. Lowest mortality was seen in the Vet-yeast supplemented group at the three challenge periods (6th, 8th and 10th week PV). The other groups showed variable results for the three periods of challenge (Table 3).

DISCUSSION

Two important challenges face fish culture in Egypt: first making fry available throughout the year, especially during and immediately after the winter season, which retard tilapia growth and production as well as fry survival. The second challenge is to recover the time (winter and early spring) lost, when there is no investment in fish production due to shortages of fry. This study aimed to improve the survival and resistance

of tilapia fry stressed during the winter season to infection through the use of immunostimulants and probiotics together with vaccination.

According to Aly et al. (2010), an improved survival among tilapia fed on diets containing immunostimulents or probiotics was recorded where garlic-supplemented diet resulted to a higher total harvest weight. It was previously stated that, immunostimulants improve the protection of fish against diseases by enhancing non-specific and humoral defense mechanisms (Sakai, 1999). Garlic has been effect against many bacteria (Ress et al., 1993), fungi (Adetumbi et al., 1986) and viruses (Weber et al., 1992).

These findings show the effect of immunostimulants on non-specific immunity by increasing the number of phagocytes or improving phagocytosis (Shoemaker et al., 1997). Garlic and other immunostimulants enhance the bactericidal activity of the fish's phagocytic cells (Sahu et al., 2007). Moreover, garlic shows positive action against several bacteria (Ress et al., 1993). Previous studies revealed that, yeast, β -1,3 glucan enhances resistance against several major bacterial pathogens (*Vibrio anguillarum*, *Vibrio salmonicida*, *Yersinia ruckeri*, *Edwardsiella tarda* and *A. hydrophila*) in fish species such as carp, *Cyprinus carpio* (Yano et al., 1991), Atlantic salmon, *Salmo salar* (Robertsen et al., 1990) and African catfish, *Clarias gariepinus* (Yoshida et al., 1995). Several researchers reported the effect of β -(1,3) D-glucan on the

nonspecific cellular and humoral defense mechanisms, including the function of phagocytes, bacterial killing activity of macrophages in rainbow trout, Atlantic salmon and catfish (Nikl et al., 1991). β -(1, 3) D-glucan induces the production of superoxide anion by macrophages (Dalmo and Seljelid, 1995) and accelerates the synthesis of cytokine-like molecules (Yoshida et al., 1995). The protective effect of echinacea preparations may be due to the activation of lymphocytes (Wagner et al., 1986). However, the immunomodulatory effects of echinacea are considered to be via non-specific activation of the immune system (Maass et al., 2005).

In the present study, although the antibody titer showed no significant difference among supplemented groups at the same time post-vaccination, they were significantly increased in the vaccinated overwintered tilapia at the end of the 6th - 8th week PV in the control group, at 4th - 8th week PV in garlic and echinacea supplemented groups and at 2nd - 10th week PV in organic green and Vet-yeast supplemented groups. Previous studies revealed that, glucan treatment in fish enhanced the expression of interleukin 1 (Fujiki et al., 2000) and complement activity (Engstad et al., 1992). Moreover, glucan accelerated the specific immune response in catfish and Atlantic salmon against vaccines for *E. ictaluri* and *A. salmonicida*, respectively (Chen and Ainsworth, 1992; Baulny et al., 1996). Earlier finding reported that, oral administration of *Lactobacillus delbreckii* sp. *lactis* and *B. subtilis*, alone or combined increased phagocytic activity in gilthead seabream (*Sparus aurata* L.) after 2 weeks of feeding (Salinas et al., 2005).

The challenge infection of the overwintered-vaccinated tilapia using *A. hydrophila* showed lowest mortality in the Vet-yeast supplemented group at three challenging periods (6th, 8th and 10th week PV). Yeast glucans revealed increase in the macrophage-chemotactic and phagocytic activities, the lysozyme and complement activities and also stimulated cytokines (IL-1, tumor necrosis factor) release and leukocyte migration (Secombes and Fletcher, 1992). Thereby, it significantly reduces fish mortality after challenge with various pathogens (Aly et al., 2015; Abel and Czop, 1992; Verlhac et al., 1996, 1998).

It may be concluded that, immunostimulants and probiotics can improve the response of overwintering tilapia fry to vaccination and resistance to diseases. Its use is recommended together with a vaccination program in order to improve the economic outcomes and to avoid environmental contamination through the widespread application of drugs and antibiotics.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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