

**Effect of some immunostimulants as feed additives on the survival and growth performance of Nile tilapia, *Oreochromis niloticus* and their response to artificial infection**

**George John, Salah Mesalhy, Mahmoud Rezk,  
Gamal El-Naggar and Mohamed Fathi**

*WorldFish Center, Regional Research Center for Africa and West Asia,  
Abbassa, Abou-Hammad, Sharkia, Egypt.*

**ABSTRACT**

Four plant based immunostimulants (*Echinacea purpurea* 0.25 and 1.0 ppt, *Allium sativum* 3 %, *Nigella sativa* 3% and *Origanum marjorana* 2 and 3% and mixture of *Allium sativum* , *Nigella sativa* 3:1, 1:3 and 3:3%, respectively ) were tested as feed additives for their effect on the survival and growth of 1600 *Oreochromis niloticus*. The mean weights of fish used in the various treatments was  $1.11 \pm 0.01$ . Four ponds were used in this experiment, each contained 10 hapas represented 10 treatments including the control that randomly distributed and each hapa contained 40 fish. The experiment was conducted in two phases ie; the summer (3 months) where tilapia fed basal diet mixed with feed additives and the winter (6 months) where tilapia feed on basal diet only. By end of first phase (summer), weight gain in fish treated with 1 ppt echinacea (E2) was significantly higher than that in the control. The observed values of the biomass in all the treatment groups were higher than that in the control group. Hematocrit values showed significant changes in all treatments except marjoram. By end of second phase (winter) phase, the observed mean final weights in all treatments were higher than the control. The mean weight gains were significantly higher than that in the control in most treated groups. Overall survival during rearing and survival in response to challenge infection were significantly higher in the groups that received immunostimulants in comparison to the control group, however it was type and dose dependant. The results suggest that immunostimulants can enhance survival especially during winter stress. Significant increase in body weight and total biomass production were seen with *Echinacea* (1.00 ppt). The results have applied value in aquaculture.

**INTRODUCTION**

Various strategies are adopted for the management of fish health. Therapeutic approaches are amongst the most direct ones, while the more efficient and aquaculture- compatible and environmentally sound means of health management involve the use of immunostimulants to enhance the general well being and health of fish. Immunostimulants and non-specific immune-enhancers mostly in the form of natural products stimulate the immune system, reduce susceptibility to diseases and protect fish from stress and diseases in

aquaculture. This reduces the dependence on chemicals or drugs and minimizes the negative environmental impact. It can also render aquaculture products more acceptable to consumers. Emerging trends show that eco-friendly approaches through the use of probiotics and immunostimulants can contribute significantly to health management in fish farming (Sakai, 1999). Aquaculture is likely to adopt the increased use of immunostimulants as feed additives since it can improve the efficiency of the system, enhance production, reduce the use of chemicals and render aquaculture products more acceptable and safe.

There is a considerable emphasis on health management in aquaculture based on prevention rather than treatment (Midtlyng, 1997). Recently a number of studies have supported the rationale in incorporating the use of immunostimulants into the overall health management plan (Rodriguez *et al.*, 2003; Sahoo and Mukherjee, 2002; Smith *et al.*, 2003; Dugenci *et al.* 2003).

A more efficient and health management could lead to reduced cost and stability in the production system and improve the economics of aquaculture operations. Sakai (1999) has conducted an extensive review on the use of immunostimulants in aquaculture. Immunostimulants can increase resistance of fish to environmental stress and are therefore suitable for use in aquaculture. They can be used in complementing the activity of vaccines. However, overdosing can lead to immunosuppression (Sakai, 1999). Immunostimulants enhance disease resistance by improving non specific defense mechanisms. Their use, in addition to other agents and vaccines is acceptable to farmers. There seems to be a wider efficacy and greater safety with immunostimulants in comparison to chemotherapeutics and vaccines.

The present study was conducted to see if medicinal plant products of Echinacea, Marjoram, Black seed and Garlic used alone or in combination had any beneficial effect both in the short term and in the long term, on the survival and growth of *O. niloticus*.

## MATERIAL AND METHODS

**The experiments were conducted in two phases:**

**i- First phase (July –September, 2003):**

*O. niloticus* fry were stocked in a quarantine tank to assess their general health condition and grown till they reached a weight of about 1.0 g. The mean weights of fish used in the various treatments ranged from  $1.11 \pm 0.01$  to  $1.16 \pm 0.01$ . Four ponds with concrete walls 50 x 5 x 1 m, length x width x depth were used as complete blocks. 10 hapas, 1m<sup>3</sup> each were installed 5 meters apart along the length of each pond. Hapas were stocked each with 40 fish chosen at random. The hapas in each pond were assigned to a control and 9 immunostimulants, at random. The water in the tanks was flushed every week and water temperature

ranged from 25-30 °C.

The immunostimulants were administered as feed additives in a basal diet prepared from local ingredients (Tables 1 and 2). These treatments were selected on the basis of earlier experiments conducted at the center. Echinacea (*Echinacea purpurea*) extract (1.5% chicoric acid grade) was procured from SEKEM Company, Egypt while crushed garlic (*Allium sativum*), dried and powdered Marjoram (*Origanum sp*); and black seed (*Nigella sativa*) were procured from the local market. The various doses of immunostimulants in crushed or powdered form were added to ingredients used in the preparation of a basal diet used as a standard feed for grow out experiments. Pellets (2 mm) were prepared using a CPM pellet machine. The feeds were stored under refrigeration to prevent deterioration in quality. Fish were fed daily at 5% body weight in three aliquots. The feeds were placed in plastic feeding trays fixed in each hapa.

Table 1. Feed ingredients in 1 Kg of the basal diet.

Ingredient	Weight (g)
Fish meal	50
Soyabean meal	460
Corn	350
Wheat flour	50
VegeTable oil	52.4
Cod liver oil	26.4
Dicalcium phosphate	10
Mineral mixture	0.7
Vitamin mixture	0.5
Vitamin C	0.3

Table 2. Design of Experiment.

Group	Treatments	Basal diet	Echinacea (ppt)	Garlic (%)	Black seed (%)	Marjoram (%)
1.	Control	+	-	-	-	-
2.	E1	+	0.25	-	-	-
3.	E2	+	1.0	-	-	-
4.	G3	+	-	3	-	-
5.	G3 Bs1	+	-	3	1	-
6.	K3	+	-	-	-	-
7.	G1 Bs3	+	-	1	3	-
8.	G3 Bs3	+	-	3	3	-
9.	M1	+	-	2	-	2
10.	M2	+	-	3	-	3

The experiment was conducted from 1<sup>st</sup> July to 30<sup>th</sup> September 2003 (90 days). The temperature ranged from 20-25 °C. On conclusion, the weight of each fish was recorded. For determining hematocrit values, 12 fish from each treatment (3 x 4 replicates) were anaesthetized with MS 222 and blood drawn from the caudal vein. Heparinized hematocrit tubes were filled up to two thirds, centrifuged for 1 min and values determined as described by Blaxhall (1972). A total of 20 fish from each treatment (5 x 4 replicates) were transferred to well aerated aquaria and challenged with 0.5 ml of a virulent strain of *Pseudomonas* sp. at  $4 \times 10^6$  cells/ml administered intraperitoneally and the mortality was recorded over 96 hours.

#### **ii-Second phase experiment (October 2003- March 2004):**

This was conducted to test the long term effect of immunostimulants administered during the summer phase. Fish were therefore not given any immunostimulants during this phase. Fish that remained from the replicates of each treatment of the summer phase were pooled separately and 20 of these were distributed in each of the four replicate hapas (1 m<sup>3</sup>) as in the earlier layout. The weight of each fish was recorded. Fish in all treatment groups including the control group were given the basal diet (Table 1) at 1% body weight in two daily aliquots during the growing period starting 1<sup>st</sup> October 2003 – 30<sup>th</sup> April 2004 (180 days). At the conclusion of the experiment the fish were weighed and the weight gain in each hapa was used for assessing the effect of immunostimulants. A total of 20 fish from each treatment group (5 x 4 replicates) were subjected to a challenge infection with 0.5 ml of a virulent strain of *Pseudomonas* sp. at  $4 \times 10^6$  cells/ml administered intraperitoneally and the mortality was recorded over 96 hours.

#### **iii- Statistical analysis was done using SAS 9.1.3.**

The experiment was analyzed as a randomized complete block design with 4 blocks and 10 treatments. The differences between treatments were assessed by t grouping. Chi square test was done to assess the effect of various treatments on mortality after the challenge infection.

## **RESULTS**

#### **i- Summer phase:**

After 90 days of rearing it was observed that the mean final weight and weight gain in fish treated with 1 ppt echinacea (E2) were significantly higher than that in the control. The observed values in all other treatments were higher than the control. Survival rates of fish treated with echinacea, black seed and marjoram were higher than the control. The survival was significantly high with E2, Bs3 and M2. Hematocrit values showed significant increase in all treatments except M1 and M2. The total fish biomass produced with 1 ppt echinacea (E2) treatment showed a significant increase compared to the control group. The observed values of the biomass in all the treatment groups were higher than that in the control group. The mortality after challenge infection was

low in all treated group in comparison with the control, it was lower in E2 and G3Bs3 than other groups (Table 3).

Table3. Mean weight gain, total biomass, hematocrit values, survival, mortality following challenge infection in *O. niloticus* treated with immunostimulants at first phase of experiment (July-Sept 2003) (Mean  $\pm$  SE).

Group	Treatments	Initial weight (g)	Final weight (g)	Weight gain (g)	Total biomass (fish)	Hematocrit value	Survival (%)	Mortality (%) on challenge
1.	Control	1.113 $\pm$ 0.006	8.647 $\pm$ 0.38 <sup>B</sup>	7.532 $\pm$ 0.388 <sup>B</sup>	270.225 $\pm$ 15.255 <sup>B</sup>	28.667 $\pm$ 0.492 <sup>B</sup>	78.125 $\pm$ 5.717 <sup>B</sup>	50
2.	E1	1.168 $\pm$ 0.002	9.511 $\pm$ 0.41 <sup>AB</sup>	8.342 $\pm$ 0.413 <sup>AB</sup>	313.85 $\pm$ 42.938 <sup>AB</sup>	31.174 $\pm$ 0.565 <sup>A</sup>	82.5 $\pm$ 5.401 <sup>AB</sup>	10 (0.0058)
3.	E2	1.128 $\pm$ 0.005	9.865 $\pm$ 0.431 <sup>A</sup>	8.736 $\pm$ 0.43 <sup>A</sup>	345.275 $\pm$ 35.174 <sup>A</sup>	31.208 $\pm$ 0.295 <sup>A</sup>	87.5 $\pm$ 4.895 <sup>A</sup>	5 (0.0014)
4.	G3	1.155 $\pm$ 0.003	9.03 $\pm$ 0.336 <sup>AB</sup>	7.875 $\pm$ 0.337 <sup>AB</sup>	304.75 $\pm$ 26.234 <sup>AB</sup>	31.042 $\pm$ 0.647 <sup>A</sup>	84.375 $\pm$ 4.828 <sup>AB</sup>	10 (0.0058)
5.	G3 Bs1	1.148 $\pm$ 0.003	8.646 $\pm$ 0.424 <sup>B</sup>	7.499 $\pm$ 0.424 <sup>B</sup>	291.8 $\pm$ 15.963 <sup>AB</sup>	30.542 $\pm$ 0.376 <sup>A</sup>	84.375 $\pm$ 1.573 <sup>AB</sup>	20 (0.0467)
6.	K3	1.125 $\pm$ 0.003	8.678 $\pm$ 0.327 <sup>B</sup>	7.553 $\pm$ 0.327 <sup>B</sup>	303.725 $\pm$ 16.768 <sup>AB</sup>	30.208 $\pm$ 0.318 <sup>A</sup>	87.5 $\pm$ 2.282 <sup>A</sup>	25 (0.1025)
7.	G1 Bs3	1.14 $\pm$ 0.003	9.294 $\pm$ 0.414 <sup>AB</sup>	8.155 $\pm$ 0.414 <sup>AB</sup>	311.35 $\pm$ 25.384 <sup>AB</sup>	31.208 $\pm$ 0.47 <sup>A</sup>	83.75 $\pm$ 2.394 <sup>AB</sup>	25 (0.1025)
8.	G3 Bs3	1.143 $\pm$ 0.004	9.261 $\pm$ 0.386 <sup>AB</sup>	8.118 $\pm$ 0.388 <sup>AB</sup>	317.2 $\pm$ 40.702 <sup>AB</sup>	30.833 $\pm$ 0.622 <sup>A</sup>	85.625 $\pm$ 5.242 <sup>AB</sup>	5 (0.0014)
9.	M1	1.158 $\pm$ 0.006	8.714 $\pm$ 0.337 <sup>B</sup>	7.552 $\pm$ 0.338 <sup>B</sup>	298.45 $\pm$ 17.97 <sup>AB</sup>	28.292 $\pm$ 0.252 <sup>B</sup>	85.625 $\pm$ 5.807 <sup>AB</sup>	20 (0.0467)
10.	M2	1.148 $\pm$ 0.005	9.24 $\pm$ 0.434 <sup>AB</sup>	8.091 $\pm$ 0.435 <sup>AB</sup>	302.6 $\pm$ 15.517 <sup>AB</sup>	29.0 $\pm$ 0.324 <sup>B</sup>	88.125 $\pm$ 3.59 <sup>A</sup>	15 (0.0181)

Mean $\pm$ SE having the same letter in the same column are not significantly different at P<0.05. Chi- Square values for comparison against basal diet treatment above are given in parentheses.

## ii- Winter phase:

After 6 months of rearing (October – March), the mean final weights of fish treated with echinacea (E1 and E2) and garlic + black seed (G3Bs1, G3Bs3) and Marjoram (M1), were significantly higher than the control. The observed mean final weights in all the other treatments were higher than the control. The mean weight gains were significantly higher than that in the control in all cases except G3 and Bs3. Survival of fish was significantly higher than the control in all treatments, highest survival was seen in E1 followed by G1Bs3 and G3Bs3. Mortality was also low in all treated groups in comparison to the control, lowest mortality was seen in E1 and K3G3 followed by E2 (Table 4).

Table 4. Mean weight gain, survival, mortality following challenge infection in *O. niloticus* treated with immunostimulants at the second phase (Oct 2003 – March 2004) (Mean  $\pm$  SE).

Group	Treatments	Initial weight	Final weight (g)	Weight gain (g)	Survival (%)	Mortality (%) on challenge
1.	Control	9.363 $\pm$ 0.398	12.340 $\pm$ 0.772 <sup>D</sup>	3.006 $\pm$ 0.766 <sup>C</sup>	43.75 $\pm$ 4.27 <sup>D</sup>	60
2.	E1	9.0 $\pm$ 0.401	14.706 $\pm$ 0.666 <sup>AB</sup>	5.727 $\pm$ 0.664 <sup>AB</sup>	83.75 $\pm$ 3.14 <sup>AB</sup>	10 (0.0009)
3.	E2	8.767 $\pm$ 0.349	15.053 $\pm$ 0.697 <sup>AB</sup>	6.314 $\pm$ 0.688 <sup>AB</sup>	63.75 $\pm$ 7.181 <sup>BC</sup>	15 (0.0033)
4.	G3	8.053 $\pm$ 0.279	12.734 $\pm$ 0.684 <sup>BC</sup>	4.684 $\pm$ 0.658 <sup>BC</sup>	73.333 $\pm$ 1.667 <sup>ABC</sup>	25 (0.0252)
5.	G3 Bs1	8.916 $\pm$ 0.331	14.630 $\pm$ 0.885 <sup>AB</sup>	5.698 $\pm$ 0.85 <sup>AB</sup>	60.0 $\pm$ 3.536 <sup>C</sup>	25 (0.0252)
6.	K3	8.475 $\pm$ 0.407	13.258 $\pm$ 0.781 <sup>B</sup>	4.783 $\pm$ 0.78 <sup>BC</sup>	66.25 $\pm$ 5.907 <sup>BC</sup>	25 (0.0252)
7.	G1 Bs3	8.809 $\pm$ 0.406	13.677 $\pm$ 0.851 <sup>ABCD</sup>	4.9 $\pm$ 0.838 <sup>B</sup>	76.25 $\pm$ 3.75 <sup>AB</sup>	25 (0.0252)
8.	G3 Bs3	8.597 $\pm$ 0.322	15.541 $\pm$ 0.812 <sup>A</sup>	6.966 $\pm$ 0.807 <sup>A</sup>	76.667 $\pm$ 8.333 <sup>AB</sup>	10 (0.0009)
9.	M1	9.218 $\pm$ 0.347	14.494 $\pm$ 0.752 <sup>ABC</sup>	5.297 $\pm$ 0.732 <sup>AB</sup>	60.0 $\pm$ 4.082 <sup>C</sup>	45 (0.3422)
10.	M2	8.338 $\pm$ 0.364	13.817 $\pm$ 0.744 <sup>ABCD</sup>	5.388 $\pm$ 0.739 <sup>AB</sup>	66.25 $\pm$ 7.181 <sup>BC</sup>	35 (0.1134)

Mean $\pm$ SE having the same letter in the same column are not significantly different at P<0.05.

Chi- Square values for comparison against the basal diet treatment are given in parentheses.

## DISCUSSION

The present experiment was conducted to evaluate the immunostimulatory effect of locally available plant based immunostimulants and assess their potential benefits in aquaculture. Previous studies have shown antibacterial and antifungal properties of garlic (*Allium sativum*) and black seed (*Nigella sativa*) against bacterial and fungal agents isolated from *O. niloticus* (Diab, 2002). However, studies to establish a link between immunostimulants and improved growth in *O. niloticus* have been inadequate.

Immunostimulants increase resistance to diseases by enhancing non-specific immune response (Sakai, 1999). Fish use a range of specific and non-specific defense mechanisms against invading pathogens (Secombes *et al* 1996- from and Yano, 1996, Dugenci *et al* 2003). Since immunostimulants confer overall advantage in terms of survival and resistance to diseases, animals receiving them can be expected to perform better in terms of growth and thereby contribute to production. Sahoo and Majumdar (2002) have shown that immunomodulators increase specific immunity and reduce mortality in immunocompromised carp. The study of disease control in crustacean farming through use of immunostimulant (Smith *et al* , 2003) and the demonstrated effect of medicinal plant extracts on rainbow trout (Dugenci *et al* 2003) lend further support to the use of immunostimulants in the health management plan in aquaculture. These are new perspectives in the use of medicinal plant derived feed additives in adjuvant therapy to prevent fish diseases.

The first phase of the present experiment reveals encouraging opportunities and research leads that have applied value (Table 3). Though the final weight and weight gain was significantly higher only in the case of fish treated with 1 ppt echinacea (E2) the observed values were higher than that of the control in most of the cases. Overall survival was significantly higher in the case of E2, Bs3 and M2 treatments and mortality on challenge was significantly less than the control ( $p < 0.05$ ) in all treatments. An enhanced health status is also signaled by the higher haematocrit values in the various treatments. The total biomass of fish showed a significant increase of 28% with the administration of 1ppt echinacea (E2) compared to the control. In all other cases, the observed values were higher than the control. Thompson *et al* (1996) observed increases in growth in fish treated with PUFA (n-3) when compared to those given PUFA (n-6) while Amar *et al* (2004) showed that there was no effect of dietary intake of carotenoids on the specific growth rate and feed : gain ratio. Similarly peptidoglycan supplemented feed resulted in better FCR and growth in black tiger shrimp when compared to those given normal diet (Boonyaratpalin *et al* 1995) but it did not influence rainbow trout growth after 60 days of oral administration (Matsuo and Miyazano, 1993). These studies show that growth enhancement is not necessarily seen in all cases involving administration of immunostimulants.

Sakai (1999) mentioned that, long term oral administration of

immunostimulants is associated with a decrease in immune response in fish. Though the causes are not clear, it is likely that regular feed back systems are activated against immunostimulation and the response reverts to the original state. Rainbow trout that were administered peptidoglycan orally for 28 days showed protection against challenge infection with *Vibrio anguillarum* but no protection was seen after 56 days (Matsuo and Miyazano, 1993). The second (winter) phase of the experiment showed that the immunostimulatory effect persisted for more than 180 days after treatment was discontinued. This is a considerably long period considering other studies and the results seem to depend on the type of immunostimulant and the concentration.

Atlantic salmon that were given diets with a high (n-3)/(n-6) PUFA ratio showed more resistance to infection compared with those given diets with a low (n-3)/(n-6) ratio (Thompson *et al* , 1996). Similarly polyunsaturated fatty acids have been shown to influence immune response as they are precursors of eicosanoids (Hwang, 1989; Kinsella *et al* 1990) and yeast administered as feed additive to sea bream showed immunostimulatory effect (Rodriguez *et al* , 2003).

The data generated in the present experiment clearly show the beneficial effect of immunostimulants in terms of enhanced survival in treated groups compared to the control groups, both during rearing season and after challenge infection.

Rearing during winter is stressful for fish. Reduced growth and survival are seen during winter. Studies by Chen *et al* (2002) have shown that changes brought about by cold stress resulted in a depression of phagocytic activity of leucocytes and lowering of immunoglobulin M levels in *Oreochromis aureus*. It was also observed that phagocytic activity could be down regulated through cortisol action and there could be a further suppression of immunity brought about by the interaction of adrenergic agonists with cortisol. Similarly Bagni *et al* (2005) observed a decrease of innate and acquired parameters during winter in sea bass *Dicentrarchus labrax* and demonstrated the usefulness of alginic acid and beta-glucans in activating innate immune responses under conditions of immunodepression caused by environmental stress. These studies seem to provide a cue to the overall lower survival in the various treatments here during the winter months (second phase) compared to the summer months (first phase) in (Table 4).

Since no memory component is involved in the enhancement of non-specific defense mechanisms, the duration of immune response induced by immunostimulants is likely to be short (Sakai, 1999). It would therefore follow that in the absence of continued administration of immunostimulants the response would come down to near normal levels. It is interesting to note that the results of the winter (second) phase suggest the lingering long term effect and benefits with the use of immunostimulants. The final weights and weight gain show significantly higher values in a majority of cases compared to the control. Since the initial weights were different in the various treatments, it is

not the final weight but the weight gain that provides a better indication of the beneficial effect of the immunostimulants tested. Mortality of the fish on challenge infection clearly showed the enhanced immune response in all treatments except M1 and M2 (Table 4).

Through the administration of immunostimulants a number of biochemical and hematological parameters can get modified and converge in the form of a heightened immune response, but these need not necessarily manifest in gross changes like increased growth. However, the combined effect of these immune responses is reflected in improved survival rates during growth and when artificially challenged with pathogens. Enhanced survival rate when coupled with increased body weights get translated into increased biomass and this is of importance in aquaculture.

Administration of immunostimulants as feed additives appears to be a practical method for enhancing the growth and survival of *O. niloticus*. It also has the potential to enhance resistance to diseases. The cost effectiveness of the treatments which depend on the efficacy and duration of the treatment must be carefully assessed before making recommendations. The long lasting effect will have a positive bearing on the cost effectiveness of the treatments. The potential to reduce cold stress related mortalities during winter will be an added advantage. The synergy between combined effect of enhanced growth and enhanced survival getting translated into substantial increases in biomass is of applied value in aquaculture.

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