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Evaluation of *Azotobacter* and *Azospirillum* Biofertilizers as a Probiotics in *Oreochromis niloticus* Aquaculture

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ABSTRACT

Using of ecofriendly biofertilizers instead of chemical ones in fish aquacultures represents an important aim to minimize water pollution, improve water quality and enhance fish growth rate. The impact of inoculation of two strains of *Azospirillum brasilense* and/or *Azotobacter chroococcum* on bacterial count, chemical characteristics of water, Specific Growth Rate (SGR), Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and histopathological changes were studied in *Oreochromis niloticus* aquaculture. Water analysis results revealed that Dissolved Oxygen (DO), Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), NPP, NO₃-N and O-PO₄ levels were significantly ($p < 0.05$) increased by treatment with *Azotobacter* bacteria while *Azospirillum* treatment gave lower levels. On the other hand mixed or single bacterial treatments increased fish SGR especially treated with *Azotobacter* (34.62% increase in growth). Although the mixed bacterial treatment improved some water parameters it showed higher ALT and AST levels and sever histopathological lesions in fish than that of single treatment. So the present study introduces the single inoculation of *Azotobacter* bacteria biofertilizer as a suitable probiotic which may be used in *Oreochromis niloticus* aquacultures.

Key words: *Azospirillum brasilense*, *Azotobacter chroococcum*, growth rate, histopathology, probiotics

INTRODUCTION

Organic and inorganic fertilizers are often used in fish aquacultures to increase nutrient levels of a water body for enhancing fish production; however their excessive use leads to change in water quality parameters (Yadava and Garg, 1992; Garg and Bhatnagar, 1996). The use of chemical fertilizers is not only costly and lost quickly but also has caused tremendous harm to the environment. Science the three vital natural resources, soil, water and air are liable to be affected by excessive use of chemicals through leaching, volatilization and by abrupt mineralization (Garg and Bhatnagar, 1996).

Biofertilizers can be defined as microbial inoculants which contain live or latent cells of selected strains of microorganisms. They are used as a supply of nitrogen and phosphorus to agricultural crops (Shehata and El-Khawas, 2003). Moreover, the biofertilizers can reduce the use of inorganic fertilizers and prevents pollution (Narula *et al.*, 1991; Lakshminarayana, 1993). The biofertilizers

include cyanobacteria, nitrogen-fixing bacteria such as *Rhizobium*, *Azotobacter*, *Azospirillum* etc. and phosphate-solubilizing bacteria such as *Bacillus* etc. (Tripathy and Ayyappan, 2005; Nagananda *et al.*, 2010).

The potential of using these biofertilizers to fix nitrogen in different aquatic systems has been demonstrated by Tripathy and Ayyappan (1998) and Tripathy *et al.* (2001). Moreover, Garg *et al.* (1998), stated that the inoculation of biofertilizers enhance plankton production, net primary productivity and fish biomass. So that biofertilizers are represented as a source of microbial inoculants which have brought hopes for many developing countries both economically and environmentally. Since they can solve problems of high inorganic fertilizers cost and ecological hazards (Gupta *et al.*, 2003).

Azotobacter and *Azospirillum* are the most common nitrogen-fixing bacteria found in different types of aquatic habitats such as lakes, rivers and oceans (Terzaghi and Terzaghi, 1986). These organisms must not only serve as biofertilizer but also as detritus processors, fish food organisms and could also be supplementing as bioremediators, bioameliorators, biofilters which in a single term could be defined as probiotics (Tripathy and Ayyappan, 2005). The using of some microbial organisms as a probiotics was showed to improve the growth and the immune response of fish (Kesarcodi-Watson *et al.*, 2008; Marzouk *et al.*, 2008).

Although the nitrogen-fixing ability of *Azotobacter* and *Azospirillum* have been reported to occur in a wide variety of aquatic systems to optimize the productivity of freshwater fish ecosystems, the effect of such biofertilizers on fish pathology and growth rate has not been investigated so far. Hence, the present study was designed with the objective of evaluating the efficacy of use of *Azotobacter* and/or *Azospirillum* biofertilizers as probiotics in *Oreochromis niloticus* aquaculture, their effect on growth rate, liver function and moreover their pathological effect on the different fish tissues.

MATERIALS AND METHODS

Experimental aquaria: The study was carried out in El-Qanater El-khayria Research Station, Egypt. Four fiberglass aquaria (1000 liter capacity) were used, cleaned and filled with freshwater. This study was conducted from 6-2010 to 10-2010.

Preparation of biofertilizers: Strains of free-living nitrogen fixer bacteria used in the present study were *Azospirillum brasilense* (Azos. R7) isolated from *Ricinus communis* and *Azotobacter chroococcum* (Azt.) isolated from *Hordeum vulgare* (Hamza *et al.*, 1994). They were grown in N-deficient Combined Carbon Sources Medium (CCM) (Hegazi *et al.*, 1998) at 32°C in a rotary shaker for three days for *Azospirillum brasilense* and five days for *Azotobacter chroococcum*.

Experimental treatments: A total of 120 *Oreochromis niloticus* fish were divided into four groups (30 of each). The first group; control group received the food without bacteria (T₁), the second group was received *Azospirillum brasilense* treatment (T₂), the third group was received *Azotobacter chroococcum* (T₃) and the fourth one was received mixture of *Azospirillum brasilense* and *Azotobacter chroococcum* (T₄). Approximately 2-5×10⁷ bacterial cells were added daily to the fish food; the fish food consisted of 20% fish meal powder, 20% soybean, 10% corn, 45% bran, 2% vitamins and elements and 3% oils.

Water analysis: Water samples were collected every 15 days to determine the chemical parameters and bacterial count.

Chemical analysis: Dissolved Oxygen (DO) was measured using the modified Winkler method, Biochemical Oxygen Demand (BOD) with the five-day incubation method and Net Primary Productivity (NPP) with the three-hour incubation method (APHA, 1995). Chemical Oxygen Demand (COD) was carried out using the potassium permanganate method (Golterman and Clymo, 1971). Colorimetric methods were used to determine ammonia, nitrite and phosphate according to APHA (1995) and nitrate according to Mullin and Riley (1955).

Bacterial count: The pour plate technique and the plate count agar (APHA, 1995) were used for the enumeration of total bacterial counts at 32°C incubation temperatures. Total diazotrophs (associative nitrogen-fixing bacteria) were counted using the surface inoculated plate method on N-deficient Combined Carbon Sources Medium (CCM) (Hegazi *et al.*, 1998).

Growth rate determination: At the end of experiment fish were harvested, weighed and the Weight Gain (WG) and the Specific Growth Rates (SGR) were calculated according to Priestley *et al.* (2006).

ALT and AST: At the end of experiment, blood samples were taken from the caudal vein of the fish by sterile syringe rinsed with heparin. Serum was separated by centrifugation of blood at 3000 rpm for 15 min and stored at -20°C until analyses. Activities of Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) were determined colorimetrically according to Reitman and Frankel (1957) using kits supplied from Biomerieux, France.

Histopathological examination: Small portions of gill, liver, spleen and intestine from control and treated *Oreochromis niloticus* fish were fixed in 10% formalin, dehydrated in series of alcohols, cleared in xylol, embedded in paraffin wax, sectioned by a microtome at 6 µm thicknesses and the tissue sections were stained with Haematoxylin and Eosin and then pathologically examined by light microscope (Carleton, 1967).

Statistical analysis: Data were statistically analyzed using analysis of variance, ANOVA (Freed *et al.*, 1990) using the MSTAT and STATISTICA (6.0) computer programs. The correlation coefficients and linear regressions among the different parameters were determined as well.

RESULTS

Water analysis: *Azotobacter* (T₃) and mixed strains (T₄) treatments were significantly (p<0.05) increased DO (8.24 and 8.72 mg L⁻¹), BOD (8.18 and 7.31 mg L⁻¹), COD (14.64 and 15.76 mg L⁻¹) as well as NPP (12.62 and 12.21 mg L⁻¹), respectively. On the contrary *Azospirillum* treatment (T₂) showed lower values of DO (6.05 mg L⁻¹), BOD (3.98 mg L⁻¹), COD (11.59 mg L⁻¹) and NPP (6.53 mg L⁻¹) compared to the control group. Regarding the soluble forms of nitrogen the treatment with *Azospirillum* showed the significant (p<0.05) lowest values of nitrite and nitrate (0.113 and 0.726 mg L⁻¹), respectively. While the highest significant (p<0.05) O-phosphate concentration (0.65 mg L⁻¹) was determined in *Azotobacter* treated aquaria (Table 1). Correlation coefficient indicated significant positive interactions (n = 16) among the NPP with DO (r = 0.79) and BOD (r = 0.71) also NO₃-N with DO (r = 0.55), COD (r = 0.78) and O-PO₄ (r = 0.59). Negative correlation between the NH₄-N with NO₃-N (r = -0.67) and COD (r = -0.58).

Table 1: Bacterial count and water analysis as affected by bacterial treatments

Treatment	Total bacteria ------(cfu mL ⁻¹)-----	Total diazot. -----	DO	BOD	COD	NPP	NO ₂ -N	NO ₃ -N	NH ₄ -N	O-PO ₄ -----
	------(mg L ⁻¹)-----									
(T ₁)	13400 ^d	10077 ^c	6.14 ^b	6.77 ^c	14.04 ^b	8.92 ^b	0.188 ^a	1.792 ^a	0.241 ^c	0.470 ^b
(T ₂)	18217 ^c	13427 ^b	6.05 ^c	3.98 ^d	11.59 ^c	6.53 ^c	0.113 ^c	0.726 ^c	0.741 ^a	0.337 ^c
(T ₃)	35650 ^a	19968 ^a	8.24 ^a	8.18 ^a	14.64 ^{ab}	12.62 ^a	0.171 ^b	2.004 ^a	0.309 ^b	0.650 ^a
(T ₄)	23700 ^b	21258 ^a	8.72 ^a	7.31 ^b	15.76 ^a	12.21 ^a	0.174 ^b	1.542 ^b	0.252 ^c	0.332 ^c

Means followed by the same letter are not significantly different (p<0.05)

Table 2: Effect of different bacterial treatments on growth rate of *Oreochromis niloticus*

Treatment	Final weight (g)±SD	WG (W _t -W _i)	SGR (% day ⁻¹)	% increase of final weight
Control (T ₁)	37.09±9.03 ^c	11.83	0.85	-----
<i>Azospirillum</i> (T ₂)	46.40±8.99 ^{ab}	21.14	1.35	25.10
<i>Azotobacter</i> (T ₃)	49.93±8.28 ^a	24.67	1.51	34.62
Mixed (T ₄)	40.90±9.83 ^{bc}	15.64	1.07	10.27

Means followed by the same letter are not significantly different (p<0.05). Initial weight (g) = 25.26±7.58, n = 15, Specific Growth Rate (SGR) = {(ln W_t - ln W_i)/t} × 100, where W_t and W_i denote final and initial weight (g) of fish, respectively, t represents duration of the experiment (days) and ln = natural log. % increase = {(treatment-control)/control} × 100

Table 3: Effect of different bacterial treatments on ALT and AST of *Oreochromis niloticus*

Treatment	ALT (UL ⁻¹)	% increase	AST (UL ⁻¹)	(%) increase
Control (T ₁)	57.30±2.23 ^d	-----	84.10±3.16 _c	-----
<i>Azospirillum</i> (T ₂)	72.78±5.32 _b	27.01	112.89±6.40 _b	34.23
<i>Azotobacter</i> (T ₃)	67.38±4.46 _c	17.58	116.81±7.74 ^b	38.88
Mixed (T ₄)	131.00±6.26 ^a	128.60	177.04±4.11 ^a	110.50

Means followed by the same letter are not significantly different (p<0.05). ALT, AST and final weight values were expressed in Mean±SD, n = 8. % increase = {(treatment-control)/control} × 100

Regarding the bacterial count, the highest bacterial count was recorded in T3, followed by T4, T2 and T1, respectively; while diazotrophic bacteria showed the highest counts in T4, followed by T3, T2 and T1, respectively (Table 1).

Growth rate: A significant increase (p<0.05) in final weight of fish and consequently in growth rate was recorded at the end of the experiment (Table 2). The highest percentage of increase was recorded in *Azotobacter* treatment (34.62%), followed by *Azospirillum* treatment (25.10%) while mixed strains treatments recorded the lowest percentage (10.27%).

ALT and AST: A significant increase in ALT and AST in both *Azospirillum* and *Azotobacter* treated fish were recorded at the end of the experiment. This increase was about 17-38% in single strain treatments (T₂ and T₃) and reach to 110-128% in mixed strains treatment (T₄) (Table 3).

Clinical signs and histopathological changes: No abnormal clinical signs, no gross lesions or dies were observed in the four studied aquaria. Slight histopathological changes in tissues of both *Azospirillum* and *Azotobacter* treated fish were detected. Gills show hyperplasia and necrosis in fish exposed to *Azospirillum* sp. and lamellar fusion accompanied by congestion in fish exposed to *Azotobacter* sp. Intestine show few mucinus degeneration associated with congestion of submucosa

of fish exposed to *Azospirillum* sp. and infiltration of inflammatory cells in the lamina propria in fish exposed to *Azotobacter* sp. Liver show congestion in the hepatic blood vessels associated with slight degenerative changes in fish exposed to *Azospirillum* sp. and infiltration by melanomacrophage cells in fish exposed to *Azotobacter* sp. Spleen show depletion in the lymphocytic elements with hyperactivation of melanophores and melanomacrophage center numbers in fish exposed to *Azospirillum* sp. and slight congestion in fish exposed to *Azotobacter* sp. (Fig. 1-4b, c).

On the other hand, the most pathological changes were recorded in mixed bacterial treated group (T₄) which represented by severe hyperplasia, hypertrophy, lamellar fusion and presence of eosinophilic granular cells in gills. Intestine showed severe hyperplasia with fusion of some mucosal layers and activation in mucous secreting cells. Liver showed necrosis, vacuolar degeneration in hepatocytes and pancreatic tissue. In addition to depletion, congestion in the splenic blood vessels, sever hyperactivation of melanophores and melanomacrophage centers in spleen and liver tissues (Fig. 1-4d).

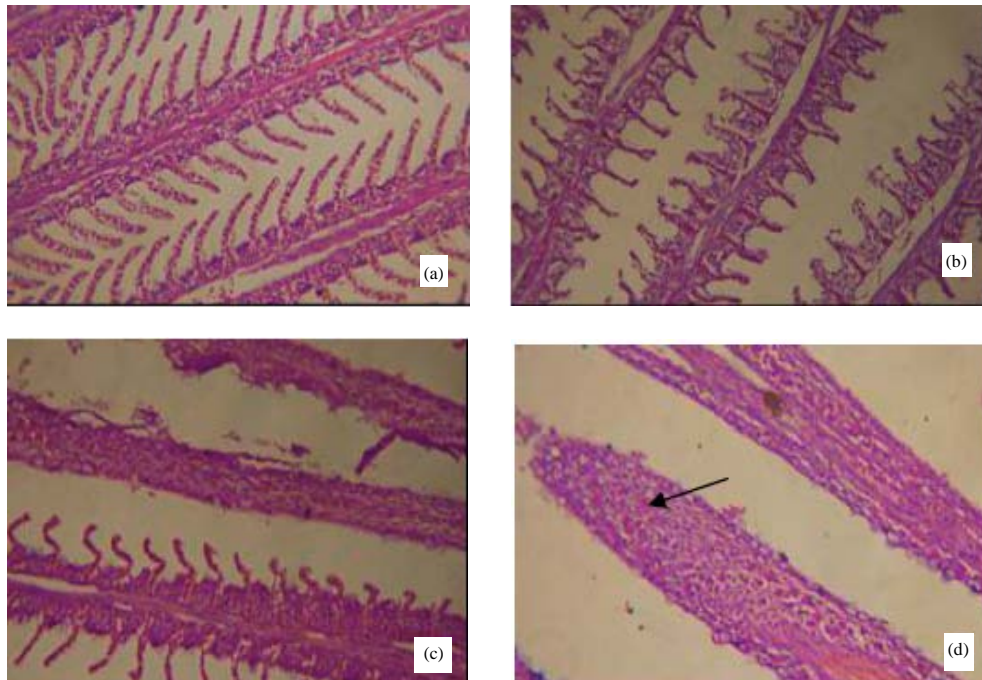


Fig. 1: Gill sections showing: (a) normal primary and secondary lamellae in fish of control group. (b) hyperplasia at the base of secondary lamellae accompanied by degenerative changes and necrosis in fish exposed to *Azospirillum* sp. (c) lamellar fusion in some primary lamellae with hyperplasia and congestion in fish exposed to *Azotobacter* sp. and (d) severe hyperplasia, hypertrophy, lamellar fusion and presence of eosinophilic granular cells (arrow) in fish exposed to mixed bacteria

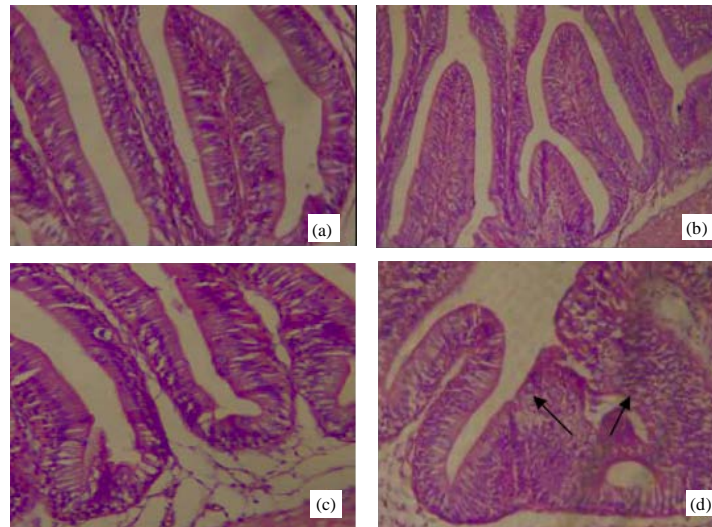


Fig. 2: Intestine sections showing: (a) intestine of control group (b) slight mucinus degeneration in the intestinal villar epithelium associated with congestion of submucosa of fish exposed to *Azospirillum* sp. (c) infiltration of mononuclear inflammatory cells in the lamina propria in fish exposed to *Azotobacter* sp. and (d) severe hyperplasia of the villar epithelium lining of the mucosa with fusion of some mucosal layers and activation in mucous secreting cells (arrows) in fish exposed to mixed bacteria

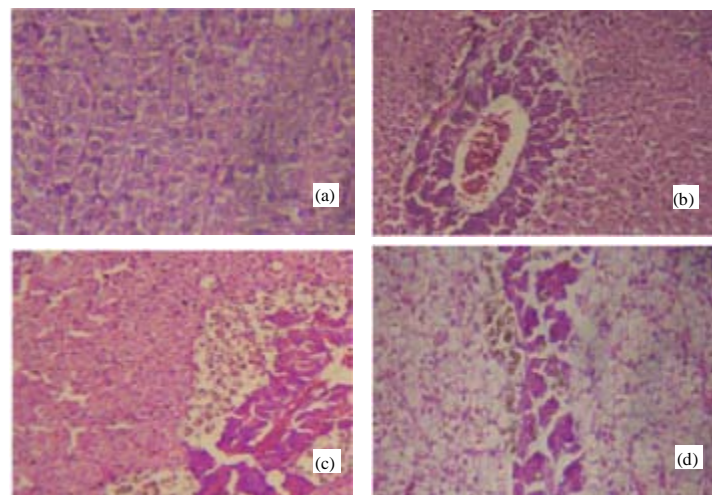


Fig. 3: Liver sections showing: (a) liver tissue of control group (b) congestion in the hepatic blood vessel associated with degenerative changes and chronic inflammatory cells infiltration in between the hepatocytes in fish exposed to *Azospirillum* sp. (c) focal necrotic area surrounding the pancreatic tissue infiltrated by melanomacrophage cells in fish exposed to *Azotobacter* sp. (d) necrosis and vacuolar degeneration in hepatocytes and pancreatic tissue with aggregation of melanomacrophage cells in fish exposed to mixed bacteria

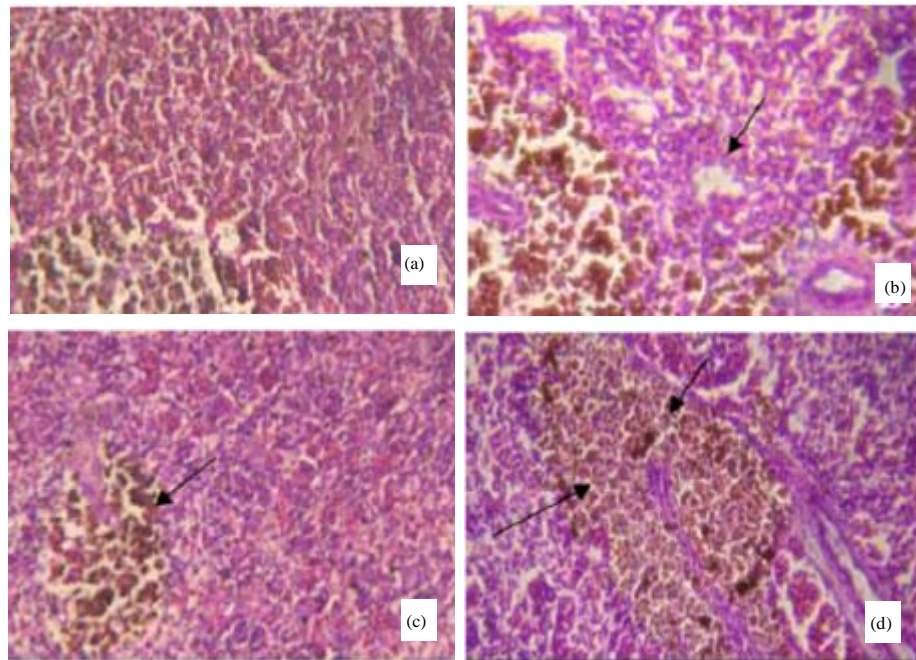


Fig. 4: Spleen sections showing: (a) spleen of control group (b) depletion in the lymphocytic elements (arrow) with hyperactivation of melanophores and melanomacrophage center numbers in fish exposed to *Azospirillum* sp. (c) congestion in between the lymphocytic tissues in fish exposed to *Azotobacter* sp. and (d) depletion, congestion in the spleenic blood vessels, sever hyperactivation of melanophores and melanomacrophage centers with fibrous connective tissue in between the lymphocytic tissue in fish exposed to mixed bacteria

DISCUSSION

Now-a-days, aquaculture has become an important economic activity in many countries; using of biofertilizers is important where chemical fertilizers impact negatively on environmental pollution and human health. *Azotobacter* and *Azospirillum* were used as biofertilizers in aquaculture; they increased the phytoplankton population and consequently the yield of fish (Garg and Bhatnagar, 1996; Garg *et al.*, 2001; Tripathy and Ayyappan, 2005). In the present study, water analysis showed that most of water parameters do not exceed the recommended water quality guidelines for the protection of cultured freshwater fish which stated by ANZECC (2000) to ensure the safety of using such biofertilizers on the fish environment.

The present study investigated that *Azotobacter* treatment was significantly increased BOD and COD; this indicates an increase of the oxi-disability and oxygen demand of water samples as well as increased the suitable components consumed by microorganisms or chemically. This may be explained by the fact that *Azotobacter* spp. in particular, are rather qualified for the bioremediation of the complex organic compounds, a mechanism for protecting their nitrogenases against high O₂ concentrations (Hegazi *et al.*, 1984) as well as the ability of *Azotobacter* to form capsular polysaccharides qualifies these particular organisms to entrap heavy metals, such as cadmium and lead ions (Pasetti *et al.*, 1996), these also illustrated increase total bacterial counts and net primary

productivity. In addition, $\text{NO}_3\text{-N}$ and O-PO_4 concentration were also significantly high in *Azotobacter* treatment as compared to the other treatments, this agreement with (Garg *et al.*, 2001) studies showed that *Azotobacter* can be used as a biofertilizer for enhancing nitrogen input and phosphate solubilization in fish ponds.

Although *Azotobacter* is a highly aerobic organism (Lakshminarayana, 1993) and its inoculation reduces DO concentration in water, our results showed that there was no decrease in DO concentration; this may be attributed to mix the bacteria strain with the fish food where Garg *et al.* (2001) observed a slight reduction in DO when *Azotobacter* were mixed with organic substrate.

The highest weight gain and specific growth rate recorded in *Azotobacter* treated fish may be attributed to the highest NPP, NO_3 and PO_4 recorded in that treatment. This indicates a high trophic status in that treatment over the other ones. And this in turn make *Azotobacter* is better used as a biofertilizer than *Azospirillum*. This was in agreement with Tripathy and Ayyappan (2005) who stated that *Azotobacter* seems to be a relatively abundant species in aquatic ecosystems due to its aerobic nature compared to the micro-aerophilic nature of *Azospirillum*. Also the inoculation of freshwater fish aquaculture ponds in India with *Azospirillum* sp. and *Azotobacter* sp. were significantly increased the phytoplankton population and consequently the yield of fish (Garg and Bhatnagar, 1996).

ALT and AST are the most sensitive tests for diagnosis of infectious diseases. Although the present study showed an increase of ALT and AST in *Azotobacter* and *Azospirillum* treated fish, there is no dangerous effect on fish health or growth. This increase may be attributed to the fact that the liver is the primary organ of detoxification; also, exposure of fish to any external stressful stimuli may cause adverse physiological reactions affecting its essential life functions as stated by Abdel-Moneim *et al.* (2008) and Dobsikova *et al.* (2009). On the other hand the marked elevation in ALT and AST in the mixed bacteria treatment revealed a serious problem in that case.

Unfortunately, there is no available data about the histopathological effects of bacterial biofertilizers treatment on fish tissue. While in present study, the treatment with *Azotobacter* sp., revealed the slight histopathological alterations in fish tissues than *Azospirillum* sp. and mixed bacterial treatment.

The prominent improve in growth in addition the hyperactivation of melanophores and melanomacrophage centers in spleen and liver of fish treated by *Azotobacter* sp., point out to the use of this bacteria as a probiotic in fish aquaculture, this agreed with several studies which demonstrated certain modes of probiotic action in the aquatic environment, they improved feed conversion ratio and feed utilization consequently increased weight gain and specific growth rate (El-Haroun, 2007; Soundarapandian and Sankar, 2008). Also, many researches showed improvement in the immune response of fishes treated with probiotics (Marzouk *et al.*, 2008; Kesarcodi-Watson *et al.*, 2008).

CONCLUSION

Based on the present study results, the usage of *Azotobacter* or *Azospirillum* biofertilizer, singly-particularly *Azotobacter*-in fish aquacultures appears to be safer on fish than using mixed bacteria treatment. However, more work need to be done to decide the extent to which it can be incorporate in fish cultures. Also, it needs more work to evaluate its use as a probiotics to promote the growth and enhance the immune response of fish.

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