

The viability of probiotics as a factor influencing the immune response in the Nile tilapia, *Oreochromis niloticus*

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ABSTRACT

The present investigation was conducted to study the effect of dietary supplementation of the probiotics *Bacillus subtilis*, *Lactobacillus plantarum*, a mixture of both bacterial isolates (*B.subtilis* and *L. plantarum*) and the yeast, *Sacchomyces cerevisiae* on the immune response of the Nile tilapia, *Oreochromis niloticus*. Two experimental diets were conducted. In the first experiment *O. niloticus* (24.55±0.03g) were fed for 60 days on four experimental diets supplemented with *B. subtilis* (10^7 CFU/g) (D1), *L. plantarum* (10^7 CFU/g) (D2) a mixture of *B. subtilis* and *L. plantarum* (D3) and the yeast *Sacchomyces cerevisiae* (D4) and a control non-supplemented diet. All of the four experimental diets showed significantly increased phagocytic activity, acid phosphatase activity, lysozyme activity and total immunoglobulin activity in blood samples of the fish as compared with the control non-supplemented diet. In the second experiment, *O. niloticus* (35.05±0.01g) were fed on a diet supplemented with three concentrations of *L. plantarum* (10^5 , 10^7 and 10^9 CFU/g) (D1, D2 and D3) respectively for 60 days. The probiotic treatment increased significantly the phagocytic activity for fish fed D2, while acid phosphatase activity was significantly increased for fish fed D3. Diets (D2 and D3) showed significantly the highest levels of lysozyme activity. No significant differences between the three diets for phenoloxidase activity, while total immunoglobulin showed significantly increased levels for fish fed D1 and D3 respectively. The results of the present study support the use of probiotics as immunostimulants in common fish diets.

Keywords: Nile tilapia, *Oreochromis niloticus*, immunity, probiotics, fish diet.

INTRODUCTION

The wide use of antibiotics and chemotherapeutics to prevent and control bacterial diseases in fish farms has led to some problems like drug resistance (Alderman and Hastings, 1998; Teuber, 2001). These antibiotics may cause

inhibition of beneficial microbiota which is normally present in the digestive tract of the fish (Sugita *et al.*, 1991). Moreover, vaccines cannot be used alone as a universal disease control measure in aquaculture (Amábile-Cuevas *et al.*, 1995) due to their limited availability in few countries and their pathogen specific protective action for certain specific bacterial and viral diseases (FAO, 2006). Hence, the use of probiotics as biological control agents or as dietary live microbial supplements in commercial fish culture to improve growth and immune function of the fish (Gatesoupe, 1999; Irianto and Austin, 2002; Kesarcodi-Watson *et al.*, 2008). These potentially probiotic cultures stimulate the growth of preferred microorganisms, out competes with potentially harmful bacteria and reinforce the organism's natural defense mechanisms. Furthermore, lactic acid bacteria (LAB) have been found to exhibit a wide range of physiological, nutritional and therapeutic effects including immunomodulation (Gill, 1998) and antibiotic resistance characteristics (Charteris *et al.*, 1998).

The dietary addition of different *Lactobacillus* species has been applied in some investigations. It has been found that the addition of a probiotic bacteria *L. rhamnosus* (ATCC 53103) to rainbow trout *Oncorhynchus mykiss* diet could reduce mortality of fish challenged with a virulent strain of *Aeromonas salmonicida* (Nikoskelainen *et al.*, 2001). The immune system response in rainbow trout, *Oncorhynchus mykiss* was also improved by the administration of probiotic bacteria *L. rhamnosus* (JCM 1136) in feed (Panigrahi *et al.*, 2004 and 2005).

Natural immunostimulants are valuable for activating the fish immune system and protecting fish against adverse conditions (Anderson *et al.*, 1995; Sakai *et al.*, 2001). Among them, the yeast *Saccharomyces cerevisiae* has been found to be a good enhancer of the fish immune system (Siwicki *et al.*, 1994). *S. cerevisiae* contains various immunostimulating compounds such as β -glucans, nucleic acids as well as oligosaccharides and it has the capability to enhance immune responses (Ortuno *et al.*, 2002) as well as promoting growth (Li *et al.*, 2004 & 2005) of various fish species.

Most studies on the use of probiotics for aquaculture have been focused on the use of single bacterium at one or more doses. However, comparative evaluation of multistrains and multispecies probiotics in fish is yet lacking. Among the questions to be answered is whether or not the immune stimulation promoted by combinations of two or more bacteria differs from that elicited by monospecies preparations. In this sense, the present study embodies two experimental trials, the first is to assess the possible modulation of Nile tilapia, *Oreochromis niloticus* immune parameters by dietary supplementation of different probiotics (*B. subtilis* NIOFSD017, *L. plantarum* NIOFSD018, either singly or a mixture containing both bacterial isolates, and the yeast *Saccharomyces cerevisiae* NIOFSD019 isolated from healthy *O. niloticus* fish in order to evaluate their effect on the immune response. The second trial is

designed to determine the optimum probiotic dose of the talent bacterial isolate selected based on the results of the first trial.

MATERIALS AND METHODS

Isolation and selection of microbial flora from *O. niloticus* gut.

Several microbial species were isolated from intestine of ten apparently healthy *O. niloticus* fish, using the method of Trust and Sparrow (1974). One ml of ten fold dilutions seeded on Tryptone Soya Agar (TSA, 1-200, Scharulu Chemie, Barcelona, Spain), De Man, Rogosa and Sharpe (MRS, 1-135, Scharulu Chemie, Barcelona, Spain) and Yeast Peptone Dextrose Agar (YPD, 1-473, Scharulu Chemie, Barcelona, Spain). The plates were incubated at $35 \pm 2^\circ\text{C}$ for 24 to 72 h. based on the microorganisms type. The anaerobic counts were determined by incubated anaerobic agar (1-371, Scharulu Chemie, Barcelona, Spain) plates at $35 \pm 2^\circ\text{C}$ for three days in anaerobic conditions using anaerobic chamber with 5% CO_2 and relative humidity of 50% (Daboor, 2008). The authenticity of the microbial cultures were verified by sub-culturing several times on the same medium and stored at 4°C for further use. A common way to select probiotic isolates was carried out according to Vine *et al.* (2004) selecting those with the ability to produce secondary metabolites.

Three selected colonies: NIOFSD017, NIOFSD018 (bacterial isolates) and NIOFSD019 (yeast) were characterized and identified following the criteria described in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) and in parallel to commercial API 50 CH and API 20 CAUX (Bio-Merieux, Marcy I Etoile, France) for bacteria and yeasts, respectively.

B. subtilis (NIOFSD017), *L. plantarum* (NIOFSD018) and *S. cerevisiae* (NIOFSD019) were grown aseptically in 10 ml of nutrient, MRS and YPD broth for 24 h. at $35 \pm 2^\circ\text{C}$. Five ml were transferred under aseptic conditions into 250 ml of nutrient, MRS and YPD broths and held on a shaker at 150 rpm for 24-48 h. at $35 \pm 2^\circ\text{C}$. The cells of each isolate were harvested by centrifugation at 10.000 rpm at 4°C for 15 min. and washed twice with phosphate buffer (PB) having pH 7.0, then dispensed in 5 ml PB.

Experiment 1: Fish and experimental design

One hundred ninety two Nile tilapia, *O. niloticus* ($24.55 \pm 0.03\text{g}$) of both sexes were used in this experiment. Fish were brought from a private fish farm at Giza, Egypt. They were transferred to the laboratory aquaria and acclimated for 14 days prior to the experiments and they were fed with basal diet. Fish were distributed in five experimental groups with each of two replicates in indoor concrete ponds at a density of 15 fish m^{-3} according to a completely randomized design. Aeration was provided by an air pump for each pond. Water was partially changed every 3 days and entirely every week. Fish were fed three times per day (3% of body weight) during this period. Water quality of ponds was analyzed following the method of APHA (1995).

The dry ingredients were mixed with corn oil and the microbial isolates were incorporated into the feed diet components according to Salinas *et al.* (2005) as shown in tables (1&2). After a desirable dough quality was obtained, diets were passed through a mincer with a die (3 mm diameter) and the resulting spaghetti-like strings were dried until the moisture levels were at approximately 10%. The diets were then stored in a -15°C freezer until being used.

In this experimental trial, the effect of different probiotic isolates including *B. subtilis* (NIOFSD017 10^7 CFUg⁻¹) (D1), *L. plantarum* (NIOFSD018 10^7 CFUg⁻¹) (D2), a mixture containing bacterial isolates (NIOFSD017 and NIOFSD018) (D3) and yeast *S. cerevisiae* (NIOFSD019 10^4 CFUg⁻¹) (D4) were used as dietary supplements for Nile tilapia in addition to the control diet (probiotic free diet) in order to determine their effect on the immune response of the fish.

Table 1: Formulation and composition of the experimental diets (dry matter basis) used to fed the Nile tilapia in the first experimental study

Ingredients	Composition (%)		Probiotic diets			
	Control	D1	D2	D3	D4	
Fish meal	30.0	30.0	30.0	30.0	30.0	
Soybean meal	22.5	22.5	22.5	22.5	22.5	
Wheat bran	38.0	38.0	38.0	38.0	38.0	
Corn oil	5.00	5.00	5.00	5.00	5.00	
Vitamin and minerals premix ¹	2.00	2.00	2.00	2.00	2.00	
Starch	2.0	2.0	2.0	2.0	2.0	
Chromic oxide	0.5	0.5	0.5	0.5	0.5	
<i>B. subtilis</i> NIOFSD017 CFUg ⁻¹		10^7				
<i>L. plantarum</i> NIOFSD018 CFUg ⁻¹			10^7			
Mixture of <i>B. subtilis</i> NIOFSD017 and <i>L. plantarum</i> NIOFSD018 CFUg ⁻¹ ²				10^7		
<i>S. cerevisiae</i> NIOFSD019 CFUg ⁻¹					10^4	
Chemical composition						
Moisture	11.20					
Crude protein	30.02					
Ether extract	10.35					
Ash content	11.85					
Crude fiber	7.10					
Nitrogen free extract	40.68					
Growth energy (kcal./kg) ³	4382.76					

1. Vitamins and Minerals premix, Kg of premix contained, vit. A (4000 000 IU), vit.D (666 666.7 IU), vit. E (3333.3 mg), vit.K₃ (333.3 mg), thiamin (333.3 mg), riboflavin (1666.7 mg), vit.B₆ (500 mg), vit. B₁₂ (3.33 mg), pantothenic acid (3333.3 mg), folic acid (333.3 mg), Biotin (16.7 mg) Niacin (10000 mg) , Iron (10000 mg), manganese (20000 mg), copper (1333.3 mg), zinc (166666.7 mg), iodine (100 mg), cobalt (33.3 mg) and selenium(33.3mg).

2. The mixture contains *B. subtilis* NIOFSD017; 0.5×10^7 CFUg⁻¹ & *L. plantarum* NIOFSD018, 0.5×10^7 CFUg⁻¹.

3. Gross Energy (Kcal / Kg) = [5.65 (CP %) + 9.45 (EE %) + 4.2 (NEF %) × 1000 / 100] (Hepher *et al.*, 1983).

D1: *B. subtilis* NIOFSD017, D2: *L. plantarum* NIOFSD018, D3: Mixture of *B. subtilis* NIOFSD017; 0.5×10^7 CFU g⁻¹ and *L. plantarum* NIOFSD018; 0.5×10^7 CFUg, D4: *S. cerevisiae* NIOFSD019.

Table 2: Formulation and composition of the experimental diets (dry matter basis) used to fed the Nile tilapia in the second experimental study

Ingredients	Composition (%)	Probiotic diets		
	Control	D1	D2	D3
Fish meal	26.0	30.0	30.0	30.0
Soybean meal	25.5	22.5	22.5	22.5
Wheat bran	38.0	38.0	38.0	38.0
Corn oil	5.00	5.00	5.00	5.00
Vitamin and minerals premix ¹	2.00	2.00	2.00	2.00
Starch	3.00	2.0	2.0	2.0
Chromic oxide	0.50	0.5	0.5	0.5
<i>L. plantarum</i> NIOFSD018 CFUg ⁻¹		10 ⁵	10 ⁷	10 ⁹
Chemical composition				
Moisture	10.75			
Crude protein	30.42			
Ether extract	9.68			
Ash content	12.30			
Crude fiber	6.50			
Nitrogen free extract	41.1			
Growth energy (kcal./kg) ²	4359.69			

1. Vitamins and Minerals premix, kg of premix contained, vit. A (4000 000 IU), vit.D (666 666.7 IU), vit. E (3333.3 mg), vit.K₃ (333.3 mg), thiamin (333.3 mg), riboflavin (1666.7 mg), vit.B₆ (500 mg), vit.B₁₂ (3.33 mg), pantothenic acid (3333.3 mg), folic acid (333.3 mg), Biotin(16.7 mg) Niacin (10000 mg) , Iron(10000 mg), manganese (20000 mg) , copper (1333.3 mg) , zinc (166666.7 mg) , iodine (100 mg), cobalt (33.3 mg) and selenium(33.3mg).

2. Gross Energy (Kcal / Kg): [5.65 (CP %) + 9.45 (EE %) + 4.2 (NEF %) × 1000 /100] (Hepher *et al.*, 1983).

D1: *L. plantarum* NIOFSD018; 10⁵CFU⁻¹g, D2: *L. plantarum* NIOFSD018; 10⁷CFU⁻¹g, D3: *L. plantarum* NIOFSD018; 10⁹CFU⁻¹g

Immunological parameters

Blood samples were scheduled after 60 days of probiotic treatment. A total of 16 fish were taken from each treatment. Blood samples were drawn from the caudal vein of the individual fish without anaesthetization after 24 hours of starvation period. The plasma and serum samples were separated, using standard procedures and preserved at -20° C prior to analysis. The plasma samples were used for the measurement of phagocytic activity, while serum samples were used for determining the acid phosphatase, lysozyme activity, phenoloxidase activity and total immunoglobulin.

Phagocytic activity

Phagocytic activity of leucocytes was determined according to Puangkaew *et al.* (2004). Briefly, a 300 µl of the leucocyte suspension in L-15 medium (Sigma-Aldrich) containing 2x10⁶ ml⁻¹ cells was seeded into a chamber slide (Lab-Tek Nalge Nunc International Co. Ltd. Illinois, USA), the cells were incubated at 15 °C for two hours and the non-adherent cells were removed. The cells were further incubated for one hour at 15 °C after adding opsonized

fluorescent latex beads (2Am; Sigma) to each chamber to maintain the cells to bead ratio of 1:10. The latex beads were opsonized by incubating 2×10^7 beads ml^{-1} with the serum of the corresponding fish. After washing and fixation, the cells were stained with Diff Quick solution (Kokusai Shiyaku Co.Ltd., Hyougo, Japan). The phagocytic activity (PA) was expressed as the percentage of phagocytising cells quantified from 300 adherent cells under microscope.

Acid phosphatase activity

Acid phosphatase activity was determined using Acid Phosphatase Assay Kit (Cayman Chemical, Catalog Number 10008051).

Lysozyme activity

Lysozyme activity in the serum was measured by agrose gel cell lysis assay, according to the method described by Schlitz (1987). Briefly lysoplates were prepared by dissolving 0.01% agarose in 0.0067 M PBS (pH 6.3) at 100 °C, when agarose temperature reached 60-70 °C, 500 mg of uniform suspension of *Micrococcus lysodeikticus* in 5 ml saline was added to one liter of agrose and mixed well, then the plates were poured, exactly 25 μl of each of the serum samples and standard lysozyme solutions were put in each poured well, then the plates were incubated at 28-30 °C for 12-18 h. and the clear zones ring diameters were measured. The diameters of sample clear zones were plotted against the standard for obtaining lysozyme concentration in unit.

Serum total immunoglobulin

Serum total immunoglobulin was analyzed through serum protein fractionation. The electrophoretic assay was applied according to the method described by Nils (1983). Briefly, serum proteins were divided into fractions of 4 bands (albumin and α , β , and γ globulin) the quantization of different fractions was conducted using Gelman DCD 16 digital computing densitometer.

Experiment 2:

In a trial to reach an ideal dose that could be suitable for Nile tilapia culture, the second experiment was conducted using different doses (10^5 , 10^7 and 10^9 CFU/g diet) of *L. plantarum* (NIOFSD018).

In this experiment, 192 specimens of *O. niloticus* (35.05 ± 0.01 g) were acclimated for living in lab aquarium and to the basal diet as in the first experimental study. Fish were distributed into four experimental groups (three replicates for each group) at a stocking density of 12 fish m^{-3} . The rearing facilities were the same as the first experiment. Fish were fed for 60 days using different doses (10^5 , 10^7 and 10^9 CFU/g diet) of *L. plantarum* (NIOFSD018) in addition to a control non-supplemented diet in a trial to reach an ideal dose that could be suitable for Nile tilapia culture. Sixteen specimens were sampled at the end of each experimental trial and blood samples were collected as described in the first experiment. The methodology employed for the determination of phagocytic, acid phosphatase activities and total immunoglobulin was the same as in experiment 1.

Lysozyme activity

Lysozyme activity was measured using the turbidimetric assay developed by Parry *et al.* (1965) and the micro plate adaptation method of Hutchinson and Manning (1996) with some minor modifications. Chicken egg lysozyme (Sigma) was used as a standard. It was dissolved in 40 mM phosphate buffer (pH 6.2) to make a solution of 2500 units/ml. A 0.6 mg/ml suspension of lyophilized *Micrococcus lysodeikticus* (Sigma) was prepared as substrate. A 25 μ l of serum sample was added to 25 μ L of buffer then 50 μ l of the substrate (*M. lysodeikticus*) was added to give a final volume of 100 μ L. The absorbance (at 450 nm) was measured after 0.5 and 4.5 min. The unit of enzyme activity was defined as a reduction in absorbance of 0.001 $\text{min}^{-1}\text{ml}^{-1}$

Phenoloxidase activity

Phenoloxidase activity was measured spectrophotometrically according to Leonard *et al.* (1985) with some modifications. This method depends on recording the formation of dopachrome from L-DOPA (L-dihydroxy phenylalanine). The 50 μ l of serum was preincubated with 50 μ L of laminarin (2mg/ml) for one hour at 20 °C before 50 μ l of L-DOPA (2mg/ml) was added. The reaction was allowed to proceed for 10 min at 20 °C, after which the absorbance was measured at 490 nm. The enzyme activity was expressed as the increase in absorbance of 0.001 $\text{min}^{-1}\text{ml}^{-1}$ serum.

Statistical analysis

Results are expressed as means \pm standard deviation (SDM). Data were statistically analyzed, using one-way analysis of variance (ANOVA). Multiple comparisons among means were made by Duncan's (Duncan, 1955) using SPSS 17 statistical program for Dalhousie University, Halifax, Canada. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Experiment 1

After 60 days of feeding the Nile tilapia, *O. niloticus* with four experimental diets (D1, D2, D3 and D4) containing four different probiotics, all the immune parameters showed significantly higher values than the control, non-supplemented diet. Fish fed D4 showed statistically the maximum phagocytic activity followed by fish fed diets D2, D3 and D1 respectively (Table 3).

Serum acid phosphatase activity was significantly increased in fish fed diets (D1, D2, D3 and D4) respectively as compared to the control diet (Table 3). The maximum increase in serum acid phosphatase activity was recorded for fish fed D3 (Table3).

All the four experimental diets showed significantly increased values in serum lysozyme activity as compared with fish fed the control diet. Although the maximum value of lysozyme activity ($342.35 \pm 20.44 \text{ Uml}^{-1}$) was recorded for fish fed diet D3, it was not significantly different from that recorded for fish fed the diets D1 and D2 (Table 3).

Feeding *O. niloticus* with four different probiotic diets increased significantly total serum immunoglobulin level. Diet D3 recorded significantly the highest values of total immunoglobulin (Table 3).

Table 3: Immunological parameters of Nile tilapia, *O. niloticus* fed various probiotic groups¹

Items	Diets				
	Control	D1	D2	D3	D4
Phagocytic activities (%)	74.5 ^c ± 1.50	77.56 ^b ± 1.55	79.0 ^a ± 1.32	78.50 ^{ab} ± 1.21	79.31 ^a ± 1.58
Acid phosphatase activities (U/ml)	6.76 ^c ± 0.22	8.14 ^a ± 0.36	8.11 ^a ± 0.17	8.27 ^a ± 0.28	7.05 ^b ± 0.09
Lysozyme activities (U/ml)	288.60 ^c ± 13.23	331.18 ^{ab} ± 19.45	330.61 ^{ab} ± 14.996	342.35 ^a ± 20.44	327.81 ^b ± 21.15
Total immunoglobulin (g/dl) ³	0.998 ^c ± 0.038	2.055 ^a ± 0.107	1.70 ^b ± 0.137	2.115 ^a ± 0.238	1.80 ^b ± 0.060

1. Values (mean ± standard deviation) in the same raw sharing the same superscript are not significantly different ($P < 0.05$), $n = 16$

2. D1; *B. subtilis* NIOFSD017, D2; *L. plantarum* NIOFSD018, D3; coculture (NIOFSD017 and NIOFSD018), D4; *S. cerevisiae* NIOFSD019, and control; microorganisms free

3. dl :deciliter

Experiment 2

The effect of *L. plantarum* (NIOFSD018) with three different doses (10^5 , 10^7 and 10^9 CFU/g diet) on the immune response (phagocytic activity, serum acid phosphatase activity, serum lysozyme activity, total serum immunoglobulin and phenoloxidase activity) of *O. niloticus* are summarized in Table 4. It is obvious that the tested immunological parameters showed varying response to the different probiotic doses. The phagocytic activity was significantly increased by increasing the probiotic dose from 10^5 to 10^7 CFUg⁻¹. However, by increasing the dose of probiotic to 10^9 CFUg⁻¹, the phagocytic activity has decreased again but it was still significantly higher than that of the fish fed the control (Table 4).

Acid phosphatase activity was significantly increased for fish fed diet D3 at the dose of 10^9 CFU g⁻¹ of *L. plantarum* (NIOFSD018), while there was non significant differences between fish fed diets D1 and D2 (Table 4).

No significant differences appeared in lysozyme activity for fish fed 10^5 CFUg⁻¹ of *L. plantarum* (NIOFSD018) (D1) and that of the control diet. On the other hand, Nile tilapia received the diets containing 10^9 and 10^7 CFUg⁻¹ of probiotic exhibited the highest average of lysozyme activity respectively (Table 4).

As shown in Table (4), the total serum immunoglobulin for fish fed different doses of *L. plantarum* (NIOFSD018) were significantly increased compared to the control group. The maximum value for total serum immunoglobulin was observed for fish fed D2. On the other hand, Phenoloxidase activity (Table 4) was insignificantly increased by each increasing in *L. plantarum* (NIOFSD018) dose.

From the previous immunological data, it is clear that Nile tilapia, fed D2 (10^7 CFUg⁻¹) had significantly the highest values of phagocytic activity and total immunoglobulin. However, no significant differences were observed in lysozyme activity or phenoloxidase activities between D2 and D3 (10^7 CFUg⁻¹). Acid phosphatase exhibited significantly higher activity in fish fed D3 than fish fed D2.

Table 4: Immunological values of Nile tilapia, *O. niloticus* fed different concentrations of *L. plantarum* (NIOFSD018¹)

Items	Treatments ²			
	Control	D1	D2	D3
Phagocytic activities (%)	72.72 ^c ±1.36	73.39 ^c ±2.12	78.5 ^a ±2.20	76.67 ^b ±2.45
Acid phosphatase activities (10 ⁻⁴ U/ml)	3.47 ^b ±0.91	5.50 ^b ±0.31	6.28 ^b ±0.07	9.25 ^a ±2.90
Lysozyme activities (U/ml)	80.00 ^b ±42.40	128.30 ^b ±49.06	889.78 ^a ±89.74	913.78 ^a ±93.71
Total immunoglobulin (g/dl)	1.6597 ^c ±0.072	2.0436 ^b ±0.121	2.207 ^a ±0.077	2.0535 ^b ±0.154
Phenoloxidase activities (U/ml)	7.93 ^a ±5.06	8.00 ^a ±3.40	8.87 ^a ±1.03	10.67 ^a ±2.47

1. Values (mean ± standard deviation) in the same column containing different superscripts were significantly different (P<0.05), n = 16 fish.

2. D1; diet having 10⁵, D2; 10⁷, D3; 10⁹ CFU/g of *L. plantarum* NIOFSD018 and Control; probiotic free diet

3. dl: deciliter

DISCUSSION

By definition, probiotics are living microorganisms, which upon ingestion in certain numbers exert health benefits on the host (Guarner and Schaafsma, 1993). Results obtained in the present study on the immune response of tilapia showed that the immunological parameters were modulated in fish fed the different probiotics. The beneficial effects of probiotics on the modulation of fish immune system have been investigated (Austin *et al.*, 1995; Stosik and Szenfeld, 1996; Rengpipat *et al.*, 2000; Heyman and Me'nard, 2002; and Taoka *et al.*, 2006, Nayak, 2010). It has been shown that the modulation of immune response by probiotics comprised different responses in a variety of domestic animals and fish, including the induction of proinflammatory cytokines, the activation of natural killer cells, production of mucosal and systemic antibodies and increasing the phagocytic lysozyme and complement activities (Miettinen *et al.*, 1996, Matsuzaki and Chin, 2000; and Panigrahi *et al.*, 2004 and 2005). Probiotics treatment provide a wide spectrum of responses and better non-specific disease protection as a result of both the enhancement of serological immunity and competitive exclusion in host's gut (Rengpipat *et al.*, 2000). It has been also reported that vaccination or immunostimulation may provide only short-term protection against specific pathogens (Sung and Song, 1996 and Sung *et al.*, 1996). In addition, the cell wall components of probiotic bacteria, such as

β -glucan, lipopolysaccharide and peptidoglycans were attributed to the immunostimulatory effects in fish (Rengpipat *et al.*, 2000, Gullian *et al.*, 2004). *B. subtilis*, when administered through feed may have an additional advantage as a promising candidate probiotic because of its spore forming ability (Kennedy *et al.*, 1998, Moriarty, 1998; and Querroz & Boyd, 1998).

Fish fed on a diet supplemented with the different probiotics (D1, D2 and D3) showed a significant increase in phagocytic activity, acid phosphatase activity, lysozyme activity and total serum immunoglobulin compared with fish received the control diet. Lactic acid bacteria (LAB) have been widely used as a dietary supplement to protect fish from various infections (Verschuere *et al.*, 2000). They are considered a safe product for feeding fish. Their ability to improve the immune response against harmful pathogens allows them to be considered as an alternative method to control various fish diseases (Gatesoupe, 1999).

Phagocytic activity is responsible for early activation of the inflammatory response before antibody production and plays an important role in antibacterial defense. Probiotics can effectively trigger the phagocytic cells in host and enhancement of phagocytic activity. Stimulation of phagocytic activity by LAB bacteria supplemented in the diet of *O. niloticus* has already been observed in the present study. Stimulation of phagocytic activity by these probiotics has been found in several fish species (Irianto and Austin, 2002; Panigrahi *et al.*, 2004; Brunt *et al.*, 2007; Pieter *et al.*, 2008). Feeding *O. niloticus* a diet supplemented with a mixture of *B. subtilis* and *L. plantarum* (D3) stimulated the phagocytic activity of the leukocytes of the fish but it was not the most effective diet. It was suggested that LAB might mediate the phagocytic activity through modulation of cell surface molecules that are involved in bacterial uptake by leukocytes (Panigrahi *et al.*, 2005).

Acid phosphatase an enzyme localized within lysosomes is important for intracellular digestion of phagocytized antigens and has been used as a marker of macrophages activation in animal modes including fish (Zeng-fu *et al.*, 2006). In the present study, it was observed that an increase in acid phosphatase activity when *O. niloticus* fed the probiotic supplemented diets which indicating that these probiotics could stimulate the immune response of the fish.

In the present study, the significant increase in lysozyme activity of fish fed different probiotics suggests an immune stimulation. Lysozyme, is an enzyme with antibacterial activity that can split peptidoglycan in bacterial cell walls particularly the gram positive species and it can cause lysis of the cells (Chipman and Sharon, 1969). Lysozyme occurs predominantly in fish mucus, serum and tissues rich in leucocytes (Ellis, 1999). Infections or invasion by foreign material could result in an increase in the lysozyme concentration in fish blood (Siwicki and Studnicka, 1987; Moyner *et al.*, 1993).

The total immunoglobulin results showed significantly higher values in fish fed with the different probiotics as compared to the control. This confirms

that the probiotic bacteria can stimulate the antibody production in fish (Panigrahi *et al.*, 2004). Serum immunoglobulins are major components of the humoral immune system which provide disease protection in animals and human beings (Watts *et al.*, 2001). Panigrahi *et al.* (2004) reported an increase in total serum immunoglobulin levels of rainbow trout, *Oncorhynchus mykiss* and the Indian major carp, *Labeo rohita*, fed diet containing *B. subtilis* (Nayak *et al.*, 2007). Yeast cells have been found to be effective in increasing the serum immunoglobulin IgM level of seabream, *Sparus aurata* by its supplementation to the diet as an immunostimulant (Cuesta *et al.*, 2004).

In this investigation, an improvement in several immunological parameters was obvious mainly due to the administration of *Saccharomyces cerevisiae* (NIOFSD019) to the diet. Yeasts are capable of specifically adhering to the intestinal mucus membrane of fish (Douglas, 1989; and Vázquez-Juárez *et al.*, 1997). *S. cerevisiae* contains various compounds such as β -glucans, nucleic acids and mannan oligosaccharides which are considered as immunostimulating compounds (Siwicki *et al.*, 1994; Anderson *et al.*, 1995; Ortuño *et al.*, 2002). It has been recognized that nucleic acids and β -1,3-glucans effectively enhanced immune functions of African catfish (Yoshida *et al.*, 1995), Atlantic salmon (Engstad *et al.*, 1992), rainbow trout (Jørgensen *et al.*, 1993; and Siwicki *et al.*, 1994) and shrimp *Penaeus monodon* (Thanardkit *et al.*, 2002).

The findings of the present study suggest a possible interaction between the two bacterial isolates (*B. subtilis* and *L. plantarum*) whereas the highest values for acid phosphatase activity, lysozyme activity and total immunoglobulin were recorded in fish fed D3. Few studies have been undertaken to test the effects of bacterial co-culture as a probiotic in fish. However, the combination of bacterial strains occupy different niches within the gut microflora environment may in turn have desirable effects on the host immune response and health of gilthead sea bream (Salinas *et al.*, 2005) and *O. niloticus* (Aly *et al.*, 2008).

In the second experimental trial, three doses (10^5 , 10^7 and 10^9 CFU/g) of *L. plantarum* were used. A higher percentage of phagocytic cells were observed in the probiotic fed groups particularly the group of fish fed with (10^7 CFU/g) bacterium dose. This could be indicating that an enhanced immune response that could protect against pathogenic microorganisms. In addition, the highest phagocytic activity was obtained with the dose of 10^7 CFU/g and not by the dose of 10^9 CFU/g. This is in agreement with the study of Panigrahi *et al.* (2004) that phagocytic activity of rainbow trout, *Oncorhynchus mykiss* fed with two doses (10^9 CFU/g and 10^{11} CFU/g) of *L. rhamnosus* were significantly higher than control. However, the authors concluded that low dose bacterium would be sufficient to elicit the phagocytic activity of the leukocytes.

The only significant increase in serum acid phosphatase activity was observed in fish fed with 10^9 CFU/g *L. plantarum* (NIOFSD018) that indicates the possible activation of this enzyme only with a high dose of this probiotic in

fish diet. Similar to present findings, acid phosphatase activity was activated in *Miichthys miiuy* only by supplementation with the highest dose (10^9 CFU/g) of *C. butyricum* (Zeng-fu *et al.*, 2006).

Lysozyme activity of *O. niloticus* fed the lower dose 10^7 CFU/g was found to be not significantly different from the value obtained by the higher dose 10^9 CFU/g. Contrary to this result, the serum lysozyme activity was significantly greater in rainbow trout, *O. mykiss* fed the higher level (10^{11} CFU/g) of probiotic bacteria *L. rhamnosus* (JCM 1136) compared with the control fish (Panigrahi *et al.*, 2004). This difference in determining the optimum dose may be due to the difference in *Lactobacillus* species or fish species or to the difference in experimental duration.

Immunoglobulin results showed an improvement in the total immunoglobulin level by increasing the dose of *L. plantarum* (NIOFSD018) in the diet up to 10^7 CFU/g after which the Ig level decreased. It is widely recognized that immunoglobulins provide disease protection to animals and humans. Some studies have also shown the effect of lactic acid bacteria in enhancing immunological factors such as Ig. In contrast to our results, the total immunoglobulin level in rainbow trout was not significantly increased by the addition of different doses of *L. rhamnosus* (JCM 1136) to the diet (Panigrahi *et al.*, 2004).

The results of the present study exhibited slight stimulation of phenoloxidase by increasing the dose of *L. plantarum* (NIOFSD018) in tilapia diet. However, in the trial of Zeng-fu *et al.* (2006), serum PO activity in *Miichthys miiuy* was significantly increased by an increase in the dose of *C. butyricum* added to the diet. Phenoloxidase (PO) is known to be an important enzyme in the defense mechanism of fish. Phenoloxidase activity is commonly used as an indicator of the immune response in shrimp and crab and the activity of this enzyme has been also detected in serum of fish, *Miichthys miiuy* (Zeng-fu *et al.*, 2006). Phenoloxidase usually exists as an inactive precursor (proPO) (Smith and Söderhäll, 1991; Asokan *et al.*, 1997), which can be readily activated to phenoloxidase (PO) by an endogenous activating system or exogenous elicitors such as trypsin, sodium dodecyl sulphate (SDS) (Asokan *et al.*, 1997) and laminarin (Leonard *et al.*, 1985). Phenoloxidase has been also found in neutrophils of *O. niloticus* (Witten *et al.*, 1998).

It is clear from the present results that mean values of phagocytic activity, acid phosphatase activity, lysozyme activity and total immunoglobulin levels increased to different degrees depending on the dose of *L. plantarum* (NIOFSD018) compared to the control group. Phenoloxidase activity was slightly increased with the increase in the dose of probiotics. However, by attaining a dose of *L. plantarum* (NIOFSD018) up to 10^9 CFU/g diet, a significant increase in acid phosphatase activity was observed. However, the other immunological parameters such as phagocytic activity and immunoglobulin level were significantly decreased by increasing the probiotic dose to 10^9 CFU/g. For lysozyme activity, no significant differences were detected

between fish fed either 10^7 or 10^9 CFU/g diet of this probiotic. This suggests that with the exception of acid phosphatase, administration of a dose of 10^7 CFU/g of *L. plantarum* (NIOFSD018) was sufficient to stimulate the immune system of tilapia and the higher dose of 10^9 CFU/g should be considered as an overdose of this probiotic.

In conclusion, the present study indicates that administration of probiotics in fish diet have stimulatory effects on the immune system of the fish and can lead to improving the health of *O. niloticus*. It can be recommended to incorporate these probiotics as dietary supplements in commercial feeds or in the form of bacterial biofilm to achieve better colonization in the digestive tract of the fish.

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