Effects of Some Feed Additives on Health and Growth of Nile tilapia "*Oreochromis niloticus*"

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ABSTRACT

Two hundred and forty Nile tilapia "*Oreochromis niloticus*" with average body weight 21.98 ± 0.26 g. were used to investigate the effect of some feed additives (*Pediococcus acidilactici* , *Bacillus subtilis natto*, *Saccharomyces cerevisiae*, mixture of Vitamin C and E and Oxytetracycline) on growth and immune responses as well as resistance to *Aeromonas hydrophila* infections. The results revealed that, final weights, weight gain, specific growth rate and Immunoglobulin levels were significantly higher ($P \leq 0.05$) in treated groups with *Saccharomyces cerevisiae*, *Bacillus subtilis natto* and vitamin mixture while control group and treated group with Oxytetracycline were significantly low. All treated groups received feed additives showed high phagocytic capacity and bactericidal activity. Low survivability was recorded after 7 days post-infection with *Aeromonas hydrophila* which was 20% in control group, 40% in group treated with Oxyteteracycline and 60% in group treated with *Pediococcus acidilactici*.

INTRODUCTION

The occurrence of diseases in fish farms is a consequence of several factors pertaining to the rearing methods and environmental condition variations. Consequently, cultivated fish can become more susceptible not only to pathogenic but also to opportunistic bacteria ***(1)***. *Aeromonas hydrophila* is a Gram negative, motile rod recorded as an opportunistic pathogen in a great variety of freshwater fish species, and can be considered to have widespread geographical distribution ***(2)***. It is one of the major disease causing pathogens responsible for a variety of fish pathological conditions, collectively named as "aeromonosis" ***(3)***. In addition, they are also responsible for enteric or, less often, extra enteric infections ***(4)***. Aquaculture industry suffers major economic losses due to this infection ***(5)***. The use of antibiotics and chemotherapeutics for prophylaxis and treatment in intensive aquaculture has been widely criticized for its negative impact ***(6)***. Research on interactions between immunity, growth and development of eco-friendly alternatives to antibiotics that may keep fish healthy such as probiotics and plant - based immunostimulants has increased ***(7)***.

The common probiotics used in aquaculture belong to Lactobacillus sp., Bacillus sp., Bifidobacterium sp., Vibrio sp., Saccharomyces sp. and Enterococcus sp. ***(8)***. Lactic acid bacteria (LAB), as a dietary supplement have been widely employed to protect fish from various infections ***(9)***. Yeast could be an appropriate organism because some strains synthesize and secrete different polyamines molecules ***(10)***, and they have strong adhesion potential to intestinal mucus in fish ***(11, 12)***. Besides polyamine production, yeast may improve fish health as antagonists to pathogens and by immunostimulation ***(13)***. These multiple beneficial effects could make yeast promising probiotics.

Vitamins are among the most important nutrients that influence the immune system. Vitamin C supplementation in diets for aquatic organisms prevented the negative effects of stress, stimulated wound healing, minimized toxicity by water contaminants, and increased immune response ***(14 - 16)***.

Therefore, the present investigation was carried out to study the effect of some feed additives on the growth, immune response and resistance of *Oreochromis niloticus "O. niloticus"* to *Aeromonas hydrophila "A. hydrophila"* challenge as a major causing of pathogens.

MATERIAL AND METHODS

1. Experimental fish

The present work was performed at Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Zagazig University, Egypt. Two hundred and forty *O. niloticus* (average initial body weight 21.98 ± 0.26 g.) were collected from Abbassa Fish Hatchery at Sharkia province, Egypt. The fish were distributed amongst 200 liter glass aquaria (each of 100 cm length X 40 cm width X 60 cm height capacity). Each was provided with dechlorinated water, aerator (Azaro, 4000 sw. Japan), and thermostatically controlled "heater and thermometer". Approximately 30% of the water was changed daily. Water quality criteria ranging from 26 to 27 °C, 6.5 to 7, 6 to 6.5 mg l^{-1}, 0.01 and 0.20 mgl^{-1} for temperature, pH, dissolved oxygen , ammonia and nitrite respectively.

2. Diets and feed additives

2.1. Basal diet

Ingredient and chemical composition of the basal diets used in the experiment were carried out *(18)* "the vitamin and mineral mixture used did not contain vitamins C and E".

2.2. Feed additives

- **Bactocell** (EGAVET, Giza, Egypt), each 1gm contains 1 x 10^9 CFU (colony forming unit) *Pediococcus acidilactici* was added to the basal diet at a rate of 1 g. kg diet $^{-1}$.
- **Biogen. S** (Samu median Co. LTD. 254- 15 Dugok- Ri SINAM-MYEON YESAN-GUN. CHUNGCHEONGNAM-DO, KOREA), each 1gm contains 1 x 10^{11} CFU *Bacillus subtilis natto* was added to the basal diet at a rate of 0.2 g. kg diet $^{-1}$.
- **Yeast max** (National Development Company NADEC, 10th of Ramadan, third industrial area, Egypt – Italy Group, Egypt), each 1 gm contains 0.25 gm *Saccharomyces cerevisiae* was added to the basal diet at a rate of 1 g. kg diet $^{-1}$.
- **Vitamins (vitamin C and vitamin E)** (ROCHE Company, France) were added to the basal diet at a rate of 400 mg Vitamin C kg diet $^{-1}$ and 300 mg vitamin E kg diet $^{-1}$.
- **Oxytetracycline 20 %** Unipharma (Universal Industrial Pharmaceutical Co. El obour City, Cairo, Egypt) was added to the basal diet at a rate of 0.6 g. kg diet $^{-1}$. The diets were dried at room temperature then transferred to plastic bags and stored at -4 °C and this preparation was repeated every two weeks.

3. Experimental design

A total of six groups were used i.e, control group (G_1) fed a basal diet and another five groups fed basal diet with various feed additives. *Pediococcus acidilactici* (G_2), *Bacillus subtilis natto* (G_3), *Saccharomyces cerevisiae* (G_4), mixture of Vitamin C and E (G_5) and Oxytetracycline (G_6). Each group had 40 *O. niloticus* with two replicates (20 fish replicate^{-1}).

The fish were adapted to experimental condition for two weeks before the start of the experiment. The actual experimental period was 2 months. All fish were fed their respective diets at a level of 3% of body weight and were fed four times daily. The weight of all fish in each aquarium was obtained biweekly and the amount of daily diet was adjusted accordingly.

4. Sampling and analytical methods

4.1.Growth performance: Fish of all replicate were counted and weighted individually after 2, 4, 6 and 8 weeks of the experiment and the growth parameters were calculated as follows: Weight gain = $W_2 - W_1$

Specific growth rate (SGR) = 100 (In $W_2 - W_1$)/ T *(19)*. Where W_1 and W_2 are the initial and final weight respectively and T is the number of days of the feeding period.

4.2.Immunological parameters: Blood samples were collected at the end of the experimental period from the caudal vein of ten fish from each treatment group (five from each replicate) using sterile syringes with EDTA. Meanwhile, another blood samples were withdrawn into Eppendorf

tubes and centrifuged at 3000 r.p.m. for 15 minutes for serum separation.

4.2.1.Humoral immunity: The serum protein concentration was estimated ***(20)*** while albumin content was estimated spectrophotometrically using a standard kit (Glaxo, India). The globulin content was estimated by subtracting the albumin content from total protein content. The actual immunoglobulin concentration in the total globulins in each experimental group including the control group were analyzed ***(21).***

4.2.2.Cellular immunity: To measure the cellular immunity represented in phagocytic capacity and/or bactericidal activity, white blood cells was separated from peripheral blood of the tested fish in the different experimental groups ***(22).*** Heat-inactivated *Candida albicans (C. albicans)* was used to determine the phagocytic capacity of the phagocytic cells in each experimental group. The phagocytic capacity of the phagocytic cells was assessed ***(22).*** An intracellular survival assay was used to measure the bactericidal activity of the WBCs of each experimental group ***(23).***

5. Challenge infection: Ten fish from each treatment groups (five from each replicate) were collected and reared in glass aquaria. They were clinically and bacteriologically examined to be free from bacterial infection. *A. hydrophila* was isolated previously from liver of morbid *O. niloticus* and studied for pathogencity in the Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Zagazig University, Egypt. A culture suspension of *A. hydrophila* was then prepared by culturing on agar for 24 hrs, collected, washed and suspended in sterile saline 0.85% and counted by McFirland standard tubes. Fish were challenged by intra -peritoneal (I/P) injection with 0.2 ml ($3x10^7$ CFU ml^{-1}) of *A. hydrophila*. The challenged fish were kept under observation for 7 days and the diseased and dead fish were used for bacterial reisolation. The mortalities and clinical findings were recorded.

6. Statistical analysis

Analyses of variance (ANOVA) and Duncan's multiple range test ***(24)*** were used to determine the differences between treatments. The mean values were significant at the level of $P \leq 0.05$. Standard errors of treatment – means, were estimated. All the statistical analyses were carried out ***(25).***

RESULTS AND DISCUSSION

Growth performance

The growth performance of *O. niloticus* is shown in Table 1 which revealed that final weights, weight gain and SGR were significantly higher ($P \leq 0.05$) in treated groups with *Saccharomyces cerevisiae* (G_4), Vitamin mixture (G_5) and *Bacillus subtilis natto* (G_3) compared to the control (G_1) and Oxytetracycline (G_6) treatments. Similar results were previously recorded ***(26)*** which suggested that the addition of probiotics mitigated the effects of the stress factors. They also improved the nutrition by detoxifying the potentially harmful compounds in feeds, by denaturing the potentially indigestible components in the diet through the action of hydrolytic enzymes (amylases and proteases) and by producing vitamins such as biotin and vitamin B12 ***(27, 28).*** Also, probiotics influence digestive processes by enhancing the population of beneficial microorganisms, microbial enzyme activity; improving the intestinal microbial balance, consequently improving the digestibility and absorption of food and feed utilization ***(29).*** L-ascorbic acid is an essential vitamin for normal growth and physiological function of fish. It functions as a general water-soluble redox reagent, on collagen formation ***(30)***, iron metabolism and hematology ***(31)*** and stress ***(32).***

Immunological responses

The results of immunological parameters analyzed showed a significant increase in concentrations of total globulins in all treated groups compared to the control group (Table, 2). Immunoglobulins were significantly higher in groups treated with *Bacillus subtilis natto* (G_3), vitamin mixture (G_5) and *Saccharomyces cerevisiae* (G_4). All groups that received feed

additives showed high phagocytic capacity compared to the control group. The cell capacity to engulf 6 yeast cell or more was higher in groups treated with *Bacillus subtilis natto* (G_3), vitamin mixture (G_5) and *Saccharomyces cerevisiae* (G_4) compared to fish groups treated with Oxytetracycline (G_6) and *Pediococcus acidilactici* (G_2). On the other hand, the bactericidal activity represented in CFU/ml was significantly higher in control group than in all treated groups (Table, 3).

The use of immunostimulants can induce protection against diseases, enhancing activities in the nonspecific defense mechanism ***(33).*** The use of *Bacillus* sp. (strain S11) provided disease protection by activating both cellular and humoral immune defenses in tiger shrimp (*P. monodon*) ***(34).*** The bactericidal activity of serum increased gradually as the quantity of *B. subtilis* was increased in the diet ***(8).***

Yeast could be an appropriate organism because some strains synthesize and secrete different polyamines molecules ***(10)***, and they have strong adhesion potential to intestinal mucus in fish ***(11, 12)***. Besides polyamine production, yeast may improve fish health as antagonists to pathogens and by immunostimulation ***(13).*** These multiple beneficial effects could make yeast promising probiotics.

Supplementation of vitamin C enhances antibody production against *Edwarsiella ictaluri* in channel catfish ***(14)*** and also enhances phagocytic activity and serum lysozyme levels in turbot ***(35).*** Vitamin E is another nutrient related with immune system function which acts as an antioxidant in biological membranes.

Resistance to *A. hydrophila* infection

The survivability observed after challenge with *A. hydrophila* was significantly higher in treated groups with *Bacillus subtilis natto* (G_3), *Saccharomyces cerevisiae* (G_4) and vitamins mixture (G_5) compared to G_1 (control) and G_6 treated with Oxytetracycline (Table, 4). This may be attributed to probiotics actively inhibiting the colonization of potential pathogens in the digestive tract by antibiosis or by competition for nutrients and space, and alteration of the microbial metabolism ***(36 - 38)***. Vitamin C supplemented diets exhibited decreased mortality rates when challenged with *A. hydrophila* compared to fish fed a vitamin C non-supplemented diet. The disease susceptibility of an organism depends on its innate and acquired resistance mechanisms and also on the ability of the pathogen to establish and proliferate ***(39).*** The numbers of oxytetracycline- resistant bacteria are usually higher in fish farms undergoing antimicrobial therapy because susceptible microorganisms are inhibited, thus allowing colonization by a resistant microflora ***(40).***

The dead challenged *O. niloticus* developed typical clinical signs and pathological condition such as loss of scales, external ulceration at site of injection, fin rot and hemorrhage at pectoral fins after 7 days post- challenge. Internally, the affected fish showed severe congestion in most internal organs. This may be attributed to the virulence factors expressed by this bacterium include hemolysins, proteases, cholinesterases, enterotoxins and adhesins ***(41, 42)*** all of which contribute to overall development of the disease in fish.

It is concluded that, the administration of feed additives especially Biogen "*Bacillus subtilis natto*", Vitamin mixture "vitamin C and E" and Yeast Max "*Saccharomyces cerevisiae*" play an important role in increasing growth, immunity and disease resistance of *O. niloticus*.

Table 1. Effect of different feed additives on growth performance of *O. niloticus*

Group	Treatment	Initial weight (g.)	Final weight (g.)	Weight gain (g)	SGR* (%)
G_1	Control	22.15 ± 0.60 ^{a}	70.14 ± 0.55 ^{e}	9.43 ± 0.55 ^{c}	1.06 ± 0.05 ^{c}
G_2	*Pediococcus acidilactici* (1 g. kg diet $^{-1}$)	22.36 ± 0.72 ^{a}	85.63 ± 1.43 ^{d}	20.62 ± 1.47 ^{b}	1.99 ± 0.12 ^{ab}
G_3	*Bacillus subtilis natto* (0.2 g. kg diet $^{-1}$)	21.63 ± 0.81 ^{a}	103.87 ± 2.08 ^{b}	21.89 ± 2.08 ^{b}	1.73 ± 0.14 ^{b}
G_4	*Saccharomyces cerevisiae* (1 g. kg diet $^{-1}$)	21.73 ± 0.80 ^{a}	121.73 ± 3.18 ^{a}	32.48 ± 3.1 ^{a}	2.27 ± 0.19 ^{a}
G_5	Vitamin C (400 mg kg diet $^{-1}$) Vitamin E (300 mg kg diet $^{-1}$)	22.28 ± 0.61 ^{a}	96.02 ± 0.64 ^{c}	24.22 ± 0.64 ^{b}	2.10 ± 0.05 ^{a}
G_6	Oxytetracycline (0.6 g. kg diet $^{-1}$)	21.71± 0.54 ^{a}	72.39 ± 0.90 ^{e}	10.95 ± 0.90 ^{c}	1.22 ± 0.08 ^{c}

Mean ± SE having the same letters in the same columns are not significantly different at P≤ 0.05.
*SGR= Specific growth rate.

Table 2. Effect of different feed additives on some humoral immunological parameters of *O. niloticus*

Group	Treatment	Total proteins g/dl	Total albumin g/dl	Total Globulin g/dl	Immunoglobulins mg/ml
G_1	Control	6.58 ± 0.04 ^{d}	3.74 ± 0.008 ^{a}	2.83 ± 0.04 ^{d}	4.04 ± 0.02 ^{f}
G_2	*Pediococcus acidilactici* (1 g. kg diet $^{-1}$)	6.73 ± 0.02 ^{b}	3.45 ± 0.02 ^{b}	3.28 ± 0.04 ^{b}	8.79 ± 0.05 ^{d}
G_3	*Bacillus subtilis natto* (0.2 g. kg diet $^{-1}$)	6.85 ± 0.02 ^{a}	2.66 ± 0.01^{c}	4.19 ± 0.04 ^{a}	12.11 ± 0.06 ^{a}
G_4	*Saccharomyces cerevisiae* (1 g. kg diet $^{-1}$)	6.82 ± 0.01 ^{a}	2.64 ± 0.02 ^{c}	4.17 ± 0.01 ^{a}	11.06 ± 0.02 ^{c}
G_5	Vitamin C (400 mg kg diet $^{-1}$) Vitamin E (300 mg kg diet $^{-1}$)	6.72 ± 0.008 ^{bc}	2.56 ± 0.02 ^{d}	4.16 ± 0.02 ^{a}	11.56 ± 0.02 ^{b}
G_6	Oxytetracycline (0.6 g. kg diet $^{-1}$)	6.64 ± 0.02 ^{cd}	3.48 ± 0.02 ^{b}	3.16 ± 0.02 ^{c}	7.10 ± 0.07 ^{e}

Mean ± SE having the same letters in the same columns are not significantly different at P≤ 0.05.

Table 3. Effect of different feed additives on bactericidal activity of *O. niloticus*

Group	Treatment	Colony Forming Unit (CFU)
G_1	Control	$51.0 \times 10^{6\ a}$
G_2	*Pediococcus acidilactici* (1 g. kg diet $^{-1}$)	$0.76 \times 10^{6\ b}$
G_3	*Bacillus subtilis natto* (0.2 g. kg diet $^{-1}$)	$0.0030 \times 10^{6\ b}$
G_4	*Saccharomyces cerevisiae* (1 g. kg diet $^{-1}$)	$0.0044 \times 10^{6\ b}$
G_5	Vitamin C (400 mg kg diet $^{-1}$) Vitamin E (300 mg kg diet $^{-1}$)	$0.0038 \times 10^{6\ b}$
G_6	Oxytetracycline (0.6 g. kg diet $^{-1}$)	$0.73 \times 10^{6\ b}$

Mean ± SE having the same letters in the same column are not significantly different at $P \leq 0.05$.

Table 4. Survivability of *O. niloticus* treated with different feed additives and challenged with *A. hydrophila* after two months.

Group	Treatment	Survival (%)
G_1	Control	$20^{\ d}$
G_2	*Pediococcus acidilactici* (1 g. kg diet $^{-1}$)	$60^{\ b}$
G_3	*Bacillus subtilis natto* (0.2 g. kg diet $^{-1}$)	$100^{\ a}$
G_4	*Saccharomyces cerevisiae* (1 g. kg diet $^{-1}$)	$100^{\ a}$
G_5	Vitamin C (400 mg kg diet $^{-1}$) Vitamin E (300 mg kg diet $^{-1}$)	$100^{\ a}$
G_6	Oxytetracycline (0.6 g. kg diet $^{-1}$)	$40^{\ c}$

Mean ± SE having the same letters in the same column are not significantly different at $P \leq 0.05$

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الملخص العربى

تأثير بعض إضافات الأعلاف على صحة ونمو البلطى النيلى

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قسم أمراض ورعاية الأسماك و *قسم البكتريولوجيا والفطريات والمناعة

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استخدم عدد ٢٤٠ سمكة بلطي نيلي بمتوسط وزن ٠,٢٦ ± ٢١,٩٨ جرام لبحث تاثير مكملات غذائية مختلفة (الساكاروميسيس و الباسيليس ساتليس و مخلوط الفيتامينات والاوكسي تيتراسيكلين و بالبيديوكوكس) علي النمو والاستجابة المناعية وكذلك المقاومة لعدوي الايروموناس هيدروفيلا. اوضحت النتائج ان هناك زيادة ملحوظة في الوزن النهائي و عائد الوزن و معدل النمو النوعي و الجلوبيولين في المجموعات التي تم معاملتها بالساكاروميسيس و الباسيليس ساتليس و مخلوط الفيتامينات. كما اظهرت كل المجموعات التي تم معاملتها بمكملات غذائية مختلفة أعلي معدلات للبلعمة و النشاط القاتل للبكتيريا بينما وجد ان اقل مقاومة (٢٠ %) لعدوي الايروموناس هيدروفيلا في المجموعات الضابطة و ٤٠% في المجموعة المعاملة بالاوكسي تيتراسيكلين و ٦٠% في المجموعة المعاملة بالبيديوكوكس.