

THE IMMUNOSTIMULANT EFFECT OF *Saccharomyces cerevisiae* AND THE IMPACT OF *Fusarium solani* INFECTION ON *Oreochromis niloticus*

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Abstract: This study summarizes the impact of *Fusarium solani* on *Oreochromis niloticus* with evaluation of the efficacy of *Saccharomyces cerevisiae* for decreasing its possible consequences on fish health was investigated. Fifty samples of fish ration were collected for isolation and identification of *Fusarium solani*. One hundred and sixty *O. niloticus* clinically healthy, average weight of 59.25±1.04 g was distributed into four groups. First group was a control negative group (C). Second group (F) was infected with *Fusarium solani* (1x 10⁴ zoospores of *Fusarium* /ml). Third group (F+S) was fed a basal diet containing *S. cerevisiae* 1 g/ kg ration daily and injected intraperitoneally with *Fusarium solani*. Fourth group (S) was fed a basal diet containing (*S. cerevisiae*) daily. The experiment extended for five weeks then all groups were challenged with *Pseudomonas aeruginosa* (3x 10⁷ CFU/ml). Bacteriological findings of the intestinal microbiota revealed significant increase (log¹⁰ CFU/ml) in total bacterial count (TBC) in the (F+S) group (4.51±0.012) and decreased gradually from the 1st week to the 5th week, while the (S) group showed slight increase in TBC than (C) group (P<0.05). The haemato-immunological parameters after 3 weeks in the (S) group showed significant increase in serum lysozyme activity and total leukocytic count. After five weeks there were a marked decrease in serum lysozyme activity and total leukocytic count in (F) group. After the challenge, there was a significant decrease in serum lysozyme activity and total leukocytic count in (F) group. Lymphocyte count and gamma globulin were significantly higher in (S) group than group (C) (P<0.05). The Pathological lesions in gills, liver, muscles in (F) group showed sever degenerative changes, necrosis and leucocytic infiltrations. The lesions in group (F+S) were milder. It could concluded that *S. cerevisiae* can be used in ration of *O. niloticus* under immune-depressive stressful condition (fungal infection) to increase their resistance.

Key words: *Fusarium solani*; *Oreochromis niloticus*; *Saccharomyces cerevisiae*; immunostimulants

Introduction

Oreochromis niloticus are the commonest cultured fish worldwide due to its high protein level, excellent palatability, and rapid growth rate (1). *Fusarium solani* is a common mold fungus that contaminates grains such as corn and wheat, where they may produce mycotoxins that are harmful to the productivity and health of different animals, such as swine, poultry, and fish (2-3). The most toxicologically important *Fusarium* mycotoxins are *trichothecenes* including *deoxynivalenol* and T-2 toxin, *zearalenone* and

fumonisin B1 (4). Ingestion of low to moderate amounts of *Fusarium* mycotoxins may impair intestinal health, immune function and/or virulence of pathogen (5). The pathological changes caused by fungal infection, usually includes granuloma formation with fungal structures and focal hepatic cellular degenerations which replaced by hyphal-like structures (6). The microbiota of fish is usually a reflection of their surrounding environment which may be the source of the microorganisms as a result for the indiscriminate waste disposal into natural

water (7). There is appreciable evidence in the importance of host microbiota to fish immunity and metabolism (8). A variety of humoral components, such as transferrin, interferon, inhibitory proteins, lysozymes together with antiproteases, type C lectins, pentraxins, natural antibodies, cytokines and chemokine's can be found in the plasma, mucus and other body fluids (9). In addition to these components, fish possess leukocytes such as macrophages, eosinophils, and others polymorphonuclear leukocytes (10). Fish also produce the immunoglobulin with a predominant class (IgM) (11). Recently, there was increasing attention towards dietary administration of functional feed additives that include probiotics, prebiotics and symbiotic for the elevation of digestive enzyme activity and nutrient digestibility (12). Probiotics were known as microbial dietary supplement that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving the nutritional and microbial balance in the intestinal tract. *Saccharomyces cerevisiae* (yeast) has growth promoter effects, immune-stimulant properties and increased protection offered of fish against pathogen infection (13). These properties might be due to the proteins present in the yeast cell wall, which composed entirely of β -glucans, *mannoproteins* and *chitin*. Both β -glucans and chitins have been noticed as powerful immunostimulants for fish and play an important role by activation of the intestinal microbiota of fish (14).

This study was designed to (i) study the pathogenicity of *Fusarium* infection in *O. niloticus* and its effect on the intestinal microbiota, health status, immune system, and pathological changes, (ii) evaluate the immunostimulant properties of *S. cerevisiae* administered by diet to reduce the drastic effects of *Fusarium solani* infection.

Material and methods

Fish Feed Samples

Fifty samples of fish ration were collected from different fish farms, Sharkia Governorate, Egypt. Each sample was thoroughly ground and mixed then diluted. The samples after dilution were cultured into Sabouraud's dextrose agar medium (SDA), Czapek's media and potato dextrose agar supplemented with 0.05 mg/L chloramphenicol (15). Then incubated at 25-28 °C for 7-10 days un-

der aseptic condition and examined daily. Post inoculation, the spores were stained with lactophenol cotton blue and examined microscopically.

Fungal Identification

The fungal isolates were identified according to macro-and micromorphological characters including colony color and pigmentations on Potato Dextrose agar (PDA) media, type of mycelium, shape and septation of micro and macroconidia (16, 17, 18).

Preparation of Spores Suspension

Fungal strains of *Fusarium solani* cultured on SDA at 25°C for 7 days, and then conidial mass was harvested by adding 20 ml sterile distilled water into each culture plate, followed by collection of the suspension in 30 ml. sterile autoclavable tubes. Suspensions were filtered through two layers of sterile medical gauze to ensure that filtrate contain fungal, concentrations of which were calculated using hemocytometer (erythrocyte counting chamber) and adjusted to 1×10^4 conidia ml in sterile distilled water.

Diet

Fish were conditioned for 15 days and fed on standard commercially pelletized fish ration as a basal diet contained 32.16% protein (Table 1) until the experiment began. Star yeast (ICC Incl. Com. Exp. Elmp. Ltda. Barazil, Belgium, USA) as a commercial feed additive was prepared (one kg contains *Saccharomyces cerevisiae* "in active dried yeast" 950 g, Beta Glucan 190 g, Mannanligosaccharides 142.5 g and a carrier "soybean meal"). The recommended dose is 1gm of star yeast/kg feed stuff. Experiment was carried out in Fish Research Center, Faculty of Veterinary medicine, Zagazig University, Egypt.

Fish and experimental design

A total of 170 healthy *O. niloticus* with an average weight 59.25 ± 1.04 g. were obtained from private fish farms in Abbassa, Abohammad, Sharkia Governorate, Egypt. The fish were kept in concrete bond (3x1x1 m) for two weeks to be acclimatized before the start of the experiment. Ten fish were randomly selected for bacteriological and parasitological examinations to ensure that they were free from natural infection. A total of 160 healthy *O. niloticus* were randomly and equally distributed into four groups each one contained 40 fish (20 fish/replicate) and treated as follow:

Table 1: Ingredients and chemical composition of the basal diet

Ingredients	Percentage %
Yellow corn	31
Wheat flour	10
Soybean (44%)	22
Fish meal (60%)	16
Poultry by-products	14
Vegetable oil	5.5
Vitamins and mineral mixture*	1.5
Total	100
Crude protein	32.16

*Vitamins and mineral mixture (Amcozink): Each kg contains 2000000 I.U vit. A, 2000000 I.U vit D3, 10 gm zinc bacitracin and calcium carbonate up to 1000 gm

First group (C) group: Fish were fed on a basal diet without any additives as control negative group for 5 weeks. Second group (F) group: fish were fed on a basal diet without any additives and each fish was injected intraperitoneally with 0.1 ml of saline contained (1×10^4 zoospores of *fusarium solani* /ml). Third group (F+S) group: Fish were fed on a basal diet containing *Saccharomyces cerevisiae* as 1gm/ kg diet and injected intraperitoneally with 0.1 ml of saline contained (1×10^4 zoospores of *fusarium solani* /ml). Fourth group (S) group: Fish were fed on a basal diet containing *Saccharomyces cerevisiae* as 1g/kg diet. The study was approved by the Committee of Animal Welfare and Research Ethics, Faculty of Veterinary.

Clinical signs, postmortem (PM) examination and mortality rate

Infected fish were clinically examined for identifying clinical signs. Freshly dead fish specimens were examined for PM lesion (19).

Sampling and total bacterial count (TBC)

Gut of 6 fish from each group after 1st, 3rd and 5th weeks from the beginning of the experiment were dissected out aseptically and placed in sterilized separate plastic petri dishes. After pooling and weighing, the samples were homogenized and suspended in sterile physiological saline (1 part of sample: 9 parts of PS) to obtain a stock solution and two consecutive decimal dilutions, from the stock solution were made. The different dilutions were cultured

onto trypticase soy agar (TSA) plates (Oxoid, England). All such plates were incubated at 37 °C for 48 hours. The growing colonies were counted, and their numbers were used to interpret the effect of *fusarium* infection and yeast treatment on gut flora (20).

Haemato-immunological examination

Blood samples were taken 1st, 3rd, 5th weeks after the beginning of the experiment and 2 weeks after challenge as 6 fish (3/ replicate) were randomly taken and anaesthetized using benzocaine 50 mg/L. Two portions of blood samples were taken from the caudal vein, the first portion was taken with syringe contained EDTA as anticoagulant for total and differential leukocytic count (21). In case of the 2nd portion, blood samples were taken without anticoagulant for separation of serum that used in determination of serum lysozyme activity (22) and for total serum protein and electrophoretic pattern analysis (23, 24) for determination of gamma globulins.

Histopathological examination

Specimens from organs of five fish per aquarium were randomly collected and fixed with neutral buffered formalin then they were processed by routine methods and embedded in paraffin wax. Sections (5 µm) were mounted on glass microscopic slides, stained with hematoxylin and eosin (H&E), covered, and examined microscopically (25).

Challenge test

After adding diet containing *S. cerevisiae* as immunostimulant for 5 weeks and experimental infection with *fusarium* species then fish were challenged with *Pseudomonas aeruginosa*. 20 fish from each group (10/replicate) were transferred to glass aquaria, and then inoculated with 0.1 ml 3×10^4 CFU/ml of *P. aeruginosa* bacterial solution intraperitoneally (26). The clinical signs, post-mortem lesions, and mortalities were recorded daily for up 2 weeks (experimental period). The experimentally infected fish were examined for the re-isolation of *Fusarium solani* and *P. aeruginosa* from external and internal organ. Positive culture was confirmed by conventional bacteriological techniques (27).

Statistical analysis

Data were statistically analyzed using SPSS program version 23. The two-way ANOVA test was performed for comparison between studied factors (*i.e.*, groups and time post infection). The ANOVA followed by post hoc test using Duncan multiple range test for comparisons between means of groups. The means followed by the same letter in each column are not significantly different from each other at the 5-percent probability level ($P < 0.05$) (28)

Results

Isolation and identification of fungi from fish feedstuff

The mycological analysis of examined fish feedstuffs demonstrated presence of five genera of fungi. The *Fusarium* spp. was the most predominant mold (80%), followed by *Penicillium* spp. (70%), and *Aspergillus* spp (60%), *Mucor* spp. (40%), and then *Rhizopus* spp. (25%). Percentages had been calculated in relation to the total number of examined samples (50 samples).

Morphological criteria of *Fusarium solani*

Colony grow rapidly on PDA with dense aerial white to cream mycelium in concentric rings, abundant, floccose, somewhat powdery in aged cultures which reverse to brownish color, showing average growth rate per day at 25°C. Micro-

conidia usually abundant, ellipsoidal, and fusiform or kidney shaped after 2-3 days in fresh isolates. Macroconidia were abundantly developed after 4-7 days with thick walled having 3 to 4 septa, straight, parallel sided for most of length. The apical cell blunt had rounded end.

Clinical signs and postmortem examinations

O. niloticus infected with *Fusarium solani* became darker with abnormal swimming pattern, haemorrhage of skin and lateral fins, erosion of tail, ulceration of skin and loss of some scales (Figure 1). Congestion in liver with enlargement of gall bladder were noticed. Moreover, the gills were severely affected appeared darker red and covered with excessive amount of muocus. The clinical signs of treated group (F+S) were more milder picture than the group (F). The fish in group (S) were healthy and active.

Mortality rates

As illustrated in Figure (2) the group fed diet containing *S. cerevisiae* (S) group recorded the lowest mortality rate. The percentage of mortality of group that fed on diet containing *S. cerevisiae* and infected with *Fusarium solani* (F+S) group was lower than the group that infected with *Fusarium solani* (F) group. After the challenge with *P. aeruginosa* infection, the mortality rates in (C) (30%) and (S) groups (24%) were less than (F) (92%) and (F+S) (55%) groups.



Figure 1: The clinical sign of infected fish with *Fusarium* spp. showed haemorrhage of skin and lateral fins, erosion of tail, ulceration of skin

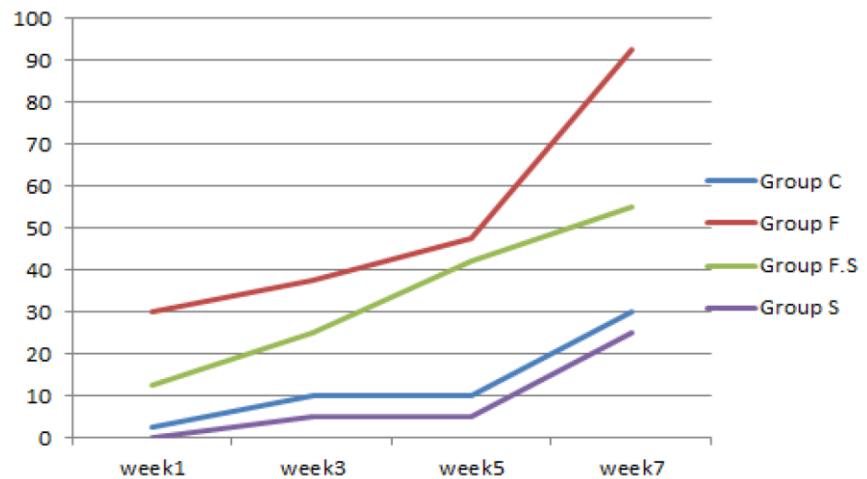


Figure 2: The mortality rate of *O. niloticus* 1st, 3rd, 5th weeks from the feeding trail and at 7th weeks post infection

Total bacterial count (TBC) in gut of fish

The (F) group which infected with *Fusarium solani* showed high TBC, that increased gradually from a mean value of (4.37 ± 0.01) CFU/ml after the 1st week to be (4.44 ± 0.01) CFU/ml after the 5th week. In the (F+S) group, the mean value of TBC decreased gradually from (4.48 ± 0.01) CFU/ml in the 1st week to reach (4.47 ± 0.01) CFU/ml at the 5th week. The total bacterial count of the examined samples in (S) group, fed on a diet supplemented with *S. cerevisiae*, showed slight increase in TBC with mean value of (4.31 ± 0) CFU/ml than the control (C)

group after 5th week of the experiment. Analysis of our data to study the group effect revealed that (F+S) group was the highest in TBC followed by (F) group, then (S) group when compared with control (Table 2).

Haemato-immunological Findings

After one week of the experiment all groups showed a significant increase in serum lysozyme activity, total leukocytic count, lymphocyte and monocyte count than control group with respect to (S) group that was the highest among of them (Table 3) ($P < 0.05$).

Table 2: Mean values of total bacterial count (\log_{10} CFU/ml) of the examined gut samples from different groups

Groups	Time	Total bacterial count (CFU/ml)			Group effect
		1 st week	3 rd week	5 th week	
C	Minimum	4.00	4.21	4.24	4.223 ± 0.02^d
	Maximum	4.28	4.26	4.30	
	Mean \pm SE	4.17 ± 0.05^f	4.24 ± 0.01^e	4.27 ± 0.01^{de}	
F	Minimum	4.34	4.34	4.38	4.397 ± 0.01^b
	Maximum	4.40	4.42	4.47	
	Mean \pm SE	4.37 ± 0.01^c	4.38 ± 0.01^c	4.44 ± 0.01^b	
F+S	Minimum	4.43	4.49	4.42	4.484 ± 0.01^a
	Maximum	4.50	4.54	4.50	
	Mean \pm SE	4.48 ± 0.01^{ab}	4.51 ± 0.01^a	4.47 ± 0.01^{ab}	
S	Minimum	4.27	4.28	4.29	4.296 ± 0.00^c
	Maximum	4.31	4.32	4.32	
	Mean \pm SE	4.29 ± 0.01^{de}	4.3 ± 0.01^d	4.31 ± 0^d	
Time effect		4.325 ± 0.03^b	4.358 ± 0.02^a	4.368 ± 0.02^a	

a, b, c, d, e, f Different superscripts indicate significant differences ($P < 0.05$). C: control negative group. F: fish infected with *fuzarium* spp not treated. F+S: fish infected with *fuzarium solani* and feeding on diet containing *S. cervesai*. S: Feeding on *S. cervesai* and not infected

Table 3: Haemato-immunological parameters of *O. niloticus* 1st, 3rd and 5th weeks after injection of *Fusarium* species and treatment with *S. Cerveisiae*

Study factors	Lysozym activity (µg/ml)	WBCs (x10 ³ /ml)	Lymphocytes (%)	Monocytes (%)	Heterophil (%)	Eosinophil (%)	
C	1 st week	357.387±22.83 ^b	17.000±1.15 ^{de}	79.000±1.15 ^e	1.333±0.33 ^d	2.667±0.67 ^{de}	1.667±0.67 ^a
	3 rd week	357.387±22.83 ^b	14.100±0.75 ^e	82.667±2.4 ^{cde}	1.000±0.00 ^d	2.333±0.33 ^e	2.000±0.58 ^a
	5 th week	357.387±22.83 ^b	17.667±1.2 ^{de}	83.000±2.31 ^{cde}	1.667±0.33 ^d	3.333±0.67 ^{cde}	1.667±0.33 ^a
F	1 st week	478.093±43.33 ^a	30.333±2.4 ^b	85.667±0.67 ^{bcd}	6.000±0.58 ^b	4.333±0.67 ^{bcd}	1.333±0.33 ^a
	3 rd week	302.933±18.26 ^{bc}	14.400±2.36 ^e	86.667±2.03 ^{abc}	2.333±0.33 ^{cd}	7.000±1.15 ^a	1.667±0.67 ^a
	5 th week	242.620±16.55 ^c	14.333±2.4 ^e	82.667±1.76 ^{cde}	2.000±0.58 ^d	2.333±0.33 ^e	1.333±0.33 ^a
F+S	1 st week	478.093±43.33 ^a	39.333±2.96 ^a	86.000±0.58 ^{abcd}	7.333±0.88 ^{ab}	5.000±0.58 ^{bc}	1.333±0.33 ^a
	3 rd week	368.800±19.77 ^b	16.933±1.05 ^{de}	91.667±1.45 ^a	2.333±0.33 ^{cd}	3.633±0.32 ^{cde}	1.000±0 ^a
	5 th week	368.800±19.77 ^b	23.333±2.73 ^{cd}	80.333±2.6 ^{de}	4.000±0.58 ^c	3.000±0.58 ^{cde}	1.333±0.33 ^a
S	1 st week	514.637±22.24 ^a	40.667±3.48 ^a	85.333±1.45 ^{bcd}	7.667±0.67 ^a	4.000±0.58 ^{cde}	2.000±0.58 ^a
	3 rd week	478.093±43.33 ^a	25.433±1.44 ^{bc}	90.667±0.67 ^{ab}	2.333±0.33 ^{cd}	4.667±0.33 ^{bcd}	2.000±0.58 ^a
	5 th week	368.800±19.77 ^b	26.667±1.45 ^{bc}	90.333±2.6 ^{ab}	2.667±0.67 ^{cd}	6.000±0.58 ^{ab}	1.667±0.33 ^a

Means within a column with different superscript small letters are significantly different (P<0.05). C: control negative group. F: fish infected with *fungarium* spp not treated. F+S: fish infected with *fungarium* spp and feeding on diet containing *S. cerevisai*. S: Feeding on *S. cerevisai* and not infected

Table 4: Immune parameters of *O. niloticus* injected with *Fusarium* species and treated with *S. cerevisiae* for 5 weeks then challenged with *Pseudomonas aerogenosa*

	Lysozyme activity (µg/ml)	WBCs (x10 ³ /ml)	Lymphocytes (%)	Monocytes (%)	Heterophil (%)	γ1 g/L	γ2 g/L
C	380.213±22.83 ^b	61.000±5.86 ^a	88.667±1.45 ^b	1.667±0.33 ^a	7.333±0.88 ^a	5.390±0.12 ^b	17.467±0.47 ^b
F	217.220±31.23 ^c	43.367±2.81 ^a	88.333±0.88 ^b	1.667±0.33 ^a	5.667±0.67 ^a	6.957±0.6 ^{ab}	12.837±0.74 ^c
F+S	403.040±0.00 ^b	59.633±6.33 ^a	93.333±0.67 ^a	2.333±0.33 ^a	5.667±0.88 ^a	8.370±0.5 ^a	20.677±1.62 ^b
S	514.637±22.24 ^a	59.367±5.88 ^a	91.667±1.2 ^{ab}	3.000±0.58 ^a	6.333±0.33 ^a	8.110±0.5 ^a	27.333±2.06 ^a

Means within a column with different superscript small letters are significantly different (P<0.05). γ1= gamma globulin 1. γ2 = gamma globulin 1. Means within a column with different superscript small letters are significantly different (P<0.05). C: control negative group. F: fish infected with *fungarium* spp not treated. F+S: fish infected with *fungarium solani* and feeding on diet containing *S. cerevisai*. S: Feeding on *S. cerevisai* and not infected

After three weeks serum lysozyme activity was significantly higher in (S) group than (F+S) and (C) group while (F) group was lower than (C) group. Total leukocytic count and lymphocyte count was significantly higher in (S) group than other groups (Table 3). After five weeks there was a marked decrease in serum lysozyme activity, total leukocytic count in (F) group than other groups with slight increase in lymphocyte count in (S) group (Table 3). When the data were statistically analyzed with respecting the interaction between time factor and the different groups, we found that serum lysozyme activity increased in all treated groups then decreased gradually to control limits except (F) group that decreased than control in the 5th week. Total leukocytic count and monocyte count increased in the 1st week then return to control limit. Lymphocyte count increased in all groups at the 3rd week then return to control limit except (S) group that persisted high. At the

end of the experiment challenge was done and after two weeks the analyzed data revealed a significant decrease in serum lysozyme activity (F) group than control group, while (S) group was the highest. Lymphocyte count and gamma globulin were significantly higher in (S) group and (F+S) treated group than other groups (Table 4).

Histopathological findings

Fish gills infected with *Fusarium solani* and fed on a basal diet (F) group after 3rd weeks post infection showed destructed gill filaments, epithelial lifting, lamellar epithelium hyperplasia and characteristic primary gill filament capillary telangiectasis. After 5th weeks post infection; Gills showed epithelial lifting, round cells infiltration and eosinophilic granular cell infiltration in the primary gill filament beside severe congestion of the central venous sinuses of the primary

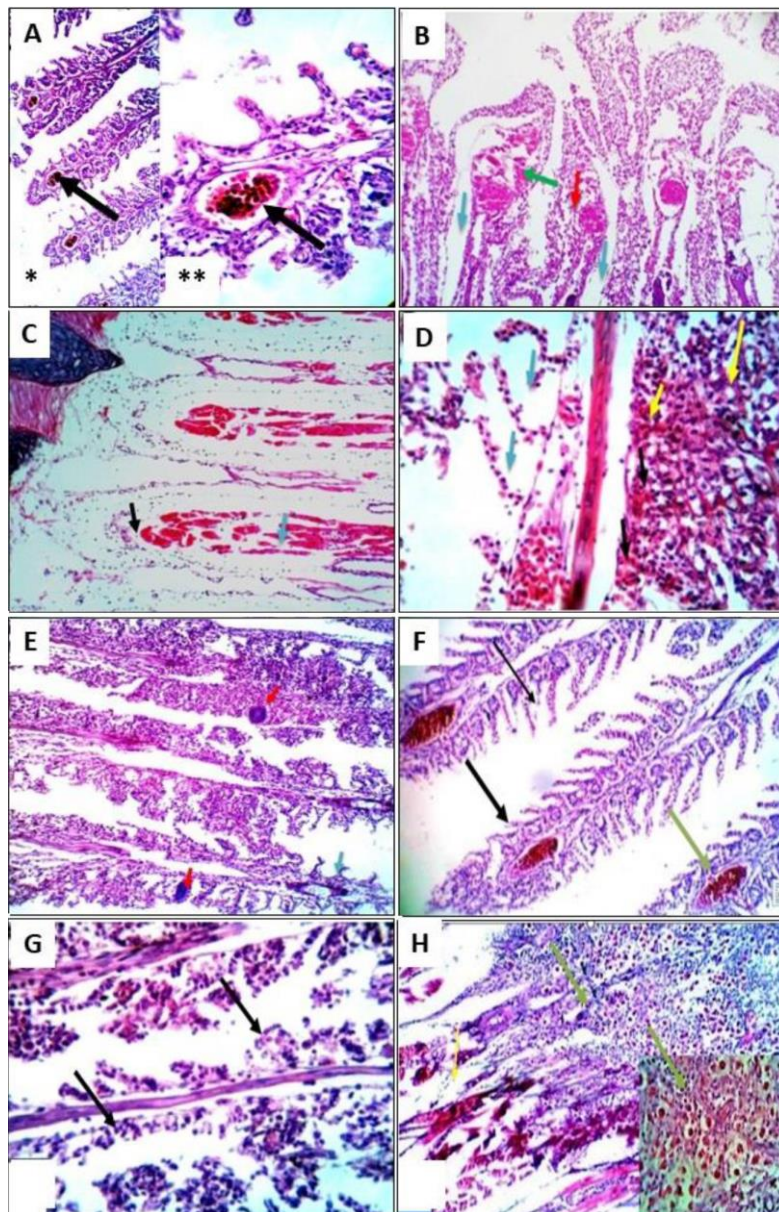


Figure 3: **A).** Gills of group (F), 3rd weeks post infection with *Fusarium* species are showing, lameller epithelium hyperplasia and capillary telangiectasis (black arrow) (A* and **) (H&E, X 200 and 400). **B).** Gills of group (F), 5th weeks post infection with *Fusarium* species are showing destroyed gill filaments, epithelial lifting (blue arrow), round cells infiltration (red arrow) and congestion of central venous sinuses (green arrow) H&E, X200). **C).** Gills in group (F), 7th weeks (after the challenge with *Pseudomonas aeruginosa*) are showing marked necrosis of gill filaments (blue arrows) and inflammatory exudate in the gill arch (black arrows) (H&E, X200). **D).** Gills in group (F), 7th weeks (after the challenge with *Pseudomonas aeruginosa*) are showing destroyed gill filaments, epithelial lifting (blue arrow), round cells infiltration (yellow arrow) beside capillary congestion (H&E, X400). **E).** Gills in group (F), 7th weeks (after the challenge with *Pseudomonas aeruginosa*) are showing filaments destruction (blue arrows), severe round cells infiltration and detection of many numbers of bacterial colonies (red arrows) (H&E, X200). **F).** Gills in group (F+S) 3rd weeks post infection with *Fusarium* species are showing partial lamellar epithelium sloughing, round cells infiltration (black arrow) and milder congestion of primary and secondary capillaries (green arrow) (H&E, X200). **G).** Gills in group (F+S) 5th weeks post infection with *Fusarium* species are showing destroyed gill filaments (black arrows). **H).** Gills in group (F+S) 7th weeks (after the challenge with *Pseudomonas aeruginosa*) are showing capillary telangiectasis (yellow arrow) infiltration of the gill arch by round cells and eosinophilic granular cells (green arrows) (H&E, X200 and 400)

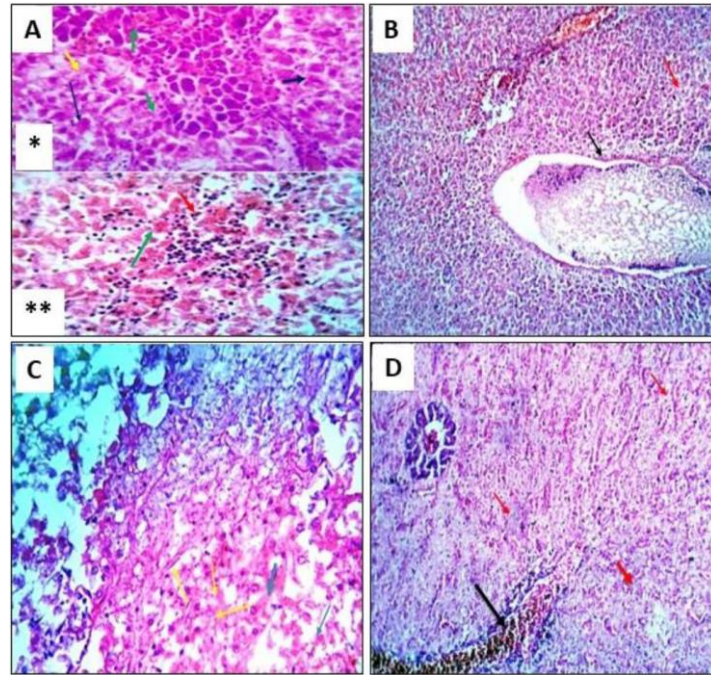


Figure 4: A*). Liver in group (F), 5th weeks post infection with *Fusarium* species is showing focal hepatic cellular degeneration (yellow arrow), apoptosis (black arrow) together with periportal aggregations leucocytic cells (green arrow). **A**).** showing necrosis and interstitial round cells infiltration (red arrow) (H&E, X400). **B).** Liver in group (F) 7th weeks (after the challenge with *Pseudomonas aerogenosa*) is showing congestion of the hepatic blood vessels (black arrow) and focal degenerative changes (red arrow) (H&E, X200). **C).** Liver in group (F), 5th weeks is showing hepatocellular necrosis and replacement by hyphal-like structures (yellow arrows) and infiltration with melano-macrophages (blue arrow) (H&E, X 400). **D).** Liver in (F+S) group, 7 weeks P.I. is showing marked congestion of the hepatic blood vessels (black arrow), degenerative and necrotic changes (red arrows) (H&E, X200)

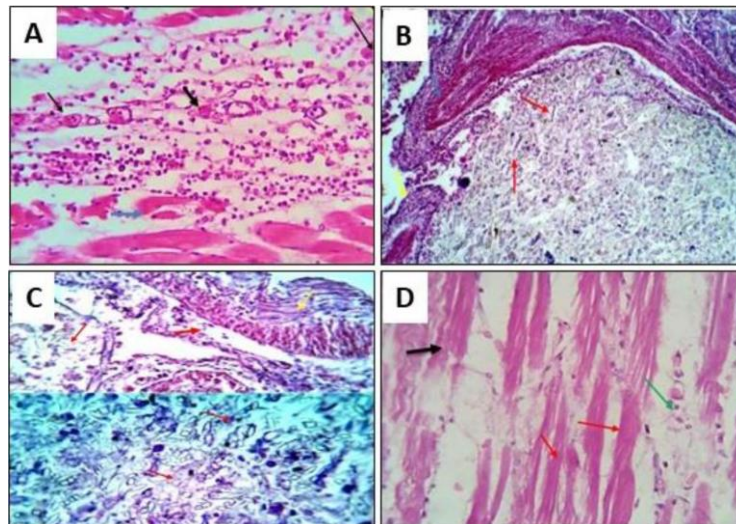


Figure 5: A). Muscles of group (F), 3rd weeks post infection with *Fusarium* species are showing focal hyalinization of the muscles, intramuscular aggregation of lymphocytes, macrophages and eosinophilic granular cells (arrows) (H&E, X400). **B).** Muscles of group (F) 7th weeks (after the challenge with *Pseudomonas aerogenosa*) are showing focal myofibril destruction (yellow arrows) and replacement by few septate fungal mycelium elements (red arrows) (H&E, X200). **C).** Muscles of group (F), 7th weeks (after the challenge with *Pseudomonas aerogenosa*) are showing focal destruction of muscle fibers (yellow arrows) and few septate fungal mycelium (red arrows) (H&E, X400). **D).** Muscles of group (F+S) 5th weeks post infection with *Fusarium* species are showing mild hyaline degeneration (black arrow) and intramuscular edema (green arrow) and focal myomalacia (red arrow) (H&E, X200)

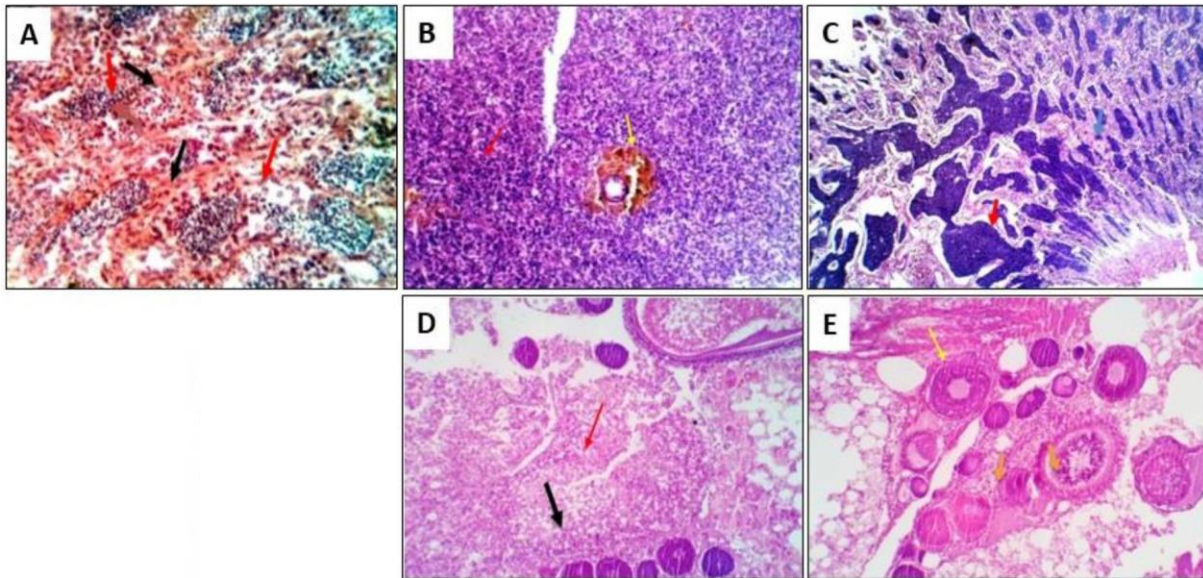


Figure 6: A). Spleen of (F) and (F+S) groups, 5th weeks post infection with *Fusarium* species are showing moderate lymphoid depletion (red arrows) and marked histiocytosis (black arrow) (H&E, X200). **B).** Spleen of (S) group are showing normal lymphocytic populations (red arrows) (H&E, X200). **C).** Testis of (F) group is showing spermatocytes filled with mature sperms (red arrows) and surrounded by thick interstitial tissue (blue arrows) (H&E, X200). **D).** Ovary of (F) group, 3rd weeks post infection with *Fusarium* species are showing necrotic degenerated follicles (red arrow) (H&E, X200). **E).** Ovary of (F+S) group, 3rd weeks post infection with *Fusarium* species is showing normal mature and growing follicles (H&E, X200)

gill lamellae. At the end of 7th weeks post infection (*Pseudomonas aeruginosa*), the gills of fish of (F) group showed marked necrosis of gill filaments, exudative inflammatory exudate in the gill arch. Filament's destruction, severe round cells infiltration and eosinophilic granular cell infiltration and detection of many numbers of bacterial colony aggregations were noticed. Fish gills which infected with *Fusarium solani* and received *S. cerevisiae* containing diet (F+S) group after 3rd weeks post infection showed milder partial lamellar epithelium sloughing, round cells infiltration in the gill arch by round cells. Meanwhile, after 5th and 7th weeks P.I., massive infiltration of the gill arch by round cells and eosinophilic granular cells and capillary telangiectasis were seen (Figure 3).

Liver of group (F) after 3rd and 5th weeks post infection with *Fusarium solani*, showed hepatocellular degeneration, apoptosis, hepato-portal aggregation of melano-macrophages and interstitial round cells aggregation. After 7th weeks, there was congestion of the hepatic blood vessels, degenerative changes, and diffuse necrotic areas. In some cases, the replacements by hyphae-like structures together with periportal aggregations

of leucocytic cells were reported. Liver of (F+S) group showed hepato-portal aggregation of melano-macrophages and interstitial round cells aggregation. Also, the liver showed marked congestion of the hepatic blood vessels focal degenerative and necrotic changes (Figure 4).

Fish muscle in group (F) group 3rd weeks post infection with *Fusarium solani* showed focal hyalinization of the muscle intramuscular aggregation of lymphocytes, macrophages, and eosinophilic granular cells. At the end of the experiment the muscle showed focal destruction of the myofibrils and replacement by septate fungal mycelial elements. The muscle in case of (F+S) group after 5th week P.I. with *Fusarium solani* showed focal intermuscular aggregation of lymphocytes, macrophages, mild hyaline degeneration, intramuscular edema and focal myomalacia (Figure 5).

Spleen in (F) group and (F+S) showed moderate to severe lymphoid depletion and marked histiocytosis (Figure 6A). The spleen in group (S) showed normal lymphocytic populations and activated melano-macrophage centers (Figure 6B).

Testis in groups (F) and (F+S) showed that seminephrous tubules filled with mature sperms

and surrounded by thick interstitial tissue (Figure 6C). The ovary in group (F) showed necrotic degenerated follicles (Figure 6D). The ovary of fish of (F+S) group was apparently normal (Figure 6E). The histological picture of (S) group that received *S. cerevisiae* as and not infected was apparently normal.

Discussion

Molds in fish feed stuffs has great consideration in motivating the mycotoxin output. *Fusarium* is a genus of family *Ascomycetes* containing a few hundred species, distributed primarily in soils and water systems (29). *Fusarium solani*, *F. oxysporum* and *F. verticillioide* have been reported as the most frequent species causing invasive fusariosis in mammals, especially in immuno-suppressed members (30). Mycotoxins resulted by *Fusarium* fungal growth caused injurious consequences for humans (31). Probiotics has important role in stimulation of the intestinal microbiota and inhibit the growth of pathogenic microorganisms (32). In the present study, *Fusarium solani* was isolated from different fish feed stuffs and examined to determine its pathogenicity on *O. niloticus*. To improve fish tolerance against fungal infection stress, it was important to increase their immunity against *Fusarium* infection by using a natural immunostimulant probiotic as *S. cerevisiae*.

At the seventh week Post infection following the challenge by *P. aeruginosa*, our results revealed that the cumulative mortality rates began to increase in group (F) than all other groups indicating the immune-suppressive effect of *Fusarium solani*. Our results were on similar way with Abd El-Ghany et al. (33) who discussed that bacterial and fungal infections were implicated in fish mortalities. Despite that water is naturally inhabited by the pathogenic bacteria, fungi and parasites, but under certain stressful factors they cause fish diseases. Differently, the cumulative mortality rates in group (S) were lower than the control negative group (C) which may be due to the impact of *S. cerevisiae* as stimulant to the non specific immune response of *O. niloticus* as previously mentioned by Ringø et al. (34). The challenge infection of *O. niloticus* using *P. aeruginosa* showed lowest mortality in the *S. cerevisiae* supplemented groups than other groups at the challenging period. That agreed with El-Boshy et

al. (35) who improved that Yeast glucans significantly reduced fish mortality rates after challenge with various pathogens. Similar to mammals, the gut microbiota of fish can be admitted as a physiological key that aids health maintenance of its host and the knowledge of its composition and exact functional role is crucial (36).

In the current investigation, total bacterial counts were estimated for 6 gut samples from each group and the results declared that the TBC was higher in the treatment groups when compared to the control (C) group which fed on basal feed only. The TBC slightly increased in the (S) group than the (C) group and fish were healthy and active. Similar observations were recorded by Bagheri et al. (37) after feeding yeast (*S. cerevisiae*) which may be imputed to the increased number of beneficial bacteria. In the (F+S) group there was a significant decrease in the mean value of TBC in relation to time that the mean value was $(4.48 \pm 0.01 \text{ CFU/ml})$ after 1st week and decreased to $(4.47 \pm 0.01 \text{ CFU/ml})$ after 5th week. Our results were partially agreed with Abu-Elala et al. (38) who discussed that probiotics facilitate the colonization and survival of live microbes in the gut and enhance the health status of the intestine. Similar results were reported by Hassan et al. (39) who concerned the effect of *Saccharomyces cerevisiae* on increasing the intestinal microbiota and its protective effects against pathogenic bacteria. Likewise, El-Nobi et al. (40) improved that intestinal microbiota can enhance the resistance and improve the gut morphology by using of different levels of *S. cerevisiae*.

On the other hand, intestinal gut samples revealed that the (F) group which infected with *Fusarium solani* showed high TBC, that increased gradually $(4.44 \pm 0.01 \text{ CFU/ml})$ at the 5th week. Our study is suggesting that *Fusarium solani* lowering the immunological parameters, and cause disturbances in the intestinal microbiota. Our findings were supported by Almada et al. (32) who reported the possibility that *F. oxysporum* might acted as the primary invader, boosting bacterial infection by tissue damage.

After one week of the experiment all groups showed a significant increase in serum lysozyme activity, total leukocytic count, lymphocyte and monocyte count than control group with respect to the (S) group that was the highest among them, that may be a response to *Fusarium* infe-

ction and immunostimulatory effect of *Saccharomyces*. As the number of leukocytes in the blood is often an indicator of the activation of the immune system. Neutrophils and monocytes are involved in the innate immune response as the first line of the defense system, Lymphocytes including T cells and B cells are the only cells in the body capable of specifically recognizing and distinguishing different antigenic determinants (epitopes). so, the number of lymphocytes is significantly increased as a part of their effector functions (41). The immune response can be modulated by β -glucans and high-M-alginate. β -glucans are glucose polymers that are major structural components of the cell wall of yeast (35). After three weeks serum lysozyme activity and total leukocytic count were significantly higher in (S) group than other groups. Lysozyme is produced mainly by macrophages and its production is induced in response to microbial components such as microbial lipopolysaccharide (LPS) and many other immunostimulants. The probiotic colonized the gastrointestinal tract and stimulated peripheral blood leucocyte counts, serum lysozyme activity and total serum immunoglobulin levels after 3 weeks the start of feeding yeast, Khaksefidi et al. and Kabir et al. [42,43] who stated a highest increase in total leukocytic count and serum lysozyme activity in the third week of feeding *O. niloticus* Bio buds (*S. cerevisiae*) (1g/kg diet) for two weeks, lymphocyte count was significantly higher in (S) group and (F+S) group that agreed and explained by Almada et al. (32) who reported that probiotic caused increases in leukocyte cell counts and marked increase in percentage of lymphocytes and monocytes. The effect may be direct on the lymphatic tissues (44) or indirect through the impact of live yeast on microbial population of gastrointestinal lumen (45,46).

After five weeks there was a marked decrease in serum lysozyme activity, total leukocytic count in (F) group than other groups that might be a result to fusarium mycotoxins that agreed with Chatterjee et al. and Qureshi et al. (46,47) that stated that *fumonisin*s and *fumonisin*-producing fungi have broad immunotoxic effects on livestock including reduced number, viability and phagocytic ability of macrophages, blockade of

dendritic cell maturation and antigen presentation, diminished antigen specific T cell responses. At the end of the experiment, challenge was done and after two weeks the analyzed data revealed a significant decrease in serum lysozyme activity and total leukocytic count in (F) group than control group that agreed with Pierron et al. (48). *Fusarium* infection may influence the host-pathogen interaction by negatively affecting the intestinal barrier function and the innate and adaptive immune response, while (S) group was the highest. Lymphocyte count and gamma globulin were significantly higher in (S) group and (F+S) group than other groups that confirm the immunostimulatory effect of *S. cerevisiae* which might be due to stimulation of antibody secreting cell response and enhancement of phagocytosis of pathogens and improvement of the host innate or acquired immune responses (49).

Histopathological examination of infected *O. niloticus* revealed different clinical abnormalities of gills, liver, muscle and spleen in addition to the appearance of mycelial septated hyphae. These results are more or less similar to those of Refai et al. (50) who isolated *fusarium* from *O. niloticus*.

In our study, the gills of fish which supplemented by basal diet and infected by *fusarium* spp. showed severely affected destructed gill filaments and filament capillary telangiectasis. By the end of 3 weeks post infection; the gills showed more severe deformities of the primary and secondary lamellae, round cells infiltration and eosinophilic granular cell infiltration. At the end experiment, the gills in group (F) showed marked necrosis of gill filaments and exudative inflammation in the gill arch and filaments destruction. On the same direction the previous studies reported that *Fusarium* molds was very harmful on fish diets lead to severe histological alternation in gills and also reported that, fungal infection was precursor for invasion by other pathogens due to lowering the fish immune system (34, 50). On the other hand, fish which supplemented by diet containing *S. cerevisiae* as seen in (F+S) group showed milder improvements of the histological lesions comparing with the previous group with no evidence of secondary infection which supported by Hassan et al. (39) who proved that *S. cerevisiae* is immunostimulant and anti-inflammatory biological supplement.

The liver of fish fed on basal diet and infected with *Fusarium* spores (F) group mainly in the 7th week P.I. exhibited severely pathologically affection due to its hepatotoxic affection, as illustrated by Abd El Aziz et al. and Hassan et al. (6, 39). The current study, showed apoptosis, hepatoportal aggregation of macrophages, necrosis and replacement by hyphal-like structures. These results is confirmed by the results of Ringø et al. (34) who discussed the infected livers showed focal hemorrhages, necrotic hepatocytes and multifocal granulomatous hepatitis where as their dysfunction and also, confirmed by marked decrease total protein and liver enzymes (ALT & AST). Supplemented fish with diet containing *S. cerevisiae* (F+S) group showed milder hepatic histopathological changes including round cell infiltration then the liver become appeared apparently normal towered the end of the experiment. These reports could be attributed to *S. cerevisiae* cell wall capture the harmful effect of mycotoxic substance into nontoxic substance (52) as well as *S. cerevisiae* mainly (*B-glucans*) has hepato-protective function (53,54). Muscle of fish of group (F) group 3 weeks P.I. showed focal hyalinization of the muscle, aggregation of round cells and eosinophilic granular cells. At the end of the experiment the muscle showed focal destruction and replacement by a few septate fugal mycelium elements (55). The muscle in the (F+S) group showed intramuscular edema and focal myomalacia. Spleen in the groups (F) and (F+S) showed moderate to severe lymphoid depletion and marked histiocytosis. The spleen in group (S) showed normal lymphocytic populations and activated melano-macrophage centers [38, 55]. The existence of melano-macrophage in the tissues of spleen and liver in the (F+S) group and (S) group explained the immunological activity of *S. cerevisiae*. Many studies supported our data and reported melanomacrophage centers aggregation with highly pigmented phagocytes found primarily in the head kidney and spleen, and occasionally the liver. The leading hypothesis is that melano-macrophage represents the primitive site of adaptive immune system activation in fish (42, 55,56). Testis in the (F) and (F+S) groups showed normal architecture. The ovary in the (F) group showed mild necrotic degenerated follicles, while the ovary in case of the (F+S) group was apparently normal. Our results suggested

that *Fusarium* infection has no harmful effects on the fish fertility. These results are partially disagreed with the results of Nashwa et al. (56) who reported vacuolar degeneration of spermatoocytes, hemorrhages in testis and characterized by germinal and asymmetry. Also, the infected fish' ovaries epithelial and testicular ducts showed necrosed nuclei and edematous.

The present results concluded that *Fusarium solani* has hazardous effect on *O. niloticus* immune response and resistance to infection. There are beneficial effects of *S. cerevisiae* in enhancing the immune resistance of fish against *P. aeruginosa* infection. It can be used as an alternative to antibiotics to improve blood immunological parameters and gut intestinal flora. The results provided data for its application as a useful fish feed additive.

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