

EVALUATION OF IMMUNOMODULATORY EFFECTS OF SOME PROBIOTICS ON CULTURED *OREOCHROMIS NILOTICUS*

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Abstract

A growing concern for the high consumption of antibiotics in aquaculture has initiated a search for alternative methods of disease control. Improved resistance against infectious diseases can be achieved by the use of probiotics. The objective of the present study was to evaluate the influence of some probiotics on the immune response of *O. niloticus*. The experimental fish were divided into three groups, the first group was fed on diet supplemented with dead *Saccharomyces cerevisiae* yeast, the second group was fed on diet supplemented with live *Bacillus subtilis* and *Saccharomyces Cerevisiae* and the third group was served as control fed on probiotic-free diet. Six weeks later the results indicated that, the fish groups which received diet supplemented with probiotics revealed significant increase in non specific immune response as detected in vitro phagocytic activity test. Histologically, the spleen and liver showed great activation of melano-macrophage centers and kupffer cells. The probiotic fed fish groups showed high resistance to the challenged pathogenic microorganisms.

Key words: Probiotics, phagocytic assay, phagocytic index and challenge test

INTRODUCTION

It is widely demonstrated that farmed fish are more susceptible to disease agents than their wild counterparts due to the artificial conditions posed by intensive rearing (Irene Salinas *et al.*, 2006). The immune system of aquatic organisms, such as fish, is continuously affected by periodic or unexpected changes of their environment. Adverse environmental situations may acutely or chronically stress the health of fish, altering some of their biochemical parameters and suppressing their innate and adaptive immune responses (Giro'n-Pe'rez *et al.*, 2007). Non specific defense mechanisms play an important role at all stages of infection. Fish, particularly, depend more heavily on these non-specific mechanisms than do mammals. Hence, in the last decade there has been increasing interest in the modulation of the non-specific immune system of fish as both a treatment and prophylactic measure against disease (Misra *et al.*, 2006). When infectious outbreaks appear they may be fought by means of chemotherapeutants, vaccines or immunostimulants. More recently, the administration of probiotics to fish through the diet has appeared as a very promising control measure in fish farms.

Probiotics are defined as microbial dietary adjuvants that beneficially affect the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract (Villamil *et al.*, 2002). Most studies with probiotics conducted to date in fish have been undertaken with strains isolated and selected from aquatic environments. There are a wide range of microalgae (*Tetraselmis*), yeast (*Debaryomyces*, *Phaffia* and *Saccharomyces*) and gram positive (*Bacillus*, *Lactococcus*, *Micrococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Streptococcus*, *Weissella*) and gram negative bacteria (*Aeromonas*, *Alteromonas*, *Photobacterium*, *Pseudomonas* and *Vibrio*) that has been evaluated as a probiotics (Gastesoupe, 1999). Several studies have demonstrated certain modes of probiotic action in the aquatic environment. Further, they improved feed conversion ratio and feed utilization, revealed adhesion capacity to the intestinal mucosa that hindered the adherence of pathogenic bacteria, produced extra-cellular antibiotic like products or iron binding agents (siderophores) that prevent the growth of some pathogenic flora. Also the probiotics achieved improvement in water quality (bioremediation) and facing the problem of red tide planktons. Concerning the immunostimulation point of view, many researches showed improvement in the immune response of fishes treated with probiotics (Watson *et al.*, 2008). The objective of this study was to clarify the effect of some microbial approved probiotics on the non specific immune response of cultured *O. niloticus*.

MATERIALS AND METHODS

● Material

1. Fish and experimental condition

Apparently healthy *O. niloticus* with an average body weight of 30 gram were obtained from private farm at Kanater, Kalubia Governorate. Fish were kept in full glass aquaria measuring (100 X 50 X 30 cm) and maintained in aerated de-chlorinated fresh water at $22 \pm 2^\circ\text{C}$ for 14 days prior to use in experiments. The health status was examined throughout the acclimatization period. Water pH was measured by using electric digital pH meter and water temperature was recorded daily using a glass thermometer.

2. Fish diet

A basal diet was prepared at Dept. of Animal production, Faculty of Agriculture, Ain-Shams University. It contained 30% crude protein, 3.7 Kcal/g of metabolizable energy, 3.4% fiber and 7.03% fat as well as vitamins and minerals in the form of dry pellets.

2. Probiotics

Two commercial products containing probiotics were used and mixed thoroughly with the prepared basal fish diet during its preparation.

➤ **Daimond-V Yeast**

Is a dried product composed of yeast and the media on which it was grown, dried in such a manner as to preserve the fermenting activity of the yeast supplied by Cedar Rapids, Iowa, USA. It is composed of *Saccharomyces cerevisiae* (*S. cerevisiae*) yeast grown on a media of ground yellow corn, hominy feed, corn gluten feed, wheat middlings, rye middlings, diastatic malt and corn syrup, and cane molasses. The recommended dose by the producer is 10 g / Kg feed.

➤ Megalo: It is composed of *S. cerevisiae* and *Bacillus subtilis* (*B. subtilis*). Each 100 grams contains:

Yeast <i>Saccharomyces cervisiae</i>	1 trillion c. f. u.
<i>Bacillus Subtilis</i>	400 million c. f. u.

The recommended dose by the producer is 1.5 g / Kg feed.

4. Stains

- a) Gram's stain: It was used for staining the bacterial smears. It was prepared as described by Ronald (2001).
- b) Field stain: It was used for the staining of blood films for the differential leukocytic count according to Tankeyul *et al.* (1987).
- c) Hematoxyline and Eosin (H & E) stain: It was used for staining the histopathological sections according to Drury *et al.* (1976).
- d) Trypan blue (Sigma): It was used in trypan blue exclusion test to examine the viability of isolated leukocytes.
- e) Giemsa stain: it was prepared according to Ronald (2001) and used to stain the phagocytic cells.

5. Chemicals and reagents

- a) Ethyl alcohol: used for disinfection and staining.
- b) Drabkin solution: It was used for determination of hemoglobin concentration according to Stoskopf (1993).
- c) Natt-Herrick solution: Natt-Herrick solution was used for red and white cell count according to Feldman *et al.* (2000).
- d) Heparin: It was used at a final concentration of 100 IU/ml as anticoagulant for determination of hematological parameters according to Jain (1986).
- e) Tissue culture medium: RPMI-1640 (sigma).
- f) Histopaque (1.077g/ml density) (sigma).
- g) Cedar oil: It was used microscopic examination.

h) Canada balsam: It was used for fixation of cover slips on the cover slides.

6. Diagnostic kits

- o Commercial diagnostic kits supplied by Stan-bio-laboratory, USA were used for determination of hemoglobin concentration, activities of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), total protein, and albumin.
- o Oxidase discs (Himedia)

7. Bacterial strain

A Pathogenic strain of *Pseudomonas fluorescence* used in the experimental infection of *O. niloticus* was supplied from International Research Center, Dept. of Aquatic Animals, Dokki, Giza.

8. ***Candida albicans***: A good identified strain was kindly supplied by the Dept. of Microbiology and Immunology, Fac. Vet. Med., Cairo University. This strain was used for phagocytosis studies.

8 Methods

1-Experimental design

Hundred *O. niloticus* were distributed into five glass aquaria and acclimatized for the experimental conditions for 15 days prior to the start. During that period fish were adapted on feeding of control diet (without any additives). Water was changed every week to maintain good water quality. Water temperature and pH were adjusted at 20-25°C and 7.4 respectively during the experimental period. The experimental design is to be seen in table (1).

Table 1. *Oreochromis niloticus* studied groups

Fish group	Fish No.	Treatment	Dose	Feeding% /fish biomass
Control	20	Basal diet	-----	3%
Group I	40	Basal diet + Diamond*	10 gm / Kg	3%
Group II	40	Basal diet + Megalo**	1.5 gm / Kg	3%

* Dead *S. cerevisiae*

** Live *S. cerevisiae* and *B. subtilis*.

During the experimental period fish were fed on diet supplemented with the feed additives in the form of dried pellets. Feeding rate was about 3% of the total biomass of fish per day. The amount of feed was corrected every 2 weeks according to the new biomass. After 6 weeks blood samples were collected on 100 IU/ml Heparin for measuring of blood parameters and the phagocytic activity test. Other blood samples were collected without anti-coagulant for serum separation to be used in measuring blood chemistry.

2. Phagocytic activity test

A. Isolation and cultivation of the peripheral blood mononuclear phagocytic cells

Leukocytes isolation was performed according to the method described by Böyum (1968) and modified by Faulmann *et al.* (1983).

Procedure

- Blood was collected from the caudal vessels by syringe moisten with heparin (100 IU/ml).
- The blood then was diluted 1:2 (volume / volume) with culture medium RPMI-1640 supplemented with 5% tilapia serum.
- Cell suspension was layered over a solution of histopaque of density 1.077 g/ml in poly styrene tubes and the gradients were centrifuged at 2500 rpm for 30 min. in cold centrifuge 4°C.
- The band of leucocytes at the interface between the solutions was harvested with Pasteur pipette and the cell suspension diluted 1:10(volume to volume) with cold RPMI-1640 supplemented with 5% tilapia serum.
- The cells were washed twice at 2500rpm for 15 min. at 4°C in RPMI-1640 supplemented with 5% tilapia serum.
- The leucocytes isolated by this methodology accounted for over 90% of those present in the original cell suspension.
- The average yield was about 4×10^7 cells/ml blood.
- When stained with May-Grünwald Giemsa, the leukocytes from peripheral blood were about 60-70% lymphocytes by morphological criteria, the remaining cells were approximately equally distributed among cells that could be classified as thrombocytes, monocytes and granulocytes.
- All cell preparation used were judged more than 90% viable by the criterion of trypan – blue exclusion test.
- The prepared cell suspensions from two treated and control fish were used in phagocytic assay at the end of each exposure period.

B-Phagocytosis assay**Preparation of *C. albicans* for phagocytosis**

- *C. albicans* was prepared as 24 hours old culture. This could be obtained by cultivation of *C. albicans* on Sabouraud's dextrose agar containing chloramphenicol at one day before the phagocytosis experiment.
- Using a platinum loop a suspension of *C. albicans* culture was made in 1 ml RPMI.
- The number of *Candida* cells was counted using the haemocytometer, then diluted for obtaining the required concentration which was 1×10^6 yeast cells/ml (the optimum concentration for phagocytosis). The dilution was achieved using RPMI.

Phagocytic activity of peripheral blood monocytes of *Oreochromis niloticus* using *C. albicans***Procedure**

- Isolation and cultivation of the peripheral blood mononuclear phagocytic cells:
- Separated peripheral blood leucocytes were adjusted to a concentration of 2.5×10^6 viable cells/ml RPMI.

The cells were cultivated according to the modified procedures described by Chu and Dietert (1989), Siwicki *et al.* (1994) and Park and Jeong (1996).

- One ml of the cell suspension of viable cells in the adjusted concentration as previously mentioned was placed in cell culture and staining chamber (CCSC) containing sterile rounded cover slips.
- The chambers were incubated for 1 hour at 37°C in a humidified CO₂ (5-10%) incubator. Adherent cells were then washed gently with warm RPMI medium for removal of non-adherent cells.

C-Evaluation of the phagocytic activity

- To each CCSC, 1 ml volume of adjusted *C. albicans* suspension was added.
- The chambers incubated in a humidified CO₂ (5-10%) incubator at 37°C incubator at 37°C for further one hour
- Cover slips were then washed gently three times with cold RBMI and stained with Giemsa stain. Under the oil immersion lens of ordinary light microscope, about 200 phagocytes were counted and differentiated as follows:

- a. Total number of adherent cells
- b. Number of cells ingesting *Candida* blastospores

The phagocytic Activity was calculated according to the

Following equations:

$$\text{Percentage of phagocytosis} = \frac{\text{No. of ingesting phagocytes}}{\text{Total number of phagocytes including non ingesting cells.}}$$

Phagocytic index = No. of ingested *C. albicans* cells / No. of Ingesting phagocytes.

3. Measuring of hepatosomatic and splenosomatic indices

At the end of experimental period five fish from each group were dissected and the viscera were exposed. The liver and spleen were taken and weighed after which the indices were calculated according these equations:

Hepatosomatic index = Weight of the liver/fish body weight.

Spleenosomatic index = Weight of spleen/fish body weight.

4. Clinicopathological examinations

A. Hematogram

- a) **Hemoglobin concentration (gm/dl):** Hemoglobin concentration was determined using the cyanomet-hemoglobin method according to Stoskopf (1993).
- b) **Packed Cell Volume (PCV%):** Packed cell volume was estimated by the micro-haematocrite method described by Decie and Lewis (1991)
- c) **Erythrocyte and leukocyte count:** A manual method for counting using a hemocytometer counting chamber and Natt-Herrick solution was carried out according to Stoskopf (1993).
- d) **Differential leukocytic count:** The stained blood film was prepared .The relative and absolute count was estimated according to Thrall (2004).

B. Biochemical parameters

- a) **Alanine aminotransferase activity (ALT) and Aspartate aminotransferase activity (AST):** Colorimetric determination of ALT and AST activity were performed according to Reitman and Frankel (1957).
- b) **Total proteins:** Assay of total proteins was carried by a test kit according to biuret method described by Weichselbaum (1946).
- c) **Albumin:** Serum samples from all experimental groups were estimated for albumin by a colorimetric method at wave length 550 nm according to Dumas and Biggs (1972).
- d) **Globulin:** Globulin was calculated by mathematical subtraction of albumin value from total proteins.
- e) **Albumin / Globulin (A/G) Ratio:** Albumin: Globulin ratio was calculated from data of albumin and globulin.

5. Challenge test. At the end of 6th week, seven *O. niloticus* from each group were challenged I/P with 0.2 ml sterile saline containing (1.5×10^8 / ml) pathogenic strain of *Pseudomonas fluorescens*, The clinical signs, P.M. lesions and mortalities were monitored for 7 days post challenge, its rate was estimated according to El-Attar and Moustafa (1996).

$$\text{Mortality \%} = \frac{\text{No. of death in a specified period}}{\text{Total population during that period}} \times 100$$

At the end of experimental period and after the challenge test, tissue samples namely, liver and spleen were collected for histopathological examination. Liver and spleen were weighted to determine both hepatosomatic index and splenosomatic index.

6. Histopathological examination

For histopathological studies, tissue specimens were obtained from liver and spleen. The tissue specimens were fixed in 10% neutral formalin, embedded in paraffin, sectioned and stained with Hematoxyline and Eosin (H & E) according to the method described by Drury *et al.* (1976).

RESULTS

1. Phagocytic assay

The phagocytosis percent and phagocytic index were significantly increased in the fish groups fed on diet supplemented with Probiotics in comparison with the control group. These values are demonstrated in table (2) and the fig. (1) Shows the phagocytic activity of blood monocytes to *Candida albican*.

Table 2. Percentage of phagocytosis and phagocytic index of different *O. niloticus* groups

Fish group	Peripheral blood monocytes				
	Total no. of phagocytes	No. of ingested phagocytes	Blastospores within phagocytes	% of phagocytosis	Phagocytic index
control	165	122	235	73.93	1.93
Group I	175	143	325	81.7%	2.27
Group II	160	133	350	83.125	2.63

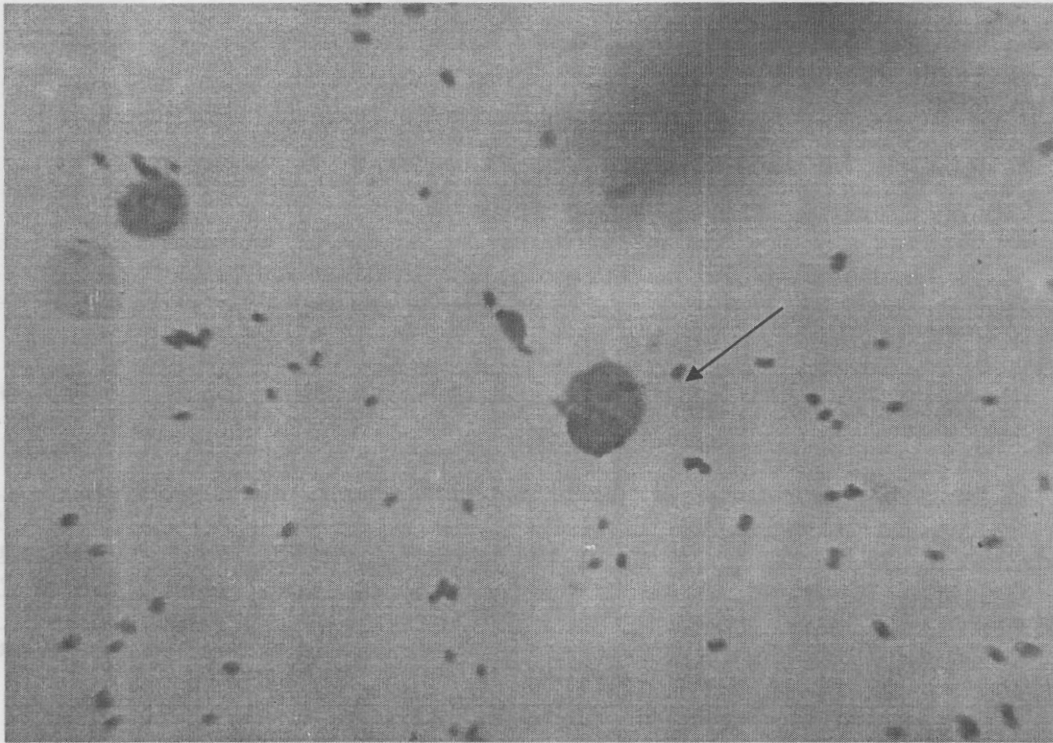


Fig. 1. *O. niloticus* monocyte engulfing one *Candida albicans* blastospore. (Giemsa stain x1000).

2. Hepatosomatic and splenosomatic indices

The statistical analysis demonstrated that there is no significant difference between splenosomatic and epatosomatic indecies in all groups table (3).

Table 3. Hepatosomatic and splenosomatic indices of *O. niloticus* after (6 weeks)

Fish groups	Hepatosomatic index (HSI)	Splenosomatic index (SSI)
Control group	1.18±0.13 ^a	0.23±0.02 ^a
Group I	1.4±0.085 ^a	0.26±0.042 ^a
Group II	1.11±0.06 ^a	0.21±0.01 ^a
F-va	2.53 [*]	0.57

Data are represented as means of six samples ± SE.

Means with the same letter for each parameter are not significantly different; otherwise they do (SAS, 2000).

* Significant difference at P<0.05 **highly significant difference at P<0.0

CLINICOPATHOLOGICAL FINDINGS**a- Hematogram:**

The results of hematogram revealed a significant increase in RBCs count, H.B. value, PCV%, WBCs and differential leukocytic count in the two groups treated with probiotics. These results are presented in table (4).

Table 4. Hematogram of *O. niloticus* groups post -treatment with probiotics

Parameters Studied groups	RBCs ($\times 10^6$ /mm ³)	Hb (g/100ml)	PCV (%)	Total WBCs ($\times 10^3$ /mm ³)	Heterophils ($\times 10^3$ /mm ³)	Lymphocytes ($\times 10^3$ /mm ³)	Monocytes ($\times 10^3$ /mm ³)
Group I	1.5 \pm 0.07 ^a	5.57 \pm 0.11 ^a	23.3 \pm 1.17 ^c	71.7 \pm 4.3 ^a	5.56 \pm 0.15 ^a	57.3 \pm 1.69 ^a	10.7 \pm 0.84 ^a
Group II	1.57 \pm 0.02 ^a	5.17 \pm 0.24 ^a	19 \pm 0.60 ^b	74 \pm 3.95 ^a	4.7 \pm 0.44 ^a	59.0 \pm 0.37 ^a	11.66 \pm 0.21 ^a
Control group	1.13 \pm 0.06 ^b	4.03 \pm 0.24 ^b	15.0 \pm 0.63 ^a	56 \pm 1.89 ^b	3.07 \pm 0.11 ^b	46.3 \pm 1.7 ^b	4.7 \pm 0.42 ^b
F- Values	18.38**	14.52**	23.93**	7.66**	21**	49**	46**

Data are represented as means of six samples \pm SE. Means with the same letter for each parameter are not significantly different; otherwise they do (SAS, 2000)

**Highly significant difference at P<0.01.

B-Biochemical parameters**Protein profile and liver enzymes**

The results of protein profile and liver enzymes showed a significant increase in total protein and globulin .and significant decrease in albumin, A/G ratio and liver enzymes (ALT and AST). These results are illustrated in table (5).

Table 5. Protein profile and activities of serum enzymes (ALT & AST) In *O. niloticus* groups post-treatment with probiotics

Parameters Studied groups	Total protein (g/100ml)	Albumin (g/100ml)	Globulin (g/100ml)	A/G ratio	ALT (u/l)	AST (u/l)
Group I	3.18 \pm 0.18 ^b	0.66 \pm 0.03 ^a	2.50 \pm 0.20 ^a	0.27 \pm 0.04 ^b	14.4 \pm 0.89 ^a	18.36 \pm 2.96 ^a
Group II	3.82 \pm 0.06 ^a	0.49 \pm 0.02 ^a	3.34 \pm 0.08 ^b	0.15 \pm 0.03 ^a	9.49 \pm 0.26 ^b	11.57 \pm 1.42 ^b
Control group	2.74 \pm 0.30 ^b	0.72 \pm 0.06 ^a	2.02 \pm 0.26 ^a	0.38 \pm 0.05 ^b	17.5 \pm 2.04 ^a	19.62 \pm 1.89 ^a
F- Value	7.1**	147**	1.54**	22**	9.8**	3.93*

Data are represented as means of six samples \pm SE.

Means with the same letter for each parameter are not significantly different, otherwise they do (SAS, 2000).

**Highly significant difference at P<0.01.

* Significant difference at P<0.05

Clinical signs and post mortum lesions after challenge with *P. fluorscens*

No apparent clinical signs were observed in both fish groups received probiotics and the control positive group after *P. fluorscens* infection revealed excess mucus and hemorrhages on the skin at the base of fins. Ulceration on the gill cover and slightly protruded reddish vent, skin darkness and tail rot were also noticed. Petechial hemorrhages were evident in the kidney, liver and submucosa of the gut and hemorrhagic distended gall bladder. Mortalities were observed in control group and group I while the group II has no mortality. These are shown in table (6) and Fig.(2).

Table 6. Mortality percent of *O. niloticus* challenged with *P. fluorscens*.

Fish group	Total number	The number of dead fish	% of survival	% of mortality
Control	7	2	71.4%	28.6%
Group I	7	1	85.7%	14.3%
Group II	7	0	100%	0%

Studied groups

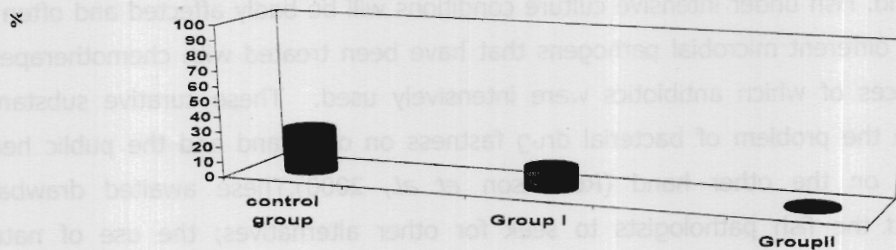


Fig. 2. Mortality percent of *O. niloticus* Challenged with *P. fluorscens*

Histopathological findings

The splenic and liver tissues of probiotic-treated fish showed great activation of melano-macrophage centers and Kupffer cells respectively as shown in Fig. (3) and Fig. (4).

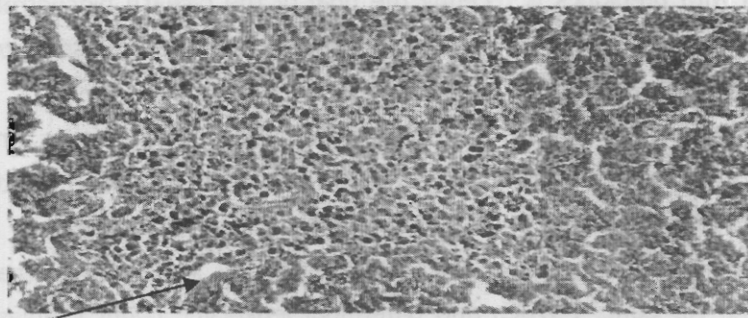


Fig. 3. Spleen of *O. niloticus* treated with living *S. cerevisiae* and *B. subtilis* shows increasing of melanin intensity in melano macrophage centers with prominent lymphocytes.(H&E stain X200).

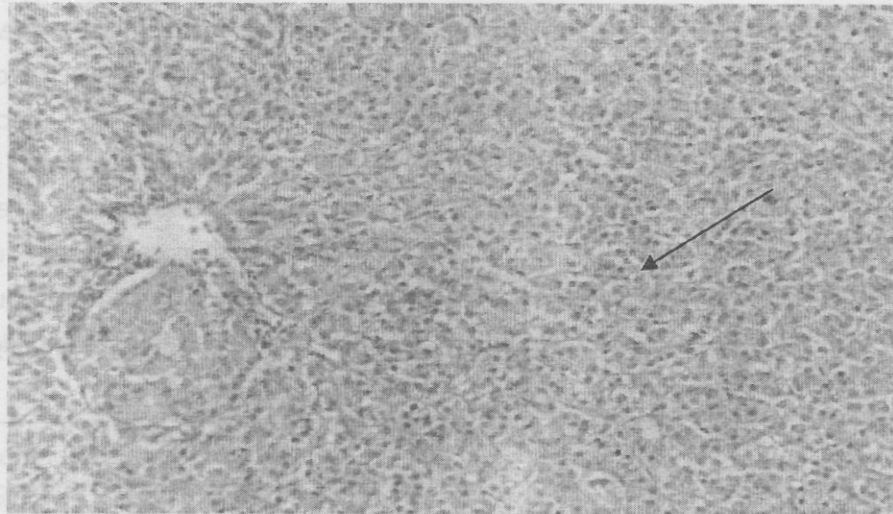


Fig. 4. Liver of *O. niloticus* treated with dead *S. cerevisiae* showing Kupffer cells aggregation with apparent normal hepatocytes. (Stain H & E -X200).

DISCUSSION

Fish culture is increasing to compensate the shortage of animal protein all over the world. Fish under intensive culture conditions will be badly affected and often fall prey to different microbial pathogens that have been treated with chemotherapeutic substances of which antibiotics were intensively used. These curative substances produce the problem of bacterial drug fastness on one hand and the public health hazards on the other hand (Robertson *et al.*, 2000). These awaited drawbacks enforced the fish pathologists to seek for other alternatives; the use of natural immunostimulants in fish culture for the prevention of diseases is a promising new development and could solve the problems of massive antibiotic use. Natural immunostimulants are biocompatible, biodegradable and safe for both the environment and human health. Moreover, they possess an added nutritional value (Jesus *et al.* 2002).

The parallel use of biological products namely the probiotic either alone or in combination with prebiotics is recently the goal of the disease biocontrol strategy in aquaculture as they improve the fish health and modify the fish associated microbial community (Gibson and Roberfroid, 1995).

This study was planned to evaluate the effect of probiotics on the blood parameters and immune response of cultured *O. niloticus*. Concerning the effect of both commercial products Diamond and Megalo on the health status of *O. niloticus*, the results indicated a positive effect represented by significant increase in RBCs count, PCV%, Hb Conc. , WBCs and differential leukocytic count (Tables, 4). These could be attributed to the fact that, the probiotics used increased the blood parameter values as a result of hemopoietic stimulation. These results supported the results of Sarma *et al.* (2003) and Rajesh *et al.* (2006). Also the obtained results in this study was confirmed by the histological pictures of *O. niloticus* groups received diets

supplemented with probiotics in which the histological structure of both liver and spleen were normal and showed hyperactivity of Kupffer cells and melanomacrophage centers with intensity of melanin pigment and the oval individual cells of *S. crevisiae* appeared to be colonized to the intact intestinal epithelial cells and scattered in the intestinal lumen. Further, yeast cells could produce a nutritional valuable ingredient represented by valuable members of vitamin B complex could explain the normal blood cells formation.

Concerning the non-specific immune stimulation in *O. niloticus* fish groups received diets supplemented with probiotics. It was clear that high non-specific immunity was developed as manifested by increased number of lymphocytes and monocytes in the differential leucocytic count as well as increase in the percent of phagocytosis and phagocytic index in table (2). The results indicated that the percent of Phagocytosis and Phagocytic index in *O. niloticus* group II which received (Megalo) was the best 83.1% and 2.63 respectively followed by *O. niloticus* in group I in which the values were 81.7% and 2.27 respectively in comparison to *O. niloticus* kept on a basal diet in which values were 73.9% and 1.9 respectively. These could be attributed to the different components of *S. crevisiae* in particularly the β -glucan, that activate the phagocytic cells and melanomacrophages in the hemopoietic organs other than increasing the size of hemopoietic organs as confirmed in the histological examination and results of hepatosomatic and splenosomatic indices. These results agreed with the results obtained by Jessus *et al.* (2002), who worked on yeast and reported the similar results and stated that, the activation mechanisms involved are known to be related to the carbohydrates derived from the yeast cell wall and β -glucans added to the feed stimulated the phagocytic function and protection after challenge with pathogenic bacteria in some fish species. Moreover, the existence of β -glucan receptor on the macrophage cell surface has been demonstrated in Atlantic salmon. The intake of chitin also increases the cellular immune response, including phagocytosis, repository burst and natural cytotoxic activity. Not only sugars but also nucleic acids, especially yeast RNA, could act as immune activators, since the growing evidence that nucleic acids from yeast sources, previously considered nutritionally non-essential for immune reactivity in mammals.

The results indicated a significant increase in total protein and decrease A/G ratio which could be attributed to the immuno-modulatory effect of *S. crevisiae* and *B. subtilis* on the liver cells which activate the anabolic capacity of the hepatocytes to produce blood proteins particularly globulin and this was also supported by the results of hepatic enzymes analysis which decreased in *O. niloticus* kept on probiotics in comparison to control group indicating a normal, positive and beneficial effect of both probiotics on the maintenance of the integrity of hepatocytes and their roles in

improvement of liver histology. These results were supported by several authors Jassus *et al.* (2002); Nayak *et al.* (2004) and Safinaz (2006).

Concerning the challenge of the *O. niloticus* fish groups (control, group and group II) with specific fish pathogen *P. fluorescens* (Table, 6).The results indicated that appearance of characteristics clinical signs and post mortem lesions in *O. niloticus* control group as early as three days post challenge as a total mortality percentage 28.6%. Ehab (1991) attributed these clinical signs to the effect of extracellular proteases produced by *P. fluorescens* which attack endothelial lining of blood vessels and parenchymatous organ causing the haemorrhagic phenomena as well as the degenerative changes. Also he reported that the tail and fin rot in Pseudomonas infection was caused by histolytic enzymes of invading bacteria and such bacteria were transmitted from fish to other by direct contact or through water and he recorded that experimental infection with *P. fluorescens* strain was successfully only when the organism was injected and not when the fish were left in an infected water. This indicated that, the penetration of the bacteria was a predisposing factor for infection.

On the other hand the *O. niloticus* in the group II kept on diet supplemented with Megalo did not show any mortality within one week post challenge and survival rate was 100% while *O. niloticus* of group I kept on diet contain dead *S. cerevisiae* showed 14.3% mortality (Table, 6). These results confirmed the immune stimulatory effect of the living *S. cerevisiae* and *B. subtilis* and also their inhibitory effect to *P. fluorescens* also the variation in the mortality ratios in both groups I and II indicated that the living yeast cells and bacteria cells are more potent than dead yeast cell in the protecting the *P. fluorescens* infection. These results supported those reported by Ehab (1991); Elattar and Moustafa (1997).

In conclusion, the results of this study revealed that *B. subtilis* and *S. cerevisiae* frequently used probiotics that are able to adhere and colonize the *O. niloticus* gut preventing the adhesion and colonization of specific fish pathogens. Also the diets supplemented with living *B. subtilis*, dead or living *S. cerevisiae* improve the non-specific immune response which reflected on the stimulation of macrophage cells and increasing their phagocetic activity. Finally, these probiotics could provide healthy and safe fish production from aquaculture replacing the Xenobiotics (antibiotics) for both fish and fish consumers.

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