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Full Length Article

Use of different *Saccharomyces cerevisiae* biotic forms as immune-modulator and growth promoter for *Oreochromis niloticus* challenged with some fish pathogens

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KEYWORDS

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Abstract The manipulation of intestinal bacterial flora through dietary supplementation of beneficial microbes is a new approach not only from the nutrition point of view but also to overcome the adverse effects of chemotherapeutants and lack of effective vaccines. The current study was performed to assess the role of *Saccharomyces cerevisiae* as a whole yeast cell (Probiotic), its extract (mannan-oligosaccharide - Prebiotic) and Pre-Probiotic mixture (Synbiotic) as growth promoters and immunostimulants in cultured *Oreochromis niloticus* (*O. niloticus*). One hundred fifty-six *O. niloticus* were divided into four groups fed on Probiotic, Prebiotic, synbiotic and basal diet for two months. Treated fish groups showed significant improvement in growth performance and activation of non-specific cellular/humoral immunological measures together with a relative enhancement of resistance against challenged bacteria. Synbiotic feed additive has proved significant enhancement of fish innate resistance against selected fish pathogens as well as positively increased the growth performance of challenged fish.

1. Introduction

Despite the fact that Egyptian aquaculture is among the highly progressing fish production systems worldwide, yet, the industry is currently facing several limitations. High feeding cost and microbial diseases are the two major limiting factors hindering our national aquaculture. [1]. Therefore, utilizing a good feeding regime together with continuous enhancement of the fish immunity, will have a great impact on growth/profitability of the reared fish throughout the entire production cycle [2].

The manipulation of gut microbiota through dietary supplementation of beneficial microbes is gaining high scientific and commercial interest. Those beneficial microorganisms are usually referred as Probiotics. They colonize and multiply in the host' gut with numerous consequent beneficial effects on both host and its ambient environment [3].The

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Table 1 Feeding regime used throughout the entire study.

Fish groups	No. of aquaria	No. of fish/group	Treatment	Dose/kg food
Control	3	39	Basal diet	Nil
Probiotic	3	39	Basal diet + BGY35®	2 g/kg
Prebiotic	3	39	Basal diet + BioMos®	2 g/kg
Synbiotic	3	39	Basal diet + BGY35® + BioMos®	2 g/kg for each form

success of probiotics, has led to development of other concepts like Prebiotics. They represent the non-digestible food ingredients that selectively stimulate the growth and/or activity of beneficial bacteria such as bifidobacteria and lactobacilli [4]. Nowadays, there is a combined form which has been referred to synbiotic, which synergize the action of both Pre/Probiotics [5].

The range of probiotics examined for use in aquaculture has encompassed both gram-negative and gram-positive bacteria, bacteriophages, yeast and unicellular algae [6]. *Saccharomyces cerevisiae* (baker' yeast) is a good model of these organisms in both whole cell or extract forms. Benefit wise, such yeast is non-pathogenic, free of plasmid-encoded antibiotic resistance genes and resistant to bile and acidic pH.

Dietary *S. cerviceae* is the most common single cell protein incorporated within aquafeeds to efficiently minimize the need for protein of animal origin [7,8]. It has been efficient in improving the growth performance and feed efficiency rate in Israeli carp [9], seabass fry [10,11], *Oreochromis niloticus* [12,13], gilthead seabream [14] and rainbow trout frys [15]. Yeast cell, has been reported to produce some energy substrates for intestinal cells, which contribute to healthy gut. Also, the dietary nucleotides of brewer yeast have several benefits including, rapid intestinal repair, improved mucosal gut flora and mucosal surfaces with relative elongation of the intestinal tract in aquatic animals [16].

The cell wall extracts of *S. cerevisiae* (glucan, mannoprotein and chitin) are valuable, natural immunostimulants with some evidence on their role as growth promotors [17]. Incorporation of mannan-oligosaccharide in feed has improved the weight gain, feed conversion ratio and microvilli density of the posterior gut in rainbow trout [18,19]. However, some authors have reported that mannan-oligosacchride had no effcect on the growth of catfish and gilthead seabream [20,21]. On the other side, supplementation of β-glucan in feed has increased the growth rate of *Labeo rohita* [22] and failed to improve the growth performance in Nile tilapia [24].

β-glucan and chitin have been described as powerful immunostimulants in fish and mammals. They stimulate the cellular and humeral non-specific defence of fish against diseases [23,24]. Interestingly, the immunostimulant effect of yeast mannoprotein has been locally utilized by gut mucosa through its adsorption capacity to intestinal pathogens with consequent dismissal in feaces [25].

Across the long chain of studies debating the entire benefit of yeast' biotic forms , the current study was planned to evaluate the effect of whole yeast cell (BGY-35® ,probiotic), its extract (mannan oligosaccharide – Bio-Mos® ,prebiotic) and the combined mixture (BGY-35®&Bio-Mos®, synbiotic) on the growth performance and immunological response against bacterial diseases in *O. niloticus*.

2. Materials and methods

2.1. Fish sampling

One hundred fifty-six apparently healthy *O. niloticus* weighing 80 ± 5 g were stocked in 12 glass aquaria (13 fish/aquarium). The fish were acclimated for 3 weeks and fed on commercial pelletized food 25% protein twice daily at 2% of their body weight. They were maintained in aerated, de-chlorinated tap water. The water temperature was adjusted at 24 °C throughout the entire experiment. Fecal matters were siphoned out once daily and water was changed every 3 days to maintain a good water quality.

2.2. Preparation of experimental feed

Standard commercial pelletized fish ration was mixed with different feed additives using gelatin as binding material and appetizer. The study design is to be seen in (Table 1).

2.3. Growth performance and conversion ratio

Growth performance was determined and feed utilization was calculated on the following basis:

- Body weight gain: Final fish weight (g) - Initial fish weight (g) [26].
- Specific growth rate: was calculated as the percentage increase in weight per fish per day as described by Poumonge and Mbonglang [27], using the following equation:

$$\mathrm{SGR}\% = \frac{LnWT - Lnwt}{T - t} \times 100$$

- Feed conversion ratio (FCR): total feed consumed by fish (g)/total weight gain by fish (g) [27].
- Protein efficiency ratio (PER): weight gain per fish (g)/Protein intake per fish (g) [27].

2.4. Histological examination

Fresh intestinal tissue specimens were sampled from all fish groups at the end of experimental period. The tissue specimens were fixed in 10% neutral buffered formalin. The formalin fixed tissues were then processed and embedded in paraffin wax. Five micron tissue sections were then stained with hematoxylin and eosin (H&E) using the method described by Ronald [28].

2.5. Haemogram and immunological measurements

At the last day of the experiment, blood samples were collected on 100 IU/ml sodium heparin for estimation of haemogram

and phagocytic activity. Another group of blood samples were collected without anticoagulant for serum separation to be used in immunological measurements.

2.5.1. Haemogram

Erythrocytes and leukocytes were counted using hemocytometer counting chamber and Natt & Herrik solution [29]. Hemoglobin concentration and packed cell volume (PCV) were estimated using cyanomethemoglobin and microhaematocrite methods [30].

2.5.2. Phagocytic assay

Phagocytic activity was adapted from the method described by Wang et al. [31]. For preparation of viable leucocytes from peripheral blood 3 ml of heparinzed blood was carefully overlaid onto equal volume of a histopaque medium (1.077 g/ml, Sigma–Aldrich chemical. St. Louis, MO, USA) on polystyrene tube and centrifuge at 3000 rpm for 20 min. The leukocytes at the interface were collected and washed twice with (RPMI-1640 supplemented with 100 IU ml^{-1} penicillin and 1 mg ml^{-1} streptomycin). The cell precipitate was re-suspended in a modified culture medium where fetal bovine serum was replaced by 0.22 µm filtered tilapia serum (RPMI1640 supplemented with 3% (v/v) of pooled tilapia serum, 100 IU ml^{-1} penicillin and 1 mg ml^{-1} streptomycin). No. of viable cells were detected using the trypan blue exclusion method and adjusted to $4 \times 10^7 ml^{-1}$ using culture medium. One milliliter of the cell suspension was placed in cell culture and staining chamber (CCSC) containing sterile rounded cover slips. The chambers were incubated for 1 h at 37 °C in a humified CO_2 incubator. Adherent cells were then washed gently with RPMI-1640 medium for removal of non-adherent cells. 1 ml volume of (1×10^6 *Candida albicans*) suspension was added. The chambers were incubated in a humified CO2 incubator at 37 °C for further 1 h. Cover slips were washed gently three times with cooled RPMI-1640 and stained with Giemsa stain. Under the microscope, about 200 phagocytic cells were counted and differentiated using the following equations:

$$\text{Percentage of phagocytosis} = \frac{\text{No. of ingesting phagocytes}}{\text{Total No. of phagocytes}}$$

$$\text{Phagocytic index} = \frac{\text{No. of ingested } C.albicans \text{ cells}}{\text{No. of ingesting phagocytes}}$$

2.5.3. Oxygen radicals (NBT reduction activity)

The production of oxygen radicals by leukocytes was assayed by assessing the reduction of nitroblue tetrazolium (NBT, Sigma–Aldrich chemical. St. Louis, MO, USA) [32]. Blood was added to equivalent volume of NBT solution (2 mg/ml), and incubated for 30 min. Further, 5 ml of the solution was added to 0.1 ml of N,N-dimethylformamide (DMF). The mixture was centrifuged at 100*g* for 15 min and the absorbance of the supernatant was measured at 550 nm in a spectrophotometer.

2.5.4. Nitric oxide assay (reactive nitrogen species)

Fifty microliter of serum was added on an equal volume of Griess reagent in flat-bottomed 96-well plate, followed with gentle shaking. The plate was kept in dark room for 15 min at room temp. Plate was read using an ELISA reader at wave length 570. The nitrite concentration was calculated by using Na-nitrite standard curve [32].

2.5.5. Lysozyme activity

Serum samples were measured using the turbidometric method as described by Esteban et al. [33]. A 25 µl of serum was added onto 175 µl (0.75 mg/ml *Micrococcus lysodeikticus*) together with the assay buffer in flat-bottomed 96-well plates. The reduction in absorbance at 450 nm was measured from 0 to 15 min. at 25 °C using an ELISA reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1} and the units of lysozyme activity were calculated using the hen egg white lysozyme standard curve.

2.5.6. Alternative complement pathway, ACH_{50}

Alternative complement pathway was adapted from the method described by Esteban et al. [33]. Briefly, 100 µl of sheep red blood cells (SRBC) adjusted to 2.5×10^8 cells ml^{-1} was added to 100 µl of 2-fold serially diluted serum in (Mg^{+2}-EGTA-HBSS) in an ELISA plate, then plate was incubated at 25 °C for 100 min with regular shaking. The plate was then centrifuged and the extent of hemolysis was determined by measuring the optical density of the supernatant at 550 nm using an ELISA reader. Total 100% hemolysis is given by optical reading of the supernatant from 100 µl of the used SRBC suspension added to 100 µl of distilled water. The degree of hemolysis (Y) was estimated and then the lysis curve for each specimen was obtained by plotting $Y/(1-Y)$ against the volume of serum added (ml) on a $\log_{10}-\log_{10}$ graph. The volume of serum producing 50% hemolysis (ACH50) determined the number of ACH_{50} units/ml obtained for each experimental group.

2.5.7. Cytokine production

Cytokine-like protein TNF-α was measured in fish serum using quantitative sandwich human TNF-α ELISA kit [34].

2.5.8. Challenge tests

At the end of experimental period, comparative challenges of different fish groups were carried out using the following patterns:

- Ten fish from each group were I.P. injected with 0.2 ml of (2×10^8 C.F.U/ml) *Aeromonas hydrophila* [35].
- Ten fish from each group were I.P. injected with 0.2 ml of (2×10^7 C.F.U./ml) *Pseudomonas fluorescens* [36].
- Ten fish from each group were stocked in a glass aquarium containing 48 h broth culture of *Flavobacterium columnare* (2×10^9 C.F.U/ml) after removing of some fish scales [37].
- Nine fish from control group were I.P. injected with sterile saline as control negative group.

The experimentally infected fish were inspected for seven days post infection. The clinical signs/necropsy findings were recorded and bacterial re-isolation was performed. The mortality percentages were calculated.

2.5.9. Electrophoretic pattern of serum protein after bacterial infections

Serum samples from infected fish were evaluated for different protein fractions one week post infection [38].

2.5.10. Serum glucose/cortisol post bacterial infections

Serum glucose and cortisol levels were determined using colorimetric and Monobind Cortisol EIA kits [39,40].

2.6. Statistical analysis

The statistical analysis of the data collected in this study was analyzed for the mean and standard error. Significance of the results was determined by conducting one way analysis of variance (*F*-test) followed by LSD test at $P < 0.05$ [41].

3. Results

3.1. Growth performance

The final fish weight, weight gain, specific growth rate and protein efficiency ratio increased significantly ($P \leqslant .05$) in synbiotic treated group followed by prebiotic and probiotic groups versus control group (Table 2).

3.2. Histological assessment

A relatively normal intestinal histology was recorded two months post *S. cerevisiae* oral administration with colonization of yeast cells (Fig. 1A and B) together with an increase in length and density of the intestinal villi.

3.3. Hematological/immunological findings

An increase in the haemogram (erythrocytic count, packed cell volume, haemoglubin concentration and total leukocytic count) and innate immunological parameters (cellular and humeral) were recorded in treated versus control groups. According to the enhancement degree implied on both immunological & haemogram, the Synbiotic mixture was the most potential biotic form followed by probiotic then prebiotic (Table 3).

3.4. Challenge tests

The results of comparative challenge tests with the pathogenic strains of *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Flavobacterium columnare* indicated that, the survivability percentages of fish groups treated with three biotic forms were significantly increased versus positive control group. The control group has demonstrated the lowest survivability/the highest mortality percent. However, a great variation in the potency between synbiotic and the other two biotic forms were very remarkable (Table 4).

3.5. Serum protein electrophoresis after microbial infections

The results revealed significant increase in total protein and β-globulin of fish group treated with synbiotic compared to the other three groups. There was no significant difference between probiotic and prebiotic treated groups. However, both biotic forms showed much higher values than control group. Gamma globulin (γ globulin) values presented a non significant difference between treated groups; while control positive group exhibited the lowest recorded value. Also, a significant decrease in albumin level of control positive group compared to the other three treated groups and the control negative group (Table 5).

3.6. Serum glucose and cortisol levels

The Cortisol level increased significantly in control positive group compared to all other remaining groups. Fish treated groups showed insignificant differences while control negative group showed the least recorded value. Moreover, the glucose level after microbial challenges showed significant increase in treated groups compared to both control positive and negative groups. The control positive group showed hypoglycemia when compared with control negative and fish treated groups (Table 6).

4. Discussion

In the past few years, the trend of environment friendly aquaculture has been evolving globally to overcome the shortage of fish vaccines and drawbacks of chemotherapeutics. Numerous biotic forms have been widely used to boost both growth

Table 2 Growth performance of *O. niloticus* at the end of experimental period.

Items	Fish groups				
	(A) Control	(B) Probiotic	(C) Prebiotic	(D) Synbiotic	*F*-value
Initial weight	82.01 ± 3.87	82.16 ± 3.84	82.14 ± 3.39	82.15 ± 4.49	.000
Final weight	102.83 ± 4.49^{a}	119.81 ± 4.76^{b}	120.48 ± 3.52^{b}	130.12 ± 5.06^{b}	6.358
Weight gain	20.84 ± 1.85^{a}	37.45 ± 2.09^{b}	38.34 ± 1.46^{b}	47.97 ± 0.69^{c}	31.26
SGR	0.41 ± 0.03^{a}	0.68 ± 0.03^{b}	0.69 ± 0.04^{b}	0.96 ± 0.13^{c}	10.171
PER	0.83 ± 0.48^{a}	1.42 ± 0.88^{b}	1.44 ± 0.62^{b}	1.74 ± 1.46^{c}	36.98
FCR	4.9 ± 0.02^{a}	3.01 ± 0.01^{b}	2.8 ± 0.07^{bc}	2.4 ± 0.01^{c}	55

Data represented as means ± SE ($n = 13$). Within rows, values with different superscripts a,b,c & d are significantly different at ($p < 0.05$) according to one way ANOVA followed by LSD test.

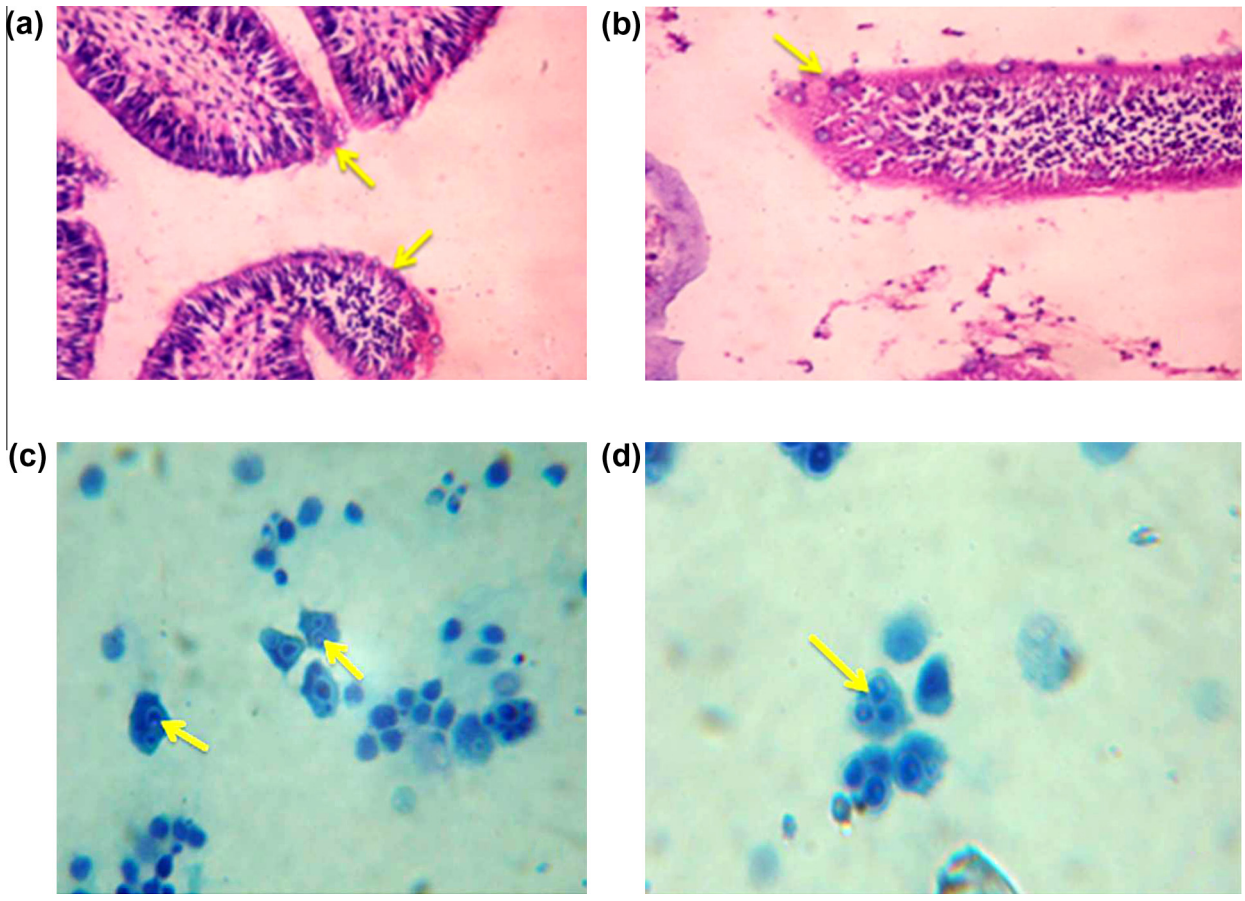

Figure 1 (A) Intestine of *O. niloticus* treated group with *S. cerevisiae* showing the colonization of yeast cells onto the epithelium of intestinal villi (H&E x400). (B) Intestine of *O. niloticus* treated group with *S. cerevisiae* showing the colonization of yeast cells onto the epithelium of intestinal villi (H&E x400). (C) Synbiotic treated Phagocytic cells engulfed more than one *C. albicans* (Giemsa stain x-1000). (D) Probiotic treated Phagocytic cells engulfed more than one *C. albicans* (Giemsa stain x-1000).

Table 3 Blood and immunological findings.

Parameter	Fish groups				
	(A)	(B)	(C)	(D)	
	Control	Probiotic	Prebiotic	Synbiotic	*F*-value
RBCs × 10^6	1.99 ± 0.04^a	2.16 ± 0.03^b	2.12 ± 0.05^b	2.28 ± 0.02^c	10.734
PCV%	26.40 ± 0.39^a	29.12 ± 0.3^b	28.78 ± 0.33^b	30.95 ± 0.13^c	41.141
Hb(g/dl)	9.03 ± 0.07^a	9.72 ± 0.21^b	9.75 ± 0.1^b	10.37 ± 0.14^c	15.421
WBCs × 10^3	41.83 ± 0.79^a	52 ± 0.86^b	51 ± 0.37b^c	53 ± 0.58^{bd}	58.009
Phagocytic index	1.52 ± 0.04^a	1.88 ± 0.04b^c	1.84 ± 0.06^b	2.00 ± 0.05^c	19.46
RNS(umol/L)	31.7 ± 0.41^a	39.57 ± 1.92^b	39.26 ± 1.58^b	44.77 ± 3.88^b	5.416
ROS	1.78 ± 0.02^a	2.02 ± 0.04^b	1.95 ±. 02^b	2.14 ±. 04^c	21.039
Lysozyme activity(µg/ml)	136.24 ± 4.12^a	154.97 ± 5.01^b	158.17 ± 1.60^b	174.68 ± 2.55^c	19.41
ACH50/ml	37.80 ± 2.38^a	133.85 ± 3.46^b	84.26 ± 3.83^c	259.44 ± 7.93^d	383.250
TNFα(pg/ml)	15.94 ± 0.13^a	134.46 ± 0.34^b	98.38 ± 2.37^c	228.03 ± 2.49^d	2532.132

Data represented as means ± SE (n = 13). Within rows, values with different superscripts a,b,c & d are significantly different at ($p < 0.05$) according to one way ANOVA followed by LSD test.

performance as well as immune response in different aquatic species. On the top list of these agents, the use of *S. cerevisiae* as a whole or extracts is globally documented.

The results of this study revealed significant increase of the weight gain and specific growth rate in all fish treated groups. This boosting potency could be attributed to the presence of *S.*

Table 4 Comparative challenge tests.

Fish groups mortality %					
Items	Control negative	Control positive	Probiotic	Prebiotic	Synbiotic
I.P. injection of (2×10^8 C.F.U/ml) *Aeromonas hydrophila*	0%	70%	40%	40%	20%
I.P. injection of (2×10^7 C.F.U/ml) *Ps fluorescens*	0%	60%	30%	40%	10%
Immersion with ($2 \times 10^{9)}$ C.F.U/ml/water pond *F. columnare* for 7 days	0%	80%	30%	50%	30%

Table 5 Serum protein electrophoresis after microbial infections.

Serum protein electrophoresis					
Fish groups	Total protein	Albumin	α	β	γ
Control + ve	1.757 ± 0.015^{a}	0.42 ± 0.05^{a}	0.25 ± 0.04^{a}	0.60 ± 0.04^{a}	0.52 ± 0.02^{a}
Control –ve	2.54 ± 0.07^{cd}	0.73 ± 0.02^{c}	0.50 ± 0.02^{b}	0.71 ± 0.03^{c}	0.60 ± 0.06^{ab}
Probiotic	2.394 ± 0.09^{b}	0.58 ± 0.01^{b}	0.22 ± 0.01^{a}	0.92 ± 0.03^{b}	0.67 ± 0.02^{b}
Prebiotic	2.302 ± 0.01^{b}	0.53 ± 0.05^{ab}	$0.29 \pm 0.03a^{a}$	0.84 ± 0.04^{b}	0.63 ± 0.01^{b}
Synbiotic	2.762 ± 0.06^{c}	0.71 ± 0.03^{c}	0.26 ± 0.01^{a}	1.11 ± 0.01^{d}	0.68 ± 0.03^{b}
F-value	35.170	12.940	13.816	38.806	4.012

Data represented as means ± SE ($n = 5$). Within columns, values with different superscripts a,b,c & d are significantly different at ($p < 0.05$) according to one way ANOVA followed by LSD test.

cerevisiae in the probiotic/synbiotic feed additives, which enhance the digestibility through providing fish with certain essential nutrients, vitamins and digestive enzymes [42].

Histologically, fish groups treated with *S. cerevisiae*, showed intestinal colonization of yeast cells with an increase in the length and density of the intestinal villi with consequent enhancement of food digestion and absorption. This hypothesis is in full agreement with similar results obtained by Gatesoupe [43] who recorded that intestinal colonization of *S. cerevisiae* on fish fry' gut accelerates the maturation of the digestive system while in older fishes stimulates the growth and metabolism. Also the dietary nucleic acids of *S. cerevisiae* have several benefits including rapid intestinal repair, improved mucosal gut flora, mucosal surface and increase the length of the intestinal tract [16].

The relative increase in growth parameters of prebiotic treated groups were similar to the results reported by Staykov et al. [18] and Stale et al. [44]. Such positive increase could be accredited to the stimulatory effects of mannan-oligosaccharide (MOS) on the beneficial *Bifidobacteria* and *Lactobacilli* with consequent improve of food digestion and assimilation [45]. The histological examination of fish intestine has demonstrated a growing enhancement of the intestinal villi' integrity, density and length. This could explain the gradual increase in weight gain of the prebiotic treated group as MOS need more time to improve the integrity of intestinal mucosa as well as to increase the density of intestinal villi. These results were consistence with the results obtained by Dimitroglou et al. [46] who indicated that the inclusion of MOS had improved the microvilli density of rainbow trout 'posterior gut. The adsorption capacity is another physiochemical property that efficiently utilized by MOS in cleaning out the fish gut from pathogens and their produced toxins [25]. Controversially, the results obtained by several authors such as Welker et al. [20] and Peterson [47] were inconsistent with our results. They had reported that the addition of MOS failed to improve the growth performance and feed conversion ratio of several fish species. The shortage of exposure time and difference of fish species could be the main difference pillar in this controversy

The results showed significant increase of the protein efficiency ratio of the fish groups treated with *S. cerevisiae* (synbiotic and probiotic) throughout the entire experiment, while the prebiotic treated group exposed significant increase at the end of second month. Reasonably, the presence of yeast cells that have been used as the most single cell protein could have induced such effect [7,8]. Further, the protease-enzyme released from aged yeast cells could have improved the protein digestibility and inhibited the intestinal bacterial toxins [48].

There was significant decrease in FCR in synbiotic, prebiotic and probiotic fish treated groups. This might be related to the presence of *S. cerevisiae* which has been literally reported to improve the enzymatic digestion of a complex polysaccharide including cellulose; organic phosphrous (phytic acid) utilization and fiber digestion [49] have the ability to produce an essential vitamin-B complex particularly Biotin and Vitamin B12. Additionally, yeast cells could have produced group of substances; namely Glutamine, Glutamic acid,

Table 6 Serum glucose and cortisol levels of different fish groups post bacterial infections.

Fish groups	Cortisol μg/dl	Glucose mg/dl
Control + ve	6.817 ± 0.93^{a}	53.33 ± 1.20^{a}
Control −ve	1.96 ± 0.29^{c}	81.33 ± 2.4^{c}
Probiotic	4.067 ± 0.41^{b}	105.00 ± 4.04^{b}
Prebiotic	3.03 ± 0.09^{b}	110.67 ± 5.81^{b}
Synbiotic	4.03 ± 0.29^{b}	114.67 ± 9.33^{b}
F-value	12.30	22.804

Data represented as means ± SE ($n = 5$). Within columns, values with different superscripts a,b,c & d are significantly different at ($p < 0.05$) according to one way ANOVA followed by LSD test.

Keto glutaric acid which can be utilized as energy substrates for intestinal cells. Ultimately, the peptide contents of yeast cells could have up regulated the fish digestive enzymes in very efficient normo-physiological way [23,25].

Blood is the patho-physiological indicator of an aquatic animal body. Therefore, blood parameters are important in diagnosing the status of fish health [30]; particularly when some additives used in feed. The highest dietary utilization and best growth rate will directly reflect on the blood parameters. An accurate assessment of fish haemogram has indicated a significant increase in erythrocytic count, packed cell volume and haemoglubin concentration in all treated fish groups. This increase in haemogram values is normally correlated to water parameters and feeding quality/quantity. Thus, the brewer yeast is a vital source of vitamin B complex as well as other haemotonic ingredients utilized in the process of haematopoisis [49,50]. The results of total leukocytic count of fish groups treated with synbiotic/probiotic showed significant increase compared to fish group treated with prebiotic and control groups. These results were fully accordant with those reported by Sang and Fotedar [51].The results could be attributed to the yeast cell wall structures mainly; β-glucan which has specific receptors on the Phagocytic cells, (heterophiles and monocytes). β- Glucan binds to receptor molecules on the surface of circulating and tissue phagocytes. Such binding will increase the phagocytic activities in engulfing, killing and digesting bacteria. Concurrently, they secrete signal molecules (cytokins) which stimulate the formation of new white blood cells. This could explain the remarkable increase in white blood cells without infection [24].

The results obtained in this study not only support the use of *Saccharomyces* yeast for better growth, and proper nutrient utilization but also support its stimulating effect on the non-specific immune response. The activation mechanisms involved could be related to the carbohydrates, derived from the yeast cell wall. Ortuñe et al. [52] stated that the yeast cell wall is constructed almost entirely of two classes of non soluble polysaccharides, mannose polymers linked with peptide (mannoprotein) and glucose polymers (glucan). The glucan and mannoprotein occur in equal amounts in the wall. The third sugar is chitin but is present only in minor amount. Not only sugar but also nucleic acid especially yeast RNA act as immune system enhancers.

Phagocytic activity is responsible for early activation of the inflammatory response before antibody production and plays an important role in antibacterial defenses. The highest Phagocytic activity and index were recorded in synbiotic and probiotic treated fish groups followed by prebiotic group (Fig. 1C and D). These findings supported those of [14,49]. These could be attributed to the stimulatory effect of yeast cell wall components, mainly β-glucan, mannoprotein; chitin and yeast RNA on the circulating and tissue macrophages. The presence of glucan and mannose are not only stimulating the phagocytosis but also increasing their destructive and killing ability [17,24].

The ability of macrophages to kill pathogenic microbes is probably one of the most important mechanisms of protection against diseases among fish. The oxygen radicals and nitric oxide are the most destructive products produced by activated macrophages. Increase of respiratory burst activity and reactive nitrogen species can be correlated with increase of oxygen and nitric oxide radicals' production and increase of killing activity [53]. In the present study, the three used biotic forms showed significant enhancement of the neutrophils/macrophages activity than in control group, which coincides with those previous publications presented by Cuesta et al. [54], Selvaraj et al. [55] and EL-Boshy et al. [56].

Lysozyme is considered as one of the important bactericidal enzymes and an indispensable tool of fish to fight infectious agents. *Saccharomyces* yeast is found to trigger the serum lysozyme level in teleost. Our research showed that the use of such biotic forms have a significant increase on the lysozyme activity in all treated fish [18,52]. Lysozyme is constitutively expressed, synthesized and secreted by neutrophils, monocytes and macrophages. The greatest concentration of lysozyme was directly proportional to the leukocytic count. In the current research, the addition of *S. cerevisiae* in fish diet has remarkably increased the leukocytic count, which in turn elevated lysozyme concentration and activity [57].

Many reliable literatures have demonstrated that alternative complement pathway (ACP) is a suitable indicator for disease resistance in fish. The statistical analysis of our achieved results in relation to ACP revealed a significant increase of ACH_{50} in the serum samples collected from synbiotic treated group followed by probiotic/prebiotic treated groups [17,52].

The last humoral innate parameter measured in this study was the cytokine production (TNF-α). The levels of TNF-α has significantly increased in whole yeast fed groups followed by the group fed on yeast extract (MOS). These positive results were in full accordance with those reported by Chansue et al. [34]. The carbohydrate components of the yeast cell wall; β-glucan attached to specific receptors on the surface of phagocytic cells; stimulate the phagocytosis and release signal molecules (cytokines), which stimulate other mediators and increase the leukocytic production [24]. Also it was detected that there are two types of receptors stimulate the phagocytosis; mannose binding receptors and glucan binding receptors. Their results indicated that β-glucan attached to glucan receptors on the circulating and tissue Phagocytic cells, while the effect of mannoprotein exerted only on the tissue macrophages as the expression of mannose binding receptor occur during monocyte-macrophage differentiation. This could explain the significant increase of TNF-α in synbiotic and probiotic treated groups, which were higher than the prebiotic treated group in all cases [17,24].

The obvious decline in fish mortalities among the three treated groups could be results of immune system activation against all kinds of pathogenic as well as opportunistic bacterial invaders. Further, the whole yeast supplement could have played a critical role in the sharp decrease of the competitor bacterial loads encircling treated fish throughout the experiment by what is called "competitive exclusion theory" [17,52,58].

Gel electrophoresis of the positive control fish serum proteins revealed a significant hypoproteinemia (hypoalbuminemia and hypoglobulinemia) due to apparent hepatic/intestinal tissue damage associated with the cytotoxic effect of bacterial toxins. On the contrary, *Saccharomyces* treated groups (synbiotic and probiotic) has revealed a significant increase of total protein and globulins especially β-globulin [59].

For decades, it has been literally established that increased blood cortisol is usually accompanied by hyperglycemia which is a common stressors' response indicator [60]. The control positive group showed significant increase in blood cortisol level with a consequent hypoglycemia. This contradictory result

could be due to the destructive effect of the bacterial toxins on the hepatic tissues with consequent retention of glycogen and inhibition of the glycogenloytic cycle. Controversially, the beneficial effects of yeast on the hepatocytes integrity have efficiently explained the remarkable hyperglycemia in treated groups with especial reference to synbiotic/probiotic [14,61].

To sum up, the efficient use of *S. cerevisiae* synbiotic forms as feed additives in cultured *O. niloticus* would have tremendous beneficial effects on both immune status as well as growth performance. Further, an economic positive outcome would be an expected end product of such beneficial effects, if these biotic forms have been efficiently/accurately added to fish diets.

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References

[1] Food and Agriculture Organization (FAO). The state of world fisheries and aquaculture 2012. Rome; 2012.
[2] Yousefian Mehdi, Amiri Mojtaba Sheikholeslami. A review of the use of prebiotic in aquaculture for fish and shrimp. Afr J Biotechnol 2009;8(25):7313–8.
[3] Balcàzar Jose luis, de Blas Ignacio, Ruiz-zarzuela Imanol, Cunningham David, verdrell Daniel, Mùzquiz José luis. The role of probiotics in aquaculture. Vet Microbiol 2006;114:173–86.
[4] RingØ E, Olsen RE, Gifstad TØ, Dalmo RA, Amlund H, Hemre GI, Bakke AM. Prebiotics in aquaculture: a review. Aquaculture Nutr 2010;16:117–36.
[5] Gatesoupe F. J. Probiotics and Prebiotics for fish culture, at the parting of the ways. Aqua feeds: formulation and beyond. 2005; 2 (3):3–5 http://www.feedware.com/index-html.
[6] Nayak S. K. Probiotics and immunity: a fish perspective''. Fish Shellfish Immunol 2010; XXX: 1–13.
[7] Tacon AGJ. Feed ingredients for carnivorous fish species: alternatives to fish meal and other dietary resources. FAO Fish Circ 1994;881:835.
[8] Oliva-Teles A, Goncalves P. Partial replacement of fishmeal by brewer's yeast (*Saccharomyces cerevisiae*) in the diets for sea bass (*Dicentrarchus labrax*) juveniles. Aquaculture 2001;202:269–78.
[9] Noh SH, Han K, Won TH, Choi YJ. Effect of antibiotics, enzyme, yeast culture and probiotics on the growth performance of Israeli carp. Korean J Anim Sci 1994;36:480–6.
[10] Li P, Gatlin MD. Evaluation of brewer's yeast (*Saccharomyces cerevisiae*) as a feed supplement for hybrid striped bass (*Morone chrysops_M. saxatilis*). Aquaculture 2003;219:681–962.
[11] Li P, Gatlin DM. Dietary brewer's yeast and the prebiotic Grobiotic™ AE influence growth Performance, immune responses and resistance of hybrid striped bass (*Morone chrysops* × *M. saxatilis*) to *Streptococcus iniae* infection. Aquaculture 2004;231:445–56.
[12] Lara-Flores M, Olvera-Novoa MA, Guzmán-Méndez BE, López- Madrid W. Use of the bacteria *Streptococcus faecium* and *Lactobacillus acidophilus*, and the yeast *Saccharomyces cerevisiae* as growth promoters in Nile tilapia (*Oreochromis niloticus*). Aquaculture 2003;216:193–201.
[13] He S, Zhigang Z, Yuchun L, Pengjun Shi, Bin Y, Einar R, Ilkyu Y. Effects of dietary *Saccharomyces cerevisiae* fermentation product (DVAQUA) on growth performance, intestinal autochthonous bacterial community and non-specific immunity of hybrid tilapia (*O. niloticus x O. aureus*) cultured in cages. Aquaculture 2009;294:99–107.
[14] Salnur Semih, Gultepe Najdet, Hossu Belgin. Replacement of fish meal by yeast (*Saccharomyces cerevisiae*): effects on Gilthead Sea bream (*Sparus aurata*). J Anim Vet Adv 2009;8(12):2557–61.
[15] Molook Pooramini, Kamali Abolghasem, Hajimoradloo Abdomajid, Alizadeh Morteza, Ghorbani Rosoul. Effect of using yeast (*Saccharomyces cerevisiae*) as probiotic on growth parameters, survival and carcass quality in rainbow trout *Oncorhynchus mykis* fry. Int Aquat Res 2009;1:39–44.
[16] Li P, Lawrence A, Castille FL, Gatlin DM. Preliminary evaluation of a purified nucleotide mixture as dietary supplement for Pacific white shrimp (*Litopenaeus vannamei*). Aquaculture Res 2007;38:887–90.
[17] Esteban MA, Rodriguez A, Mesguer J. Glucan receptor but not mannose receptor is involved in the phagocytosis of *Saccharomyces cerevisiae* by seabream (*Sparus auratus* L.) blood leucocytes. Fish Shellfish Immunol 2004;16:447–51.
[18] Staykov Y, Spring P, Denev S, Sweetman J. Effect of a mannan oligosaccharide on the growth performance and immune status of rainbow trout (*Oncorhynchus mykiss*). Aquaculture Int. 2007;5(2):153–61.
[19] Dimitroglou A, Davies S, Sweetman J. The effects of dietary mannan oligosaccharides on the intestinal histology of rainbow trout (*Oncorhynchus mykiss*). Biochem Physiol A 2008:S63.
[20] Welker TL, Lim C, Yildirim-Aksoy M, Shelby R, Klesius PH. Immune response and resistance to stress and *Edwardsiella ictaluri* challenge in channel catfish, *Ictalurus punctatus*, fed diets containing commercial whole-cell yeast or yeast subcomponents. J World Aquaculture Soc 2007;38:24–35.
[21] Arkadios Dimitroglou, Merrifiel Daniel Lee, Spring Peter, Sweetman John, Moate Roy, Davies Simon John. Effect of Mannan oligosaccharide (MOS) supplementation on growth performance, feed utilization, intestinal histology and gut microbiota of gilthead seabream, *Sparus aurata*. Aquaculture 2010;300:182–8.
[22] Misra CK, Das BK, Mukherjee SC, Pattnaik P. Effects of long term administration of dietary β-glucan on immunity, growth, and survival of *Labio rohita* fingerlings. Aquaculture 2006;255:82–94.
[23] Whittington R, Lim C, Klesius PH. Effect of dietary β-glucan levels on the growth response and efficancy of *Streptococcus iniae* vaccine in nile tilapia, *O. niloticus*. Aquaculture 2005;248:217–25.
[24] Raa J. The use of immune-stimulants in fish and shellfish feeds''. In: Cruz -Suárez, L. E., Ricque-Marie D., Tapia-Salazar M., Olvera-Novoa, M.A. y Civera-Cerecedo, R., (Eds.). Avances en Nutrición Acuícola V. Memorias del V Simposium Internacional de Nutrición Acuícola. 19–22 Noviembre, 2000. Mérida, Yucatán, Mexico.
[25] Newman, K. Form follows function in picking MOS product. Feedstuffs, 2007; 27, No 4, January 22.
[26] Annet, C.S.A model to facilitate optimal aquaculture production by quantitatively relating fish growth to feed and other environmental resources. Ph.D., thesis, Michigan State University, USA.1985.

[27] Pouomong V, Mbonglang J. Effect of feeding rate on the growth of tilapia (*O. niloticus*) in earthen ponds. Bamidegh 1993;45:147–53.
[28] Roberts J. Ronald Fish Pathology". (3rd edition) W.B. SAUNDERS London, Edinburgh, New York, Philadelphia, St. Louis, Sydney, Toronto 2001.
[29] Stoskopf, M. K. Fish medicine. Ed., W.B. Sainders Company, London. 1991.
[30] Decie SIV, lewis SM.Practical haematology. (VII Edn).1991; J and A Chuechill Ltd., Livingston, London, Melbourne and NewYork.
[31] Wang T, Yongxin S, Liji J, Yongping X, Li W, Tongjun R, Kailai W. Enhancement of non-specific immune response in sea cucumber (*Apostichopus japonicas*) by Astragalus membranaceus and its polysaccharides. Fish Shellfish Immunol 2009;27:757–62.
[32] Divyagnaneswari M, Christybapita D, Dinakaran Michael R. Enhancement of non specific immunity and disease resistance in *Oreochromis mossambicus* by *Solanum trilobatum* leaf fractions. Fish shellfish Immunol 2007;23:249–59.
[33] Esteban MA, Cuesta A, Ortuño J, Meseguer J. Immunomodulatory effects of dietary intake of chitin on gilt head seabream (*Sparus aurata* L.) innate immune system.. Fish shellfish Immunol 2001;11:303–15.
[34] Chansue N, Endo M, Kono T, Sakai M. The stimulation of cytokine-like proteins in Tilapia (Oreochromis niloticus) orally treated with β-1,3-glucan. Asian Fisheries Sci 2000;13:271–8.
[35] Badran AFM, Eissa IAM. Studies on bacterial diseases among cultured freshwater fish, *O. niloticus* in relation to the incidence of bacterial pathogens at Ismailia Governorate. Egypt. J Vet Med Ass 1991;51(4):837–47.
[36] El-Attar AA, Moustafa M. Some studies on tail rot disease among cultured Tilapia fish. Assiut Vet Med 1996;35:155–62.
[37] Soad, S. A. S. Studies on the bacterial diseases of O. niloticus reared in concrete ponds". M.V.Sc., Thesis, faculty of Vet. Medicine, Zagazig University 1999.
[38] Mahran, K.M.A. Clinicopathological and serological studies on Trichinella spiralis infection M.V. Sc. thesis, Department of clinical pathology, Faculty of veterinary medicine, Cairo University 2002.
[39] Trinder P. Determination of blood glucose using 4-aminophenazone. J Clin Pathol 1959;22:246.
[40] Yallow R, Berson S. Introduction and general consideration. In: Odell WD, Doughady WH, editors. Principles of competitive protein binding assays. J. b. Philadelphia: J. b. Lipincott Co.; 1971. p. 1–19.
[41] SAS Statistical analysis, SAS. STAT user guide version 6.2, SAS instit. INC., cary, NC.2000.
[42] Swain SK, Rangacharyulu PV, Sarkar S. Das KM Effect of a probiotic supplement on growth, nutrient utilization and carcass composition in mrigal fry. J Aqua 1996;4:29–35.
[43] Gatesoupe FJ. Live yeasts in the gut: natural occurrence, dietary introduction and their effects on fish health and development. Aquaculture 2007;267:20–30.
[44] Refstie Stale, Baeverfjord Grete, Ripman Rudi, Elvebø odd. Effect of dietary yeast cell wall β-glucan and MOS on performance, gut health and Salmon lice resistance in Atlantic Salmon"Salmo salar" fed sunflower and soybean meal". Aquaculture 2010;305:109–16.
[45] Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota introducing the concept of prebiotics. J nutr 1995;125:1401–12.
[46] Dimitroglou A, Davies S, Moate R, Spring P, Sweetman J The beneficial effect of Bio-Mos on gut integrity and enhancement of fish health. Presented at Alltech's Technical Seminar Series held in Dublin, November 2007.
[47] Peterson CB, Tyler CB, Bruce BM. Effects of Bio-Mos® on growth and survival of channel catfish challenged with *Edwardsiella ictaluri*. J World Aquaculture Soc 2010;41(1):149–55.
[48] Sultan WJ. Partical backing. 4th edn. USA: AVI publishers Inc., Co.; 1986, pp 713.
[49] Tewary Arup, Bidhan Patra C. Oral administration of baker's yeast (*Saccharomyces cerevisiae*) acts as a growth promoter and immunomodulator in Labeo rohita (Ham.). Aquaculture Research Dev 2011;2:1–7.
[50] Rajesh K, Subhas CM, Kurcheti PP, Asim K. Pal Evaluation of *Bacillus subtilis* as a probiotic to Indian major carp *Labeo rohita*. Aquaculture Res 2006;37:1215–21.
[51] Sang HM, Fotedar R. Effects of dietary beta 1,3-glucan on the growth, survival, physiological and immune response of marron, *Cherax tenuimanus* (smith, 1912). Fish Shellfish Immunol 2010;28(5–6):957–60.
[52] Ortuño J, Cuesta Alberto, Rodriguez Alejandro, Angeles Eesteban M, Meseguer Jose. Oral administration of yeast, *Saccharomyces cerevisiae*, enhances the cellular innate immune response of gillhead seabream, *Sparus aurata* L.. J Vet Immunol Immunopathol 2002;85:41–50.
[53] Sharp GJ, Secombes CJ. The role of reactive oxygen species in the killing of the bacterial pathogen *Aeromonas salmonicida* by rainbow trout macrophages. Fish Shellfish Immunol 1993;3:119–29.
[54] Cuesta A, Rodrigue, Stalinas I, Meseguer J, Esteban MA. Early local and systemic innate immune response in the teleost gilthead seabream after intraperitoneal injection of whole yeast cells. Fish Shellfish Immunol 2007;22:242–51.
[55] Selvaraj V, Sampath K, Sekar V. Administration of yeast glucan enhances survival and some non-specific and specific immune parameters in carp (*Cyprinus carpio*) infected with *Aeromonas hydrophila*. Fish Shellfish Immunol 2005;19:293–306.
[56] El-Boshy Mohamed, El-Ashram Ahmed M, Abdel Hamid Fatma M, Gadalla Hossam A. Immunomodulatory effect of dietary *Saccharomyces cerevisiae*, β-glucan and Laminaran in mercuric chloride treated Nile tilapia (*Oreochromis niloticus*) and experimentally infected with *Aeromonas hydrophila*. Fish shellfish Immunol 2010;28:802–8.
[57] Yano T. The non-specific immune system humoral defence. In: Iwama GK, Nakanishi T, editors. Fish Physiology, vol. 15. NY: Academic press; 1996.
[58] Verschuere L, Rombaut G, Sorgeloos P, Verstraete W. Probiotic bacteria as biological control agents in aquaculture Microbiology and Molecular biology Review 2000;64(4):655–71.
[59] Andrews S.R., N.P. Sahu A.K. Pal S.C. Mukherjee, Shivendra Kumar. Yeast extract, brewer's yeast and spirulina in diets for *labeo rohita* fingerlings affect haemato-immunological response and survival following *Aeromonas hydrophila* challenge Research in veterinary science Article in press.
[60] Barton BA, Iwama GK. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annu Rev Fish Dis 1991;10:3–26.
[61] José Gil Barcellos L, Luiz CK, Laura BR, Irineo F, Rosmari MQ, Leonardo C, Jaqueline C, Auren BS, Michele F, Luciana DL, Luciana DL, Terra Silvia. Haematological and biochemical characteristics of male jundiá (Rhamdia quelen Quoy & Gaimard Pimelodidae): changes after acute stress. Aquaculture Res 2003;34:1465–9.