Role of dietary vitamin C and yeast *Saccharomyces cerevisiae* as an Immunostimulants and probiotics on cultured *Oreochromis niloticus*

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Abstract: The aim of this study to determine the impact of dietary vitamin C and yeast *Saccharomyces cerevisiae* as an Immunostimulants on cultured *Oreochromis niloticus,* present study was carried out on three groups each 20 fish The first 2 groups received a supplemented diet with vitamin C and yeast *Sacchromyces cerevisiae,* while the remaining group served as a control one. The 1st group fed a diet containing vitamin C 500 mg / kg diet while the 2nd group fed a diet containing yeast *S .cerevisiae* 1.0 g / kg diet for two weeks. Present study revealed that Phagocytic activity and phagocytic index of *O. niloticus* fed on diet contain vit. C and yeast *S. cerevisiae* for 2 weeks were significantly elevated than the control group also lyzozyme concentration and serum albumin, globulin and A/G ratio.
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Key words: vitamin C- yeast *Saccharomyces cerevisiae* - *Oreochromis niloticus*- Immunostimulants- Phagocytic activity- phagocytic index – lyzozyme-albumin-globulin- A/G ratio.

Introduction:

The innate immune system is of prime importance in the immune defence of fish. It is commonly divided into 3 compartments: the epithelial/mucosal barrier, the humoral parameters and the cellular components. The epithelial and mucosal barrier of the skin, gills and alimentary tract is an extremely important disease barrier in fish, being constantly immersed in media containing potentially harmful agents. As well as providing physical and mechanical protection the fish mucus contains several immune defence parameters including antimicrobial peptides, complement factors and immunoglobulins (Aranishi and Mano 2000; Smith et al. 2000; Ellis 2001; Hatten et al. 2001; Fast et al. 2002; Suzuki et al. 2003; Magnadottir 2006; Whyte 2007; Subramanian et al. 2007; Subramanian et al. 2008). The humoral parameters are either expressed as cell receptors or as secreted soluble forms. These include the complement system, which is well developed in fish and comprises the alternative, lectin and classical pathways. The three pathways can terminate in membrane attack complex and cell lysis or enhance phagocytosis by opsonisation of the pathogen and activation of the adaptive immune response through the classical pathway (Nonaka and Smith 2000; Boshra et al. 2006). Another method for disease prevention has been investigated, such method is the immunostimulant therapy. It based on non specific stimulation of the immune system, causes an overall immune response that hastens recognition of foreign proteins, restimulates the normal immune response after a period of immune suppression (**Campos *et al.*, 1993; and Sordello *et al.*, 1997**).

Immunostimulants stimulate the macrophage immune – force of the fish to eliminate unwanted pathogens in their blood stream.Furthermore, it also relieves allergies that may occur due to depressed functionality of the immune system by supplementing the fish's diet with these immune stimulants. The use of Immunostimulants for prevention of diseases in fish is considered an important attractive and promising field (**Friedmann *et al.*, 2000**).

Several studies were performed on the yeast *Saccharomyces cerevisiae* as Immunostimulants and concluded that addition of *S. cerevisiae* to the common fish diet activates phagocytic activity and phagocytic index (**Ortuno *et al.*, 2002 and Abdel-Tawwab *et al.*, 2008).** The role of vitamin C as an antioxidant is also important for inactivating harmful free radicals produced through normal cellular activity. The antioxidant function of vitamin C could in part, at least, enhance immunity by maintaining the functional and structural integrity of important immune cells **(Alves**

***et al.*, 2007 and Affonso *et al.*, 2007)** Thus, the present study aimed to investigate application of dietary vitamin C (ascorbic acid) and yeast *Saccharomyces cerevisiae* as an Immunostimulants on cultured *Oreochromis niloticus*.

Materials and methods :

Fish:

A total number of 90 apparently healthy Nile tilapia *Oreochromis niloticus* collected from a private fish farm. The average body weight was 80 ± 5g. They were transported alive to the laboratory, three fully prepared glass aquaria measuring 50x50x100 cm^3 were used and provided with aerating devices and thermostatic heaters (for challenge experiment), aquaria were filled with de-chlorinated water, each contains 20 fish. From which 30 fish were used for experimental infection. The diet was formulated to contain 30 % crude protein composed of fish meal, soybean meal, wheat bran, yellow corn, wheat flower, cod liver oil, vitamins and mineral mixture. The diet formulated in small pellets and was provided at 3 % of the body weight as described by **Eurell *et al* (1978).**

Experimental design:

Seventy five of cultured *Oreochromis niloticus* fish were divided into three groups (each 20 fish) and acclimated for one week, three fully prepared glass aquaria measuring 50x50x100 cm^3 were used for holding fish during the experiment. The first 2 groups received a supplemented diet with vitamin C and yeast *Sacchromyces cerevisiae,*while the remaining group served as a control one. The 1st group fed a diet containing vitamin C with dose; 500 mg / kg diet while the 2nd group fed a diet containing yeast *S .cerevisiae* 1.0 g / kg diet for two weeks. Samples of blood and serum were taken for the followed four weeks.

Vitamin C (Ascorbic acid)**:** from Adwia company**.**

Yeast (*Saccharomyces cerevisiae*): (sigma) Company.

Both Vitamin C and yeast were dissolved in oil then mixed well with the diet and stored in refrigerator (4°C) till used .

Bacterial strain:

A well identified virulent *Aeromonas hydrophila* strain was freshly sub-cultured for experimental infection.

Yeast strain:

A well identified *Candida albicans* Strain and the refreshment was displayed by Sabauroud's dextrose broth (**Oxoid 1982**).

Feeding regime:

Either control or vit C & Yeast supplemented diets were administrated at a feeding rate of 300 g diet biomass / day for two weeks, and then fed with free diet with the same feeding rate till the end of the experimental period.

Sampling and blood collection:

Blood samples were collected on days 7, 14, 21, and 28. five fish randomly collected from each treatment groups and control group, part of them used for whole blood collection using anticoagulant (EDTA 10%) and the other half used for serum collection without adding anticoagulant.

Whole blood collection:

Whole blood was collected from the caudal vessels according to **Rowley (1990)** which used for Assessment of cellular immune response through:

Phagocytosis assay: (phagocytic activity and phagocytic index).

Phagocytosis was determined according to **Kawahara *et al.* (1991)**

Phagocytic activity (PA) = Percentage of phagocytic cells containing yeast cells.

Phagoytic index (PI) =

$$\frac{\text{Number of yeast cells phagocytized}}{\text{Number of phagocytic cells}}$$

Lysozyme assay:

Lysozyme assay was determined according method described by **Ellis (1990)** with minor modification as follow, 15 ml 1% (W/V) agarose gel in 0.07 M NaH_2Po_4 / Na_2HPo_4 buffer, pH (6.2) containing 50 µg/ml ***Micrococcus lysodictecus*** were prepared and poured onto 10×10 cm diameter clean glass plates. Rows of 3mm diameter holes in agarose were punched. Two fold serial dilutions of (**HEWL**) original concentration 1.6 µg/ml in phosphate buffered saline were added to wells as standard.The test samples were applied to wells in 25µl volumes at 25°C and then lifted for 17 h (over-night) at the same temperature. The diameter of cleared zones for both standard and tested samples was measured. The concentration of lysozyme in the sample was the read off as standard graph compiled from the (**HEWL**).

Determination of serum total protein:

Serum total proteins were determined according to **Doymas *et al.* (1981)** using commercial kits produced by Pasteur Lab (France).

Disease resistance (experimental infection):

Studying the effect of vitamin C and yeast *S. cerevisiae* on disease resistance was done. Fish were challenged with *A.hydrophila* strain using 24 hrs broth culture. As follow, Two weeks after the last administration of medicated diets, ten (10) fish from each treated group were injected I/P with 0.2ml /24 hrs broth culture of *A.hydrophila* strain containing 3×10^7 viable cells/ml. 20 fish from control group were collected, 10 fish were injected I/P with *A.hydrophila* strain containing 3×10^7 viable cells/ml, the other 10 were injected I/P with 0.2 ml phosphate buffered saline (PBS). Mortalities were recorded daily for 7 days, clinical signs and Postmortem findings were also monitored and recorded.

Results

Assessment of cell mediated immune response.

1- Effect of vitamin C and yeast *S. cerevisiae* supplemented diet on phagocytosis assay :

Phagocytic activity and phagocytic index of *O. niloticus* fed on diet contain vit. C and yeast *S. cerevisiae* for 2 weeks were shown in table (1).

1- vitamin C supplemented diet :

Phagocytic activity displayed significant increase in the1st , 2nd and 3rd week than the control group but in the 4th week there was non significant difference while the phagacytic index revealed significant increase in the four weeks than the control group .

2- yeast *S. cerevisiae* supplemented diet:

Phagocytic activity revealed significant increase in the1st , 2nd and 3rd weeks than the control group while in 4th week there was significant increase than the control group and that fed diet contain vitamin C while phagocytic index revealed significant increase comparing to the control group in the four weeks, there was also significant increase than the group fed on diet contain vit. C .

2- Effect of vitamin C and yeast *S. cerevisiae* on protein gram of *O. niloticus* fed for 2 weeks :

Serum total protein, serum albumin, serum globulin, and A/G ratio of *O. niloticus* fed on diet contain vitamin C and yeast *S. cerevisiae* for 2 weeks was shown in table (2).

1- vitamin C supplemented diet :

Serum total protein and globulin were showed significant increase in the1st , 2nd and 3rd weeks than the control group but in the 4th week there was non significant increase while albumin revealed significant increase for 2 weeks only than the control group while A/G ratio displayed significance for the first week only .

2- yeast *S. cerevisiae* supplemented diet:

Serum total protein and globulin were revealed significant increase along the four weeks of the experiment than the control group while globulin and A/G were revealed significant increase in the1st, 2nd and 3rd weeks than the control group there were non significant difference than the group fed on vit. C supplemented diet in total serum protein, albumin, globulin while A/G ratio there was significant difference than the group fed on vit. C .

3- Effect of vitamin C and yeast *S. cerevisiae* on the lysozyme concentration of serum of *O. niloticus*:

Lysozyme concentration of serum of *O. niloticus* fed on diet contain vitamin C and yeast *S. cerevisiae* for 2 weeks shown in table (3) .

1- vitamin C supplemented diet :

In the 1st,2nd and 3rd week there was significant increase than the control group while in 4th week there was non significant increase than the control group .

2- yeast *S. cerevisiae* supplemented diet:

In the 1st,2nd and 3rd week there was significant increase than the control group while in 4th week there was non significant increase than the control group there was non significant difference than the group fed diet contains vit.C .

4-Disease resistance (immunocompetance test):

Mortality rates of *O.niloticus* fed on vit. C and yeast *S. cerevisiae* for 2 weeks shown in table (4) . There was a decrease in mortality rates in the group fed on yeast *S. cerevisiae* compared to control (+ve) group challenged with *A. hydrophila* and fed on

diet without vit.C and yeast *S. cerevisiae* which reached its highest level on *O. niloticus* (90%). It was followed by the group fed on diet contained vit. C (40%) followed by group fed on diet contained yeast *S. cerevisiae* (20%) whereas no mortalities were recorded in control (-ve) group .

Clinical signs and postmortem findings:

The clinical pictures and postmortem of experimentally included fish of group fed on diet with vit. C and group fed on diet with yeast *S. cerevisiae* and control groups were nearly similar but varied in the severity of developed lesions. They included poor appetite, loss of equilibrium with erratic movement in some fish. Abdominal distension and finally loss of all reflexes just prior to death. Also presence of congestion and hemorrhage on fins, under the dorsal fin, caudal peduncle, body sides and ventral abdominal wall with hemorrhagic and protruded anal opening . Internally, congestion of all internal organs with yellowish serous fluid in the abdominal cavity was found enlarged liver with hemorrhagic patches, distended gallbladder with bile, enlarged and congested spleen. Kidney appeared swollen and congested while gills varied from pale anemic in some cases to congested in other cases .

Table (1) Showing effect of vit C and yeast *S.cerevisiae* on phagocytosis assay in *O. niloticus* :

Treatment / week	control		Vit. C		Yeast	
	phagocytic activity %	phagocytic index	phagocytic activity %	phagocytic index	phagocytic activity %	phagocytic index
1	20.00 ± 0.81 A	3.58 ± 0.15 A	29.50 ± 1.20 a	8.70 ± 0.23 aB	33.50 ± 1.30 a	13.25 ± 0.37 ab
2	20.10 ± 0.90 A	3.60 ± 0.09 A	28.10 ± 1.31 a	6.50 ± 0.16 aB	28.70 ± 1.25 a	10.64 ± 0.25 ab
3	20.20 ± 0.80 A	3.63 ± 0.08 A	25.70 ± 1.05 a	5.85 ± 0.15 aB	28.20 ± 1.10 a	8.34 ± 0.18 ab
4	20.10 ± 0.85 A	3.61 ± 0.10 A	23.50 ± 0.90 B	4.90 ± 0.10 aB	26.70 ± 0.82 ab	7.75 ± 0.12 ab

Small letters (a) and (b) represent a significant change to capital latters A and B respectively (by LSD using ANOVA at $p \leq 0.05$)

Table (2) Showing effect of vit C and yeast *S.cerevisiae* on proteinogram, in *O. niloticus*:

Treatment / weeks	control				Vit. C				Yeast			
	serum total protein (g/dl)	serum albumin (g/dl)	Serum globulin (g/dl)	A/g ratio	serum total protein (g/dl)	serum albumin (g/dl)	Serum globulin (g/dl)	A/g ratio	serum total protein (g/dl)	serum albumin (g/dl)	Serum globulin (g/dl)	A/g ratio
1	A 5.81 ± 0.07	A 4.10 ± 0.07	A 3.71 ± 0.08	A 3.27 ± 0.02	a 6.49 ± 0.12	a 4.42 ± 0.10	a 4.07 ± 0.07	a 3.13 ± 0.03	a 6.75 ± 0.14	a 4.52 ± 0.10	a 4.23 ± 0.10	a 3.10 ± 0.03
2	A 5.81 ± 0.08	A 4.11 ± 0.08	A 3.70 ± 0.09	A 3.26 ± 0.04	a 6.32 ± 0.10	a 4.35 ± 0.17	a 3.97 ± 0.08	3.18 ± 0.03	a 6.58 ± 0.11	a 4.41 ± 0.07	a 4.17 ± 0.08	a 3.16 ± 0.02
3	A 5.83 ± 0.09	A 4.12 ± 0.06	A 3.70 ± 0.07	A 3.28 ± 0.03	a 5621 ± 0.10	4.31 ± 0.09	a 3.93 ± 0.06	3.20 ± 0.02	a 6.42 ± 0.12	a 4.37 ± 0.08	a 4.05 ± 0.08	a 3.19 ± 0.03
4	A 5.78 ± 0.08	4.10 ± 0.07	A 3.68 ± 0.06	3.25 ± 0.03	6.01 ± 0.09	4.23 ± 0.07	3.78 ± 0.07	3.23 ± 0.02	a 6.26 ± 0.09	4.30 ± 0.08	a 4.96 ± 0.08	3.22 ± 0.04

ll Small letters (a) and (b) represent a significant change to capital latters A and B respectively (by LSD using ANOVA at $p \leq 0.05$)

Table (3) Showing the effect of vit C and yeast *S.cerevisiae* on serum lysozyme concentration µg/ml of *O. niloticus*

Treatment. / week	control	Vitamin C	Yeast
1	375.75 ± 8.50 A	399.56± 11.23 a	436.70±12.15 a
2	373.65 ± 9.53 A	398.18 ±11.62 a	422.64±11.17 a
3	373.20 ± 9.24 A	396.53 ± 9.17 a	399.25±10.05 a
4	376.50 ± 10.62 A	388.64 ± 9.50 a	392.40±10.15 a

Small letters (a) and (b) represent a significant change to capital latters A and B respectively (by LSD using ANOVA at $p \leq 0.05$ Each value represents mean ± SE; n=5).

Table (4) Showing results of experimental infection in *O. niloticus* by *A. hydrophila* fed on vit C and yeast *S.cerevisiae*:

Fish group	No. of fish	Type of inoculation	Dose	Died fish during 7 days after injection							No. of survived fish	Survival %
				1	2	3	4	5	6	7		
Control (-ve)	10	0.2 ml PBS*	---	0	0	0	0	0	0	0	10	100
Control (+ve)	10	0.2 ml A. hyd**.	$3x10^7$	6	1	1	0	0	0	0	1	20
Yeast S. *cerevisiae*	10	0.2 ml A. hyd.	$3x10^7$	0	1	1	0	0	0	0	8	80
Vit. C	10	0.2 ml A. hyd.	$3x10^7$	2	0	1	0	1	0	0	6	60

*PBS= phosphate buffer saline **A. hyd.= *Aeromonas hydrophila*

Discussion :

concerning the effect of vit. C and yeast on phagocytic activity and phagocytic index, the present study revealed that vit. C and yeast were significantly increased for 3 weeks than the control group, the result nearly agree with the result obtained by **Nevien (2005)** who reported that *O. niloticus* fed on *S. cerevisiae* supplemented diet showed increased phagocytic activity and index, similar results were obtained by **Siwicke *et al* (1994 a)** who observed an increase of some cellular activities in rainbow trout after feeding *S. cerevisiae* at a dose of 27 g/kg diet for one week. The enhanced cellular activity could be attributed to the presence of glucan receptors on the cell surface of blood monocytes, macrophages and neutrophiles which mainly responsible for phagocytic activity and phagocytic index. These finding go hand by hand with the results obtained by **Yoshida *et al.*, (1995)** in African catfish who reported an increase in phagocytic activity and index after feeding with ß – glucan supplemented diet at a dose of 1 g/kg diet for 45 days. The cellular activity peaked after 2-3 weeks of feeding then decreased slightly till dropped to baseline level at 45 day.

Simillary **Jeney *et al.*, (1997)** on rainbow trout support our results as they determined that feeding of different doses of ß – glucan for 4 weeks caused significant increase in monocytes and neutrophiles count and activity which reach their peak at low and medium doses also phagocytic activity and index enhanced in all groups this may attributed to the mode of action of glucan on phagocytic cells which activated after engagement of ß – glucan to its specific receptors on surface of phagocytic cells supporting this hypothesis (**Cook *et al.*, 2001**)

On the other hand, the result of present study revealed that vit. C affect significantly on phagocytic activity and index. This result agree with that of **Kumari and Sahoo (2006)** who reported that feeding of vitamin C for Asian catfish (*Clarias batrachus*) significantly enhanced phagocytic activity compared to their

respective control, similar results obtained earlier in other fish species **(Findlayand Munday 2000, Sahoo and Mukherjee 2001 a,b, Kumari *et al.*, 2003 and Kumari and Sahoo 2005b)** also **Blazer (1982)** who reported that feeding of rainbow trout at doses of 120, 1200 mg/kg diet for 12 weeks significantly enhanced phagocytosis at 1200 mg/kg diet, also **Robertsen *et al.*, (1995)** demonstrated that, feeding of turbot with vit. C (800, 2000 and 4000 mg/kg diet) for 18 weeks leading to enhanced phagocytic activity at dose of 800 mg/kg while phagocytic activity and serum lysozyme were markedly increased at dose of 2000 mg/kg diet. In contrast, no effect on phagocytic activity on 4000 mg/kg diet.

Regarding to the concentration of total protein in serum present result revealed that dietary vit. C increase total protein of *O. niloticus* for 3 weeks while yeast *S. cerevisiae* affect significantly along four weeks of the experiment. The results nearly agree with the result of **Nayak *et al.*, (2007)** who reported that dietary supplementation of vit. C in the form of ascorbyl polyphosphate and probiotic bacterium "*Bacillus subtilis*" and their combination for Indian major carp, rohu (*Labeo rohita Ham*) fingerlings fed for a period 60 days. serum protein and globulin content were significantly higher than the control group and higher in the group of vit. C than the other groups. They attributed that to the production of oxygen radicals in the ascorbyl polyphosphate group which in turn increase the metabolic activity increasing serum total protein and globulin.

Also **Tongjun *et al.*, (2007)** reported that the administration of dietary vit. C to Japanese eel (*Anguilla japonica*) revealed that significant increase of total serum protein than the control group when fed at a dose 762 mg/kg diet they suggesting that vitamin C can interact with other antioxidants such as vit. E as they function as water – soluble and lipid – soluble chain breaking antioxidants, They protect lipids, protein and membranes of cells from oxidative damage. It also scavenges oxygen radicals in aqueous phase, (**Niki 1987 and Mccay, 1985**). On the other hand the present study revealed that yeast was of better effect on concentration of serum total protein these was in agreement of **Abdel – Tawwab *et al.*, (2008)** who reported that total serum protein significantly increased when *O. niloticus* fed on commercial live baker's yeast *S. cerevisiae* when fed 1 g/kg diet also albumin and globulin were significantly increased than the control group. This may be attributed to that baker's yeast is a source of nucleic acid and ß – 1.3 glucans which have been recognized to be effectively enhance immune function of African catfish (**Yoshida *et al.*, 1995**) Atlantic salmon (**Engstad *et al.*, 1992**) rainbow trout (**Jorgensen *et al.*, 1993 a; Siwicki *et al.*, 1994**).

Cocerning to the effect of vit. C and yeast on lysozyme concentration in serum, the present study displayed the effect of both vit. C and yeast *S. cerevisiae* as the same on cultured *O. niloticus* increasing significantly for 3 weeks. The result nearly agree with which obtained by **Tongjun *et al.*, (2007)** who reported that dietary vitamine C for japanisi eel *Anguilla japonica* at the dose 762 mg/kg diet for 3 weeks showed significantly higher lysozyme activity of mucus and serum compared to control group Also **Ai *et al* (2004)** who reported that post dietary administration of vit. C for Japanese seabass *Lateolabrax japonicus* at 489 mg/kg diet for 8 weeks there was significant increase of lysozyme activity in serum with the increase of dietary ascorbic acid. Moreover **Robertsen *et al.*, (1995)** who reported that feeding of turbot with vitamin C (800, 2000 and 4000 mg/kg diet for 18 weeks) leads to enhanced phagocytic activity at dose of 800 mg/kg whole phagocytic activity and serum lysozyme were markedly increased at dose of 2000 mg/kg diet **Verlhac *et al.*, (1998)** observed that feeding of rainbow trout with vit. C supplemented diets at doses of (150 and 1000 ppm) caused a significant increase in lysozyme activity at dose of 1000 ppm while 150 ppm was non effective. This may be due to the role of vitamin C as an antioxidant is important for inactivating harmful free radicals produced through normal cellular activity. The antioxidant function of vitamine C could in part, at least enhance immunity by maintaining the functional integrity of important immune cells (**Chew, 1995**).

On the other hand, **Jorgensen *et al.*, (1993 b)** reported that rainbow trout injected with ß – 1.3 glucan showed enhanced bactericidal activity of head

kidney macrophages with increased serum lysozyme concentration lysozyme and complement activity can also be activated by several immunostimulants as glucan (**Engstad *et al*., 1992**) vitamine C (**Wagboo *et al* 1993**) moreover, **Sakai *et al*., (2001)** reported that the nucleotides from brewer's yeast RNA were capable of enhancing the phagocytic and oxidative activities of kidney phagocytic cells, serum lysozyme in common carp.

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