

## THE INFLUENCE OF SOME PROBIOTICS ON THE GROWTH PERFORMANCE AND INTESTINAL MICROBIAL FLORA OF *O. NILOTICUS*

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### **Abstract**

The objective of the present study was to understand the influence of some types of probiotics on the growth parameters and the intestinal microbial flora of *O. niloticus*. The fish were divided into three groups, the first one fed on diet supplemented with dead *Saccharomyces cerevisiae* yeast, the second group fed on diet supplemented with live *Bacillus subtilis* and *Saccharomyces cerevisiae* while the third group was served as control fed on basal diet. After six weeks the results indicated that, the fish groups received probiotics supplemented diets revealed significant improvement in growth parameters (body Weight gain, feed conversion ratio, protein efficiency ratio, protein efficiency ratio ). On the other hand, the examination of intestinal microbial flora pre – and post- addition of probiotics showed that, the intestinal of the second group at the end of experimental period showed failure in re-isolation of some pathogenic bacteria. Dead *S. cerevisiae* supplemented diet has no effect

**Key Words:** Probiotic, *S. cerevisiae*, *B. subtilis*, intestinal flora, growth parameters

### **INTRODUCTION**

As a negative impact to the success of aquaculture, increased intensification has led to higher disease outbreaks. Bacterial diseases are the major cause of economic losses affecting fish farms. To combat these diseases, widespread use of broad-spectrum chemotherapeutics has led to drug resistance problems in aquaculture and created human health hazards. In order to rectify this situation, greater emphasis has been placed on improved husbandry through better nutrition, improving water quality and lowering stocking densities, using of vaccines and biological control agents which depend on the fact of microbial antagonism (Robertson *et al.*,2000).The bacteria present in the aquatic environment influence the composition of the gut biota and vice versa as the host and micro-organisms share the eco system(Verschuere *et al.*,2000).So it is preferable to give probiotics to the fish in larval stage, because the larval forms of most fish and shellfish are released in the external environment at an early ontogenetic stage, these larvae are highly exposed to gastro intestinal-associated disorders, because they start feeding even though the digestive tract is not yet fully developed and the immune system is still incomplete 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 have been evaluated as a probiotics (Gastesoupe, 1999). Several studies have demonstrated certain modes of probiotic action in the aquatic environment. 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). From the fore mentioned data this study was planned to evaluate the effect of some probiotics on the fish growth and the intestinal bacterial flora especially the pathogenic one.

## MATERIALS AND METHODS

### 1 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°C ± 2 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.

### 3. Probiotics

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

#### ➤ **Diamond-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 *Saccharomyces cerevisiae*                      1 trillion c. f. u.

*Bacillus Subtilis*    400 million c. f. u.

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

### 4. Media

#### A. Liquid media (broth)

- a. Selenite- f- broth (Oxoid)
- b. Brain heart infusion broth (Oxoid)

#### B. Solid media (plating agar media)

- a. MacConky agar (Oxoid)
- b. Hekton enteric agar (H.E.) (Oxoid).
- c. Xylose lysine desoxycholate (XLD) (Oxoid).
- d. Pseudomonas agar base with supplement (Oxoid).
- e. Aeromonas agar base with supplement (Himedia).

f. Sabourad's dextrose agar medium: Sabourad's dextrose agar medium containing chloramphenicol was used for cultivation of *C. abicans*. (oxid)

#### C. Semi solid media

Soft agar (0.4 gram) was used to detect the bacterial motility as well as for the preservation of all isolates of bacteria (Cruikshank *et al.*, 1975).

All these media were used for isolation and identification of the bacterial flora in the fish gut.

## 2 Methods

### 1-Experimental design.

One 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*.

### 2-Growth parameters

**a- Growth weight:** estimated biweekly throughout the experimental period

**b- Body weight gain:** Final fish weight (g) - Initial fish weight (g) ( Annet , 1985).

**c- Specific Growth Rate %:** It was calculated as the percentage increase in weight per fish per day as suggested by Pouomonge and Mbonglang (1993), using the following equation:

$$\text{SGR \%} = (\ln \text{WT} - \ln \text{Wt}) / (\text{T} - \text{t}) \times 100$$

Where:

SGR%=Percentage increase in body weight per fish per day.

Ln WT=natural log of weight at time T.

Ln Wt= natural log of initial weight.

T=time T, t=initial time, Ln=Natural Logarithm.

**d- Feed Conversion Ratio (FCR)**

$$\text{FCR} = \frac{\text{Total feed consumed by fish (g)}}{\text{Total weight gain by fish (g)}}$$

As reported by De Silvia and Anderson (1995).

**e- Protein Efficiency Ratio (PER)**

$$\text{PER} = \frac{\text{Weight gain per fish (g)} \times 100}{\text{Protein intake (g)} \times 100}$$

As reported by De Silvia and Anderson (1995)

**3-Microbiological examinations****a. Isolation of bacteria from the gut of the fish pre- and post- oral administration of probiotics according to Himabindu *et al.* (2004)**

Two fish from each group were collected and externally disinfected with alcohol 70%. The gut was removed carefully and was divided into three parts (anterior, middle and posterior). Each part was transferred to the brain heart infusion broth (BHI) and Selenite-F broth (as enrichment of *Salmonella spp.*). The broth was incubated at 27°C for 24 hours. Mc Conky agar, H.E., XLD, Aeromonas agar base with supplement and Pseudomonas agar base with supplement were prepared and inoculated by streaking from the broth. Bacterial colonies were subjected for further purification to obtain the same colonies on each plate.

**B-Bacterial identification**

- Staining reaction (Gram positive and Gram negative bacteria).
- Oxidase test: used to differentiate between oxidase positive and oxidase negative bacteria.
- Detection of the bacterial motility using semisolid media (soft agar).
- Biochemical identification.

**RESULTS****1- GROWTH PARAMETERS**

At the end of experimental period both groups received probiotics supplemented diets revealed significant increase in the body weight gain (B.W.), specific growth rate (SGR), protein efficiency ratio (PER) and condition factor (K) which represented the relationship between the body weight and body length. A significant decrease in feed conversion ratio (FCR) in comparison with control group was found. These results are demonstrated in table (2) and Fig. (1).

Table 2. Growth parameters of *O. niloticus* after (6 weeks)

Studied groups	Body weight gain (g)		Specific growth rate (g/day)	Feed conversion ratio (FCR)	Protein efficiency ratio (PER)	Condition factor (k)
	Initial weight	Weight gain				
Control group	27±0.86 <sup>a</sup>	5.8±0.25 <sup>b</sup>	0.0046	5.8655	23.868	1.61±0.03 <sup>c</sup>
Group I	25.6±1.51 <sup>a</sup>	10.7±1.58 <sup>a</sup>	0.0074	3.0146	46.44	2.01±0.05 <sup>a</sup>
Group II	27.6±1.42 <sup>a</sup>	12.3±1.45 <sup>a</sup>	0.0096	4.7379	49.517	1.8±0.07 <sup>b</sup>
F-values	0.62	7.34**				15.75**

Data are represented as means of twenty samples ± S

Means within the same column, with the same letter for each parameter are not significantly different, otherwise they do (Duncan's multiple range test, 1955)

\*\* Highly significant difference (P < 0.01)

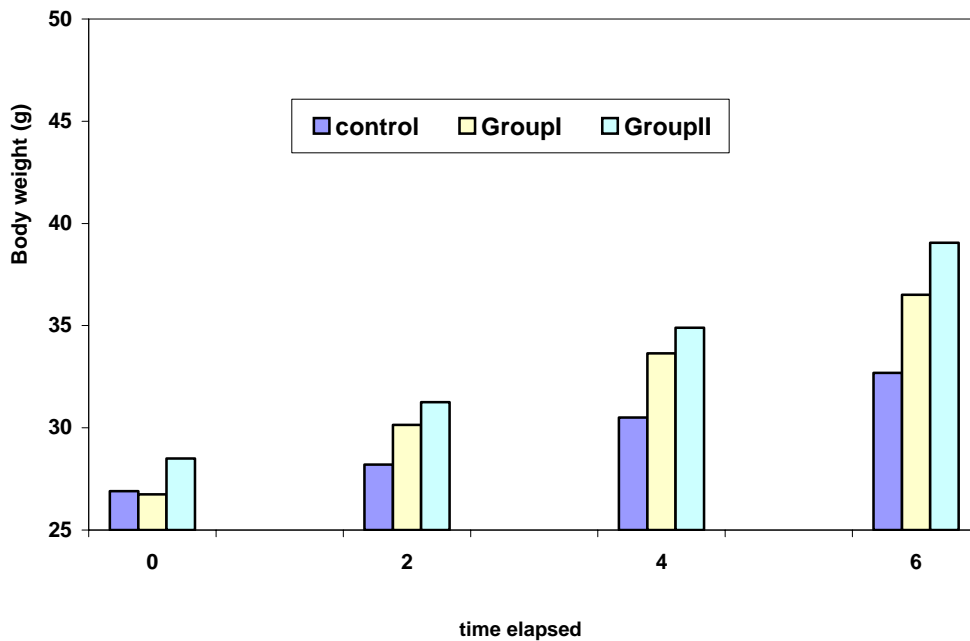


Fig. 1. Average body weight of *O. niloticus* during 6 weeks

## 2 - INTESTINAL BACTERIAL FLORA PRE- AND POST- USING OF PROBIOTICS

The results of isolation and identification of bacterial organisms from *O. niloticus* intestinal tract pre and post application of diets supplemented with probiotics revealed the presence of colonies with different morphological characters on different solid media. The pure colonies were identified by their different biochemical criteria which denoted the presence of different bacterial flora.

The results of bacterial identification pre treatment revealed the presence of *E.coli* (motile form), *E.coli* (non motile form), *Salmonella spp.*, *Klebsiella spp.*, *Morganella morganii*, *E.tarda*, *Aeromonas sobria* and *P. fluorescens*. On the other hand the intestinal bacterial flora post treatment with probiotics revealed the same organisms of the pre-treatment in *O. niloticus* received dead *S. Cerevisiae* while in those received live *S. Cerevisiae* and *B. subtilis* ,failure in resolution of lactose fermenting *E.coli*, *Salmonella spp*, *Klebsiella spp.* and *P. fluorscens* was proved.

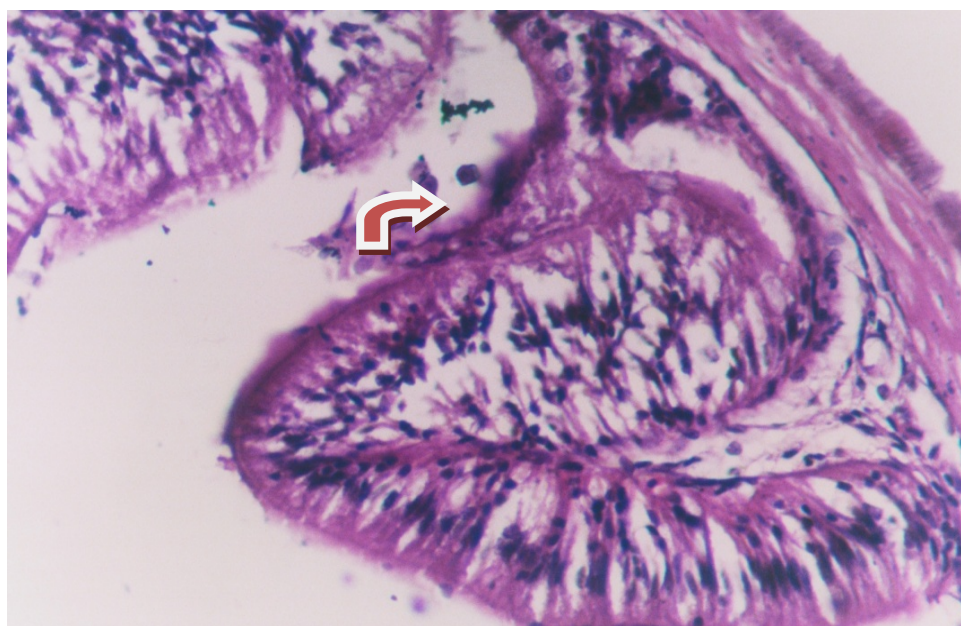


Fig. 2. Intestine of *O. niloticus* treated with living *S. cerevisiae* and *B. subtilis* showing many oval individual cells of yeast colonizing the intact intestinal epithelium and free in the lumen (H & E stain X 400).

## DISCUSSION

Fish cultures are increasing to compensate for 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 evoke the differential aspects of using probiotics in *O. niloticus* from the growth performance point of view as well as their effect on the immune response of treated fish to pathogenic bacteria.

Concerning the growth performance of *O. niloticus* treated with two commercial products containing dead *S. cerevisiae* (Diamond- V yeast) and living *S. cerevisiae* with *B. subtilis* (Megalo) the results revealed that both groups received probiotic-supplemented diets showed higher growth rate than those kept on a basal diet, suggesting that the addition of probiotics enhanced the growth performance and feed utilization and mitigated the effects of population density in the glass aquaria which is the main growth-inhibiting factor in intensive aquaculture systems.

The statistical analysis of different growth parameters of *O. niloticus* at the end of experimental period (Table, 2) indicated a significant increase in the body weight gain (W.G.) between the three different groups. *O. niloticus* in group II kept on diet supplemented with (Megalo) were the fast grower followed by the *O. niloticus* in the group I received diet supplemented with (Diamond –V yeast) in comparison to control group. The specific growth rate (SGR) takes almost the same pattern of W.G. in which *O. niloticus* in the group II have the highest SGR followed by *O. niloticus* in group I in comparison to control group. These were also true for protein efficiency ratio (PER) and Condition factor (CF) in which the *O. niloticus* in both groups treated with probiotic supplemented diets exceeded the value of control group. Only the feed conversion ratio (FCR) of *O. niloticus* kept on a basal diet (control) was higher than other two groups receiving the diets supplemented with probiotics which in turn represented a positive aspect of probiotic supplemented diets. The best FCR values observed with probiotic-supplemented diets suggested that, the addition of probiotics improved feed utilization, in practical terms this means that probiotic used can



decrease the amount of feed necessary for animal growth which could result in production cost reduction. Similar results have been reported by Lara-flores *et al.* (2003).

The PER results indicated that supplementing diets with probiotics significantly improved protein utilization in tilapia. This contributes to optimizing protein use for growth which is the most expensive feed nutrient. The improvement in the biological value of the supplemented diets in these treatments with high population and low dietary protein demonstrated that the probiotics supplements performed more efficiently in stress situations. This agreed with the results obtained by Ringo and Gatesoupe. (1998).

The previous results of growth parameters, the results indicated a positive acceptable effect of the used probiotic mainly *S. cerevisiae* and *B. subtilis* (Megalo) and dead *S. cerevisiae* (Diamond-V yeast). The obtained results could be attributed to the ability of *B. subtilis* to adhere to the intestinal mucosa of *O. niloticus* producing a wide range of relevant digestive enzymes (amylase, lipase and protease) which have the ability to denature the indigestible components in the diets, the ability to detoxify the potentially harmful components of feed and the ability to produce a lot of essential vitamin B. complex members particularly Biotin and vitamin B12, the matter of which resulted in high food utilization and an increase in digestibility of different diet components. These results supported those of Kennedy *et al.* (1998) who used *B. subtilis* in the food of common snook, *Centropomus undecimalis* and found that these probiotic bacteria increased the food absorption by enhancing the protease level and consequently gave a better growth. Also El-haroun *et al.* (2006) in his study with Biogen® as food additive containing *B. subtilis* came to the conclusion that, this organism germinates in the intestine of fish, using a large numbers of sugar (carbohydrates) and produces a wide range of digestive enzymes (amylase, lipase and protease) which have a beneficial effects including higher growth rate and higher feed efficiency. Also the incorporation of *S. cerevisiae* as a probiotic in fish diet was investigated by a lot of researchers in which similar results were obtained.

Abd El-halim *et al.* (1989) founded that the addition of living yeast in diet improve the performance of *O. niloticus*. Also Scholz *et al.* (1999) reported that *S. cerevisiae* improved the growth and survival of sea bass fry. They attributed this action to adherence of *S. cerevisiae* cells to the gut and the secretion of amylase enzymes which shared in the increased digestibility of the diet. On the other hand, the increased growth performance of *O. niloticus* treated with commercial products Megalo and Diamond-V yeast containing living *S. cerevisiae* with *B. subtilis* and dead *S. cerevisiae* respectively could be also attributed to the inhibition of some intestinal

bacterial flora and increasing the non-specific immunity of the treated *O. niloticus*. The adherence capacity of *S. cerevisiae* and *B. subtilis* to the intestinal mucosa inhibits the attachment of the other intestinal bacteria to these binding sites and so preventing the disease occurrence with its negative impact on the fish growth. These results supported the results of Esteban *et al.* (2001) who reported that the cell wall constituents of *S. cerevisiae* play a significant role in stimulation of innate immune response and protect the fish against infection. Raa (2000) reported also that mannose rich proteins from yeast are belonging to the category of compounds which adhere to receptors used by pathogenic microbes and so prevent their colonization in the fish gut. He reported also that the yeast cells could also produced group of substances namely Glutamine, Glutamic acid, Keto glutric acid which are known to be energy substrates for intestinal cells and which contribute to healthy gut. Finally he reported also about the peptides contents of yeast cells which regulate the digestive enzymes secretion in fishes.

The results of microbial profile of the different parts of the intestinal tract (anterior, middle and posterior) of *O. niloticus* pretreated with a basal diet revealed the presence of a lot of bacterial organisms as manifested by the different colonial morphology in different laboratory selective media. The main detected bacterial organisms were isolated on Mac-Conky agar, H. E. media and XLD agar media which are regarded as specific isolation media for different members of Enteriobacteriaceae also Pseudomonas agar base with its supplement and Aeromonas agar base with its supplement where used as specific selective isolation media to the most common fish pathogenic bacteria namely *Aeromonas* and *Pseudomonas Spp.* The bacteriological profile of the different parts of the intestinal tract of *O. niloticus* receiving a live *S. Cervisiae* and *B. subtilis* (Megalo) revealed a more or less different profile concerning disappearance of some members of Enteriobacteriaceae and *Pseudomonas Spp.*

The bacterial profile of *O. niloticus* received diet supplemented with dead *S. Cervisiae* (Diamond-V yeast) was similar to the control group and the biochemical identification of the bacterial organisms from *O. niloticus* kept on a basal diet as proved by traditional biochemical reactions appeared to be belonging to the *Enteriobacteriaceae* namely *E. coli* (motile form), *E. coli* (non-motile form), *Salmonella spp.*, *Klebsiella spp.*, *Morganella morganii* and *E.tarda*. Also the specific fish pathogenic bacteria *P. fluorscens* and *A. sobria* where detected. Coming to the group I kept on (Diamond-V yeast) supplemented diet, all of these organisms where reisolated. The *O. niloticus* in the group II received diet supplemented with (Megalo) the *E. coli* (motile form) *Salmonella spp.* *Klebsiella spp.*, and *P. fluorscens* were not detected. The results

of microbial assay of the different gut parts (anterior, middle and posterior) of *O. niloticus* in the different groups indicated the inhibition capacity of *B. subtilis* and living *S. cerevisiae* and this could be attributed to the ability of these probiotic bacteria and yeast to bind the intestinal mucosal cell receptors for some members of *Enterobacteriaceae* and *Pseudomonas* bacteria. Wang *et al.* (1999) came to the finding that the incorporation of *Bacillus Spp.* in the aquaculture could improve water quality by influencing the water born microbial population and by reducing the number of pathogens in the vicinity of farming species. From the fact that the bacterial profile of fish intestinal tract are usually a reflection of microbial organisms in the environment. This could explain that *B. subtilis* incorporated in Megalo inhibited the pathogenic *Salmonella spp.*, *Klebsiella spp.* and *P. fluorescens* from the fish water environment as well as fish gut. Also Verschuere *et al.* (2000) came also by the conclusion that gram positive *Bacillus Spp.* are efficient in converting organic matter in aquaculture back to CO<sub>2</sub> and so decrease the bacterial load in fish environment. Further these results could be attributed to the bactericidal or the bacteriostatic substances produced by *B. subtilis* and *S. crevisiae* that inhibit the growth of other bacteria as reported by Pybus (1994).

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