# Role of probiotics in improving growth performance, immunity and controlling *Aeromonas hydrophila* in the Nile tilapia *Oreochromis niloticus*

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## ABSTRACT

The tilapias were treated with of *Enterococcus faecium* and *Bacillus coagulans* at a final concentration of ($10^7$cfu/g) ($10^7$cfu/g). 18 aquaria with 6 replicates for treated and controls were used. After 75 days, the tilapias supplemented with the probiotic showed significantly better final weight, body length, specific growth rate, weight gain, feed intake, feed conversion ratio and protein efficiency ratio than those fed on the basal diet (Control). After 75 days from experiment start, each experimental fish group was divided into two groups; the first group was infected with *Aeromonas hydrophila* while the second group without infection. The best Hb, Htc, MCV and RBCs content was recorded by fish group fed on the *E. faecium* supplemented diet comparing to fish group fed on the basil diet and infected with *A. hydrophila* and the same trend was observed for mortality rate. The highest WBCs value and differential count were recorded fish groups infected with *A. hydrophila*. Results also showed that, fish fed on the diet supplemented with *E. faecium* followed by *Bacillus coagulans* recorded the lowest ($P< 0.001$) AST value (16.3 u/L) and (17.23 u/L), while control positive *Aeromonas hydrophila* group showed the highest ($P>0.001$) values being 29.6 u/L.

**Keywords**: Probiotics growth, performance, *Aeromonas hydrophila, Oreochromis niloticus*

## INTRODUCTION

The culture of commercial tilapia, *Oreochromis niloticus*, is one of the most rapidly expanding industries in Egypt. However, both factors including the disease and pollution cause massive mortality in the leading fish countries (Wang *et al*., 2005). The diseases that brought the most impact to the industry include viral infections and bac-teriosis. Conventional approaches to control diseases with chemicals include use of antimicrobial drugs, pesticides, and disinfectants (Gomez-Gil *et al*., 2000). Unfortunately, the abuse of such antimicro-bials in disease prevention and growth promotion can lead to the evolution of resistant strains of bacteria (Esiobu *et al*., 2002).

Therefore, the research of probiotics for aquatic animals is increasing with the demand for environment-friendly aquaculture (Vine *et al*., 2006). To our knowledge, the first application of probiotics in aquaculture was relatively recent (Kozasa, 1986), but the interest in such safe and high effective function is increasing rapidly (Gatesoupe, 1999). The microorganisms used as probiotics, including yeasts, Bacilli, lactic acid bacteria, Pseudomonads and so on, have been evaluated in aquatic animals (RingΦ and Gatesoupe, 1998; Irianto and Austin, 2002a; Hong *et al*., 2005, Vine *et al*., 2006); Kim and Austin 2002 a,b; Wang and Xu, 2006; Balcázar *et al*., 2007a,b). Among lactic acid bacteria, including some *Enterococcus faecium* (*E. faecium*) strains are non-pathogenic, with an ability to produce lactic acid and bacteriocin (Herranz *et al*., 2001). Probiotic preparations of *E. faecium* preparations have received more interest in animal management, because they can stimulate immune system and protect animals from gastrointestinal diseases (Taras *et al*., 2006).

However, the probiotic effect of *E. faecium* on the growth performance and immune

response has not been extensively investigated in tilapia (*O. niloticus*).

The aim of this study was to analyze the effect of a probiotic bacterium, *Enterococcus faecium* on growth performances and immune responses of the Nile tilapia (*O. niloticus*).

## MATERIALS AND METHODS

The present study was carried out at the Laboratory of Fish Nutrition, Faculty of Agriculture, Benha University, Egypt with colrperation of Regional Center for Food & Feed (RCFF), Agriculture Research Center. The experimental started on July 1st 2013 and continued until October 1st of the same year (90 days). It was aimed to assess the role of *Enterococcus faecium* and *Bacillus coagulans* bacteria a probiotic with special emphasize an its role in cultured *Oreochromis niloticus* as a growth promoter and immune stimulant agent beside its role during challenge with *Aeromonas hydrophila.*

All-male Nile tilapia, *O. niloticus* fry (6±0.03g) were obtained from private farm, Kafr El-Sheikh**,** Egypt. Fish were acclimated to the experimental conditions for two weeks**,** during acclimation period, fish were fed a control diet at a level of 3% of biomass**.** Settled fish wastes with one half of water were siphoned daily and water volume was replaced by aerated tap water from the storage tank. The experiment was conducted in Eighteen glass aquaria.

**Design of the experiment**

The present study was carried out to assess the role of *Bacillus coagulans* and *Enterococcu faecium* as a probiotic on growth performance, feed utilization and the effect of infection with *Aeromonas hydrophila* of *O. niloticus*. Therefore three treatments were tested in sex replicates aquaria for each. Each aquarium was stocked with 15 fish and supplied by air pump. During the last 15 days fish of three aquaria from each treatment were infected with pathogenic bacteria, *Aeromonas hydrophila* to study the effect of the tested probiotics. The different treatment were illustrated in Tables 1 and 2.

Table1: Experimental design of the present study

| Group | Treatment | Probiotic dose (ml/kg diet) |
|---|---|---|
| T1 | *Enterococcus faecium* | 20ml/kg ($10^7$cfu/g) |
| T2 | *Bacillus coagulans* | 20ml/kg ($10^7$cfu/g) |
| T2 | *Control* | Negative |

Table 2: Infection with *Aeromonas hydrophila* during the last 15 days of the experiment

| Treatment | Probiotic | Probiotic dose (ml/kg diet) | *A. hydrophila* |
|---|---|---|---|
| T1 | *E. faecium* | 20ml/kg ($10^7$cfu/g ) | Negative |
| T2 | *B. coagulans* | 20ml/kg ($10^7$cfu/g ) | Negative |
| T3 | Control | Negative | Negative |
| T4 | *E. faecium* | 20ml/kg ($10^7$cfu/g ) | Positive |
| T5 | *B. coagulans* | 20ml/kg ($10^7$cfu/g ) | Positive |
| T6 | Control | Negative | Positive |

## Preparation of Experimental diets:

Three isonitrogenous (340 g/kg crude protein) and isocaloric (3500 kcal/kg metabolizable energy) experimental diets were formulated and the proximate chemical composition of the experimental diets is presented in Table 3. The first is the basal diet supplemented with 20ml/kg ($10^7$cfu/g) of *Enterococcus faecium*, the second is the basal diet supplemented with 20ml/kg ($10^7$cfu/g) of *Bacillus coagulans* and the third is the basal diet without probiotic (control diet). All dry ingredients of the fish meal, soybean meal.

Table 3: Composition of the basal diet (g/kg) and chemical analysis % (dry matter basis ) for Tilapia fish

| Ingredients | Control |
|---|---|
| Fish meal (60%CP) | 160 |
| Soybean meal (44% CP) | 320 |
| Corn yallow (10% CP) | 370 |
| Gultain (60% CP) | 40 |
| Wheat flour | 70 |
| Soybean oil | 20 |
| Vita & M1 [1] | 20 |
| *Chemical analysis* % | |
| Dry matter | 91 |
| Crude protein | 34 |
| Crude lipid | 8 |
| Ash | 6 |
| Crude fiber | 5.3 |
| NFE[2] | 46.7 |
| ME[3] | 3504 (Kcal/Kg) |

1- Vitamin and mineral mix (mg or g / Kg diet): MnSO4, 40 mg; MgO, 10 mg; K2SO4, 40 mg; ZnCO3, 60 mg; KI, 0.4 mg; CuSO4, 12 mg; Ferric citrate, 250 mg; Na2SeO3, 0.24 mg; cholecalciferol, 4000 IU; α-tocopherolacetate, 400 mg; menadione, 12 mg; thiamine, 30 mg; riboflavin, 40 mg and pyridoxine, 30 mg.

2- NFE (Nitrogen free extract) =100-(crude protein + lipid + ash +fibre content).

3- Metabolizable energy (kJ g-1), calculated based on the physiological fuel valuesaccording to (Brett 1971) .

**Probiotics strains:**

Bacterial strains of *Enterococcus faecium* and *Bacillus coagulans* were obtained from food safety lab, Regional Center for Food and Feed (RCFF), Agriculture Research Center (ARC), and were kept at -20$^{o}$C until the start of the experimental. *Enterococcus faecium* was propagated (Table 4) and incubated at 44$^{o}$C for 48 hours, and the growth was harvested, then washed three times and resuspended in Brain Heart Infusion Broth. The suspension incubated at 37$^{o}$C for 24 hours. Counting the colony forming unit per ml transfer an aliquot of prepared sample ($10^{-1}$) to a tested tube contains 9 ml folds of Buffered peptone water (Table 4) from which 1 part is taken to another test tube containing 9 ml folds of the Buffered peptone water to have a final dilution of $10^{-3}$. Continue in this manner till reaching to level $10^{-7}$ microorganisms per ml taking into account good mixing with vortex in each step. One empty and pre-sterilized petri dish is inoculated with a known amount of each dilution before adding about 15ml of molten SBA (Table 4) previously cooled at 45$^{o}$C. Mix the inoculum and the medium thoroughly. Incubate the inverted dishes at 44$^{o}$C for 2 days. Selected average values between 10-100 colonies and report the result multiplied by the dilution factor. *Enterococcus faecium* colonies are pink –red in color.

**Preparation of *Bacillus coagulans* suspension:**

*Bacillus coagulans* was propagated in to MRS (Table 4) and incubated at 37$^{o}$C for 48 hours, and the growth was harvested, then washed three times and resuspended in Brain Heart Infusion Broth. The suspension incubated at 37$^{o}$C for 24hours to have a final concentration $10^{7}$microorganisms per ml. Counting the colony forming unit per ml transfer an aliquot of prepared sample ($10^{-1}$)to a tested tube contains 9ml folds of Buffered peptone water from which 1 part is taken to another test tube containing 9ml folds of the Buffered peptone water to have a final dilution of $10^{-3}$. Continue in this manner till reaching to level $10^{-7}$ microorganisms per ml taking into account good mixing with vortex in each step. One empty and pre-sterilized petri dish is inoculated

with a known amount of each dilution before adding about 15ml of molten MRS (Table 4) previously cooled at 45°C. Mix the inoculum and the medium thoroughly. Incubated the dishes in inverting position at 44°C for 2 day. Selected average values between 10-100 colonies and report the result multiplied by the dilution factor.

Table 4: Description of media used in isolation of *E. faecium* and *Bacillus coagulans*

| Media | Ingridients per (g /l) | pH |
|---|---|---|
| Buffered peptone water (Biolife)[1] | Peptomeat 10 g<br>Sodium Chloride 5 g<br>Disodium Phosphate 3.5 g<br>Monopotassium Phosphate 1.5 g | 7.0±0.1<br>at 25°C |
| SlanetzBartley Agar(SBA)[2] (LAB M) | Tryptose 20.0 g<br>Yeast Extract 5.0g<br>Glucose 2.0 g<br>Dipotassium hydrogen phosphate 4.0 g<br>Sodium azide 0.4g<br>2,3,4 Tetrazolium chloride 0.1g<br>Agar 12.0g | 7.2 ±0.2<br>at 25°C |
| MRS Agar (BIOLIFE)[3] | Enzymatic digest of casein 10g<br>Beef extract 10g<br>Yeast extract 4g<br>Glucose 20g<br>Di-potassium Hydrogen Phosphate 2g<br>Sodium Acetate 5g<br>Tri-ammonium Citrate 2g<br>MagnesiumSulphateheptahydrate 0.2g<br>ManganousSulphatetetrahydrate 0.05g<br>Agar 15g<br>Tween 80 1g | 7.2± 0.2<br>at 25°C |
| Brain Heart Infusion Broth (LAB M)[4] | Brain-Heart Infusion solids (porcine) 17g<br>Tryptose 10g<br>Glucose2g<br>Sodium chloride 5.0 g<br>Disodium hydrogen phosphate 2.5g | 7.4 ± 0.2<br>at 25°C |

[1]The required quantity was prepared as mentioned by the manufacturer;[2,3]and [4]the required quantity was prepared as mentioned by the manufacturer then poured in sterile Petri dishes.

**Probiotic supplemented diets:**

The probiotic test diets T1 and T2 were prepared by gently spraying the required amount of bacteria suspension on the control diet and mixing it part by part to obtain a final probiotic concentration ($10^7$cfu/g). The probiotic test diets T1 and T2 were packed in sterile poly propylene containers and stored at 4°C for viability studies. Storage period over 14 days period. New diets were prepared bi-weekly to ensure that high probiotic levels remind in the diets for the duration of the trial (Yun-Zhang *et al.,* 2010).

**Feeding system**

Fish were fed the experimental diets at a rate of 3% of live body weight twice daily at 8.00 and 16.00 hours. Fish in each aquarium were sampled biweekly and feed amounts were adjusted according to the new fish biomass. Dead fish were daily recorded and removed. The feeding period in the experiment lasted 90 days.

**Water quality**

Water temperature was recorded daily at 1.00 pm using a mercury thermometer. Dissolved oxygen (DO) was measured at 07.00 am using YSI model 56 oxygen meter (YSI Company, Yellow Springs Instrument, Yellow Springs, Ohio, USA). Total ammonia and nitrite were measured twice weekly using a DREL, 2000 spectrophotometer (Hash Company, Loveland, CO, USA). A pH was estimated on morning by using a pH meter (Orion pH meter 400, Abilene, Texas, USA). Water temperature ranged from 27.20 to 29.25°C; dissolved oxygen (DO) ranged between 5.32 and 6.81 mg/l; pH values ranged between 8.04 and 8.30

and total ammonia ranged from 0.18 to 0.2 mg/l for the different treatments during the entire experimental period (90 days) of the study. All tested water quality criteria (temperature, pH value, DO and total amonia) were suitable and within the acceptable limits for rearing *O. niloticus* fingerlings (Boyd, 1990). A photoperiod of 12-h light, 12-h dark (08:00–20:00 h) was used via fluorescent ceiling lights supplied the illumination.

**Growth performance and feed utilization parameters**

Records of live body weight (BW/g) and body length (BL/cm) of fish were measured in all fish for each pond and registered every 14 day (two weeks) during the experimental period. Growth performance parameters were measured by using the following equations:

Condition factor (K)=($W/L^3$) x 100; Where: W = weight of fish in grams and L = total length of fish in "cm"

Weight gain (WG) = final weight (g) – initial weight (g)

Specific growth rate (SGR): $SGR = \frac{LnW2 - LnW1}{t} x\ 100$

Where: Ln = the natural log; $W_1$ = first fish weight; $W_2$ = the following fish weight in grams and t = period in days.

Feed conversion ratio (FCR): FCR = Feed ingested (g)/Weight gain (g)

Protein efficiency ratio (PER): PER = Weight gain (g)/Protein ingested (g)

Survival rate: (Z/X) × 100 where: Z is the surviving fish number and X is the initial fish number.

**Infection with *Aeromonas hydrophila*:**

The goal of this experiment was to evaluate the resistance of Nile tilapia (*Oreochromis niloticus*) fed on *Enterococcus faecium* and *Bacillus coagulans* for 75 days to *Aeromonas hydrophila*. This bacteria was chosen because it is considered the major economic problem and a great loses of fish is done cause in warm aquaculture. Solvent tolerant strains of *Aeromonas hydrophila* used in the present study were obtained from Microbiological Resources Center (MIRCEN), Faculty of Agriculture, Ain Shams Univ., Cairo, Egypt. Bacteria were grown on250 ml brain Heart infusion broth (BHI) in (Table 4) for 18 hour at incubation 30±1.0$^o$C. From food safety lab, (RCFF). Each fish group was subjected to infection with *Aeromonas hydrophila* (250ml in BHI broth) by immersion for 30 minute in separated aquaria of 35 liters chloride free tape water at 24 ± 1$^o$C. Post infection fish were observed all over the experimental period (15 days). Clinical signs, post mortem findings and mortality rate were recorded at once.

**Blood sampling:**

At the end of the experiment, blood samples collected from the caudal vein of fish in clean tube with 10% EDTA solution to determine hematocrit (Htc), hemoglobin (Hb) and differential leukocytes (WBCs), blood samples of the other fish were collected also from the caudal vein of fish and the same treatment in clean dry centrifuge tubes, kept for 15 minutes and centrifuged at 3000 rpm for 10 minutes, then kept frozen at -20°C for determination of blood chemistry, asparatate amino transferase (AST) and alanine amino transferase (ALT) (Bradford, 1976).

**Hematological Parameters**

Hematocrit (Htc) was determined by the micro hematocrit method as described by Reitman and Frankel (1957). Whereas, (Hb) was determined by the total hemoglobin kit which is a standardized procedure of the cyano met hemoglobin method (Martins *et al.,* 2004).

**Blood chemistry**

Asparatate aminotransferase (AST) and Alanine aminotransferase (ALT) activities were determined according to the method described by (Reitman and Frankel 1957). Serum creatinine and uric acid were measured by calorimetric method and enzymatic detremenation methods respectively as described by (Henry, 1974).

**Differential leukocytes:**

Total count of differential leukocytes (WBCs) was carried out by the indirect method Martins *et al.,*(2004) differential counting of leucocytes in the smears stained was carried by Giemsa /May-Grunwald. Total leukocytes number was calculated by the formula:

Leukocytes/µl=(Leucocytes number in the smear × erythrocytes number/ µl)/2,000 erythrocytes counted in the blood smear.

**Proximate composition**

At the initiation and termination of the trail a random sample of five individual fish were sampled from each aquarium, then oven-dried 105◦C for 24h, ground, and stored at−20°C for subsequent analysis. Proximate analysis was conducted on emental diets and fish samples. Dry matter, crude protein, crude lipid and ash contents were all determined by the standard (AOAC, 1995). Dry matter was determined after drying the samples in an oven (105°C) for 24h. Crude protein was determined by micro-Kjeldhal method, N×6.25(using Kjeltech auto analyzer, Model 1030, Tecator, Höganäs, Sweden) (AOAC, 1995 method number 984.13) and crude fat by Soxhlet extraction with diethyl ether (40- 60°C) (AOAC, 1995 method number 920.39). Ash was estimated by incineration at 550°C for 12 h (AOAC, 1995 method number 942.05). Crude fiber content of de-oiled SFM, YFSFM and BFSFM was determined using the method of Van Soest *et al*., (1991). Nitrogen-free extract was computed by taking the sum of values for crude protein, crude lipid, crude fiber, ash then subtracting this sum from 100.

**Statistical analysis**

All data are presented as means± (SE). Growth, hematology and blood chemistry data were analyzed using one way ANOVA, followed by Duncan's multiple range tests which was used to compare differences among individual means, with statistical software SAS ANOVA procedure (Statistical Analysis System, (2004). A probability of 0.05 was utilized to account for the statistical difference between the means. Before the analysis, percentage data were normalized by arcsine-transformation.

## RESULTS AND DISCUSSION

**Growth performance and feed utilization**

Results in Table (5) indicated that final body weight (BW), body length (BL) weight gain (WG) and specific growth rate (SGR) of *O. niloticus* fed the basal diet supplemented with *Enterococcus faecium* (T1) and fish group fed the basal diet supplemented with *Bacillus coagulans* (T2) significantly compared to fish fed the control diet (T3). The diet supplemented with *E. faecium* (T1) showed the highest significantly ($P<0.05$) BW, WG and SGR when compared to the diet supplemented with *Bacillus coagulans*. Such increase in the growth in aquatic animals that were fed probiotics supplemented diets may be attributed to the improved digestive activity due to enhancing the synthesis of vitamins and enzymatic activities (Ding *et al*. 2004); consequently, improving digestibility and growth performance.

Since the first use of probiotics in aquaculture, a growing number of studies have demonstrated their ability to increase the growth rate and welfare of farmed aquatic animals (Lara-Flores *et al*., 2003; Carnevali *et al*., 2004; Macey and Coyne, 2005; Wang *et al*., 2005; Wang and Xu, 2006; Wang, 2007). Here, for the first time, an enhancement of the growth rate of the tilapia, *O. niloticus*, one of the most important farmed species for the world, was as a result of supplemented the aquaria water with probiotics ( Table 5). Similar results were reported by Bogut *et al*. (1999) and Noh *et al*. (1994) for carp with the probiotic Streptococcus faecium. A similar finding was also obtained by Shiri Harzevili *et al*. (1998), who investigated the effect of the probiotic *Lactococcus lactis* AR21 on the growth performance of rotifers and showed that addition of *Lactococcus lactis* AR21 to tank water resulted in enhanced growth of the rotifers. These results suggest that the increased growth performance might be attributed to improved immune responses.

Table 5: Effect of probitics supplemented diets on growth performance and feed utilization of Nile tilapia

| Items | Experimental Diets | | | ±SE |
|---|---|---|---|---|
| | T1 | T2 | T3 | |
| **Body weight** | **19.27$^{a}$** | **16.64$^{b}$** | **12.45$^{c}$** | **±0.190** |
| **Body length** | 10.81$^{a}$ | 10.02$^{b}$ | 9.28$^{c}$ | **±0.150** |
| **Condition factor** | 0.02$^{a}$ | **0.021$^{a}$** | **0.020$^{a}$** | ±0.001 |
| **Weight gain** | 12.93$^{a}$ | 10.36$^{b}$ | 6.14$^{c}$ | ±0.200 |
| **Specific growth rate** | 3.40$^{a}$ | 3.11$^{b}$ | 2.40$^{c}$ | **±0.010** |
| **Feed intake** | 22.17$^{a}$ | 21.0$^{b}$ | 18.50$^{c}$ | **±0.100** |
| **Feed conversion ratio** | 1.73$^{C}$ | 2.80$^{b}$ | 3.01$^{a}$ | ±0.010 |
| **Protein efficiency ratio** | 1.72$^{a}$ | 1.41$^{b}$ | 0.98$^{c}$ | **±0.040** |

Means within the same row sharing the same superscript are not significantly different (P<0.05).

Probiotics have been shown to produce digestive enzymes such as amylase, protease, lipase which may enrich the concentration of intestinal digestive enzymes. In addition, probiotics inhibit the colonization of potential pathogens in the digestive tract by antibiosis or by the competition for nutrients and the alteration of the microbial metabolism (Gatesoupe, 1999). It also improves the nutrition by detoxifying the potentially harmful compounds in feeds by producing vitamins such as biotin and vitamin $B_{12}$ (Hoshino *et al*. 1997), and by stimulating host immunity (Gibson *et al*. 1997). Soltan and El-Laithy (2008) and Hassaan *et al*., (2014) indicated that supplementation of basal diet with *B. subtilis*, significantly (P<0.001) improved BW, BL, WG and SGR of *O. niloticus*. Similarly, the application of *E. faecium* as a probiotic was found to enhance the growth performance of Nile tilapia, *O. niloticus* (Wang *et al*. 2008). Al-Dohail *et al*. (2009) also illustrated that African catfish *Clarias gariepinus* that were fed the *Lactobacillus acidophilus* showed a better growth performance than the control fish group.

Results of Table ( 5) indicated that, supplementation of the basal diets with each of *E . faecium* (T1) or *Bacillus coagulans* (T2) significantly increased feed intake, specific growth rate (SGR), protein efficiency ratio PER) and improved feed conversion ratio (FCR) compared with *O. niloticus* fed the basal die. In practical terms, this means that the use of probiotics can decrease the amount of feed necessary for animal growth which could result in a reduction in the production cost. Several studies on probiotics have been published in recent years which suggested that, probiotics provide nutritional benefits in diets for tilapia fingerling (Ferguson *et al*. 2010).

Gastrointestinal bacteria take part in the decomposition of nutrients, provide the microorganisms with physiologically active materials, such as enzymes, amino acids,

and vitamins (Bairagi *et al*. 2004; Wache¢ *et al*. 2006; Wang and Xu, 2006; Wang, 2007), and thus facilitate feed utilization and digestion. This may account for the enhanced FCR and PER by dietary *Enterococcus faecium* (T1) and *Bacillus coagulans* (T2) supplementation in the present study and previous studies (Bagheri *et al*., 2008; Soltan and El-Laithy, 2008; Hassaan *et al*., 2014).

**Hematological indices**

Haemoglobin (Hb), hematocrit (Ht) red blood cells (RBCs) and white blood cells (WBCs) of *O. niloticus* significantly increased when the basal diet supplemented with *Enterococcus faecium* (T1) and fish group fed the basal diet supplemented with *Bacillus coagulans* compared to fish fed the control diet (T3). The diet supplemented with *E. faecium* (T1) showed the highest significant (P<0.05) Hematological indices compared with the other experimental diets (T2 or T3) and the same trend was also observed when the the three *O. niloticus* groups (T1, T2 and T3) challenged with *Aeromonas hydrophila* during the last 15 days of the experiment (T4, T5 and T6). When the three fish groups (T1, T2, T3) were challenged with *Aeromonas hydrophila*, fish group fed the basal diet supplemented with *Enterococcus faecium* or *Bacillus coagulans* and challenged with *Aeromonas hydrophila* (T4 and T5, respectively) during the last 15 days significantly improved Hematological indices compared to fish group fed the basal diet for 75 days and then challenged with *Aeromonas hydrophila* for 15 days.

Hematology is an important factor that could be considered for the fish diet quality assessment. Ologhobo (1992) reported that one of the most common blood variables consistently influenced by diet are the hematocrit (Ht) and hemoglobin (Hb) levels. Probiotics and prebiotics have been used alone and together in various animals including the synbiotic, in tilapia (Abd El-Rhman *et al*. 2009), which reported positive effects on haematological parameters. On the other hand, *O. niloticus* fed diet supplemented with *B. subtilis* (Soltan and El-Laithy 2008) or supplemented with *Pediococcus acidilactici* Ferguson *et al*. (2010) showed some variation (but not significant) in Hb and Ht content among the control and fish that were fish groups fed diet enriched with probiotics. Also, Marzouk *et al*. (2008) reported that both fish groups fed the diet supplemented with dead *Saccharomyces cerevisae* yeast and both of live *Bacillus subtilis* and *S. cerevisae* showed significant (P<0.05) increase in the Ht level when compared to fish fed the control diet.

Table 6: Effect of probitics supplemented diets on hematological indices of the Nile tilapia

| Treatment | Hemoglobin (g/dl) | Hematocrit (%) | Red blood cells (10^6) | main corpuscular volume |
|---|---|---|---|---|
| T1 | $8.90^{a}$ | $29.66^{a}$ | $2.90^{a}$ | $69.66^{a}$ |
| T2 | $8.20^{c}$ | $27.63^{b}$ | $2.46^{b}$ | $64.06^{b}$ |
| T3 | $8.06^{bc}$ | $24.2^{c}$ | $2.33^{bc}$ | $57.23^{c}$ |
| T4 | $8.36^{b}$ | $23.86^{c}$ | $2.70^{ab}$ | $62.80^{b}$ |
| T5 | $8.03^{bc}$ | $23.66^{c}$ | $2.60^{ab}$ | $63.30^{b}$ |
| T6 | $7.76^{c}$ | $21.53^{d}$ | $2.03^{c}$ | $66.63^{ab}$ |
| Standard error | ±0.16 | ±0.66 | ±0.26 | ±1.23 |

**White blood cells and differential count**

Means of WBC count as affected by treatment effects are given in Table 7. These means indicated that fish treated with only **co**ntrol infected *Aeromonas hydrophila* T4 ($12.26^{5}$) followed by T5 ($12.13^{5}$) were the highest WBC count compared to all treatments, as well as the control group T3 ($8.83^{5}$) in addition, fish treated with only *Bacillus coagulans* T2 ($9.43^{5}$) appeared to follow the same trend mentioned above while there showed a significant difference compared with negative control T3 ($7.6^{5}$). The *A. hydrophila* T4

($12.26^5$) had increased significantly WBC count compared with all treatments. The same trend was observed also for lymphocytes, monocytes, neutrophill and eosinophils percentages (Table 7). Obtained results are in agreement with those obtained by Zhou *et al.,* (2010) who found that the use of *B. coagulans* improved immunity. Also Panigrahi *et al.,* (2004) recorded that, in aquaculture the dose of probiotics usually varies from$10^6$cfu/g feed. The optimum dose of a probiotics can vary with respect to host and also type of immune parameters. Song *et al.,*(2006) recorded high serum lysozyme, phagocytic activity of head kidney leucocytes and complement activities in *O. mykiss* fed for 30days with *Lactic rhamnosus* strain at $10^{11}$ cfu /g feed but not at a dose of $10^9$ cfu /g feed. Furthermore, stimulation of a particular immune response with respect to different tissue/organ also varies with dose. Sharifuzzaman and Austin (2009) recorded highest cellular and humoral immunity at two weeks of feeding probiotics. Sampath *et al.,* (1998) and Irianto and Austin (2002b) recorded that hematological parameters of fish are used as indicators of their physiological state and their study has become widespread in the control of pathologies and manipulation of stress in fish farming.

Table 7: Effect of probitics supplemented diets on hematological indices of Nile tilapia.

| Treatment | white blood cell (10^5) | Mnocytes (%) | Lymphocytes (%) | Nutrophils (%) | Eosinophils (%) |
|---|---|---|---|---|---|
| T1 | $9.43^b$ | $22.10^c$ | $71.33^a$ | $1.86^b$ | $3.50^b$ |
| T2 | $9.43^b$ | $23.66^b$ | $65.33^b$ | $2.10^b$ | $2.70^b$ |
| T3 | $7.60^c$ | $25.20^a$ | $62.66^c$ | $1.50^b$ | $2.83^b$ |
| T4 | $12.26^a$ | $23.30^b$ | $72.64^a$ | $2.00^a$ | $3.33^b$ |
| T5 | $12.13^a$ | $24.20^b$ | $66.66^{ab}$ | $4.00^a$ | $3.50^b$ |
| T6 | $11.90^a$ | $25.30^a$ | $63.33^c$ | $3.50^a$ | $4.16^a$ |
| Standard error | ±0.31 | ±1.50 | ±1.79 | ±0.27 | ±0.19 |

Results of differential leukocytes of Nile tilapia in the present study showed that lymphocytes percentage revealed the major constituent of WBCs in Table,7. Shimei *et al., (2012)* indicated that *fish fed on Bacillus coagulans* had the highest total leukocyte count (WBC), respiratory burst activity, phagocyte activity and lysozyme activity. Also with Yongjian *et al.,* (2014) indicated that *B. coagulans* as a dietary probiotic for the fish could improve growth performance, meat quality, and induce a positive modulation on immune response. Akhil *et al.,* (2014) showed that *Bacillus coagulans* supplemented diets significantly affected fish growth performance, non-specific immunity and protection against *A. hydrophila* infection.

Higher counts (%) of phagocyte cells (Nutrophils and Monocytes) and lymphocytes are also indicative of infection in fish. Probiotics interact with the immune cells such as mononuclear phagocyte cells (Monocytes and macrophages) and poly morphonuclear leucocytes (Nutrophils) and to enhance innate immune responses (Irianto and Austin, 2002b). Probiotics also actively stimulate the proliferation of lymphocytes (both B and T cells) and further immunoglobulin production in fish (Al-Dohail *et al.*, 2009). Also with Mehrim (2011) indicated that mono-sex Nile tilapia *O. niloticus* fed the basal diet supplemented with 3g Biogen $Kg^{-1}$ diet for 14 weeks significantly ($P \leq 0.05$) showed an increase WBCs count.

Aly *et al*., (2008) reported that, the increase in the total leucocytes cells (TLC) was significant in O.niloticus fed Bacillus spp. in comparison with the control group. Although the number of nutrophils had non-significant increase in these treated groups in comparison with the control, the increase in TLC for O. niloticus fed Bacillus spp. resulted mainly from the increase in lymphocytes and monocytes. Duc *et al*., (2004) suggested that B. pumilus induces bacteriocin like activity against other bacilli; this finding supports the hypothesis that the organism has a probiotic effect. Alessandro *et al.,* (2013) indicate that the fish technique is a potential tool to

characterize the dynamics of potential probiotic bacteria and their efficiency in the control of pathogenic bacteria. The results showed that the *E. faecium* were better transactions impact on the bacteria *A. hydrophila* on tilapia fish.

**Metabolism enzyems**

Alanine aminotransferase (ALT) and aspertat aminotranferase (AST) enzymes are important liver enzymes. They indicators for liver health and function through controlling the transferring amino group function of alpha-amino acids to alpha-keto acids. Large amount of ALT and AST are released into animal blood, mostly during liver cell damage (Kumar *et al*. 2011).

As shown in Table 8 ALT and AST relatively improved by supplementation the basal diet with probiotics , *Enterococcus faecium* (T1) and *Bacillus coagulans* (T2) compared to fish fed the control diet (T3) specially when fish groups (T1, T2, T3) infected by *A. hydrophila* (T4, T5 and T6, respectively). The diet supplemented with *E. faecium* (T1) showed the lowest significant ($P<0.05$) ALT and AST levels whereas T6 showed the highest significant AST and ALT levels.

As shown in Table 8, ALT and AST values decreased with supplementation the basal diets with probiotics *(E. faecium* and *B. coagulans*). Soltan and El-Laithy (2008) found that, ALT and AST levels significantly decreased when Nile tilapia fed diets supplemented with probiotics compared to control group. Similarly, Wache¢ *et al*. (2006) observed a decrease in the activity of AST, ALT and lactate dehydrogenase in *O. niloticus* after being fed with diet containing *Pseudomonas spp*. and a mixture of *Micrococcus luteus* and *Pseudomonas spp*. Similar results were also observed in *Cyprinus carpio* fed the extract of Cyanobacteria (Palikova *et al*. 2004). Marzouk *et al*. (2008) found that, fish groups fed on diets supplemented with dead *Saccharomyces cerevisae* yeast and both of live *Bacillus subtilis* + *S. cerevisae* revealed a significant ($P<0.05$) decrease in ALT and AST when compared to the control group that fed on probiotic-free diet.

Table 8: Effect of probitics supplemented diets on liver enzymes of Nile tilapia

| Treatment | Alanine amino transferase ALT µ/L | Aspartate amino transferase AST µ/L |
|---|---|---|
| T1 | $82.13^{d}$ | $16.30^{c}$ |
| T2 | $82.60^{d}$ | $17.23^{b}$ |
| T3 | $83.76^{c}$ | $17.60^{b}$ |
| T4 | $83.73^{c}$ | $17.33^{b}$ |
| T5 | $85.26^{b}$ | $17.76^{b}$ |
| T6 | $94.00^{a}$ | $29.60^{a}$ |
| Standard error | ±0.19 | ±0.19 |

**Proximate analysis**

Results outlined in Table (9) showed the proximate analysis of *O. niloticus* fed the diet supplemented with *E. faecium* (T1) and fish group fed the basal diet supplemented with *B. coagulans* (T2), basal diet (T3) and *O. niloticus* fed the diet supplemented with *E. faecium* (T4) and challenged with *A. hydrophila*, fish group fed the basal diet supplemented with *Bacillus coagulans* (T5) and challenged with *A. hydrophila*, fish fed the basal diet and challenged with *A. hydrophila* (T6).

Our results indicated that the probotics tested in the present study significantly increased protein and ash content and decreased lipid content of experimental *O. niloticus* compared to fish group fed tha basal diet and the same trend was also when the previous fish groups T1, T2 and T3) were challenged with *A. hydrophila* (T4, T5 and T6, respectively. Ye *et al*. (2011) in Japanese flounder showed an increase in the body protein content in fish fed a FOS, MOS and/or *B. clausii*-containing diet when

compared to the control, body lipid content demonstrated an opposite trend to body protein content.

Results of the present study are in agreement with those obtained by Eid and Mohamed (2008); Mehrim (2011) for tilapia and El-Haroun, (2007) for catfish. Also, Soltan and El-Laithy, (2008) indicated that, *O. niloticus* fed diet supplemented with *B. subtilis* recorded high level of dry matter and lipid content than other control group and had no effect on ash content. Also, Eid and Mohamed (2008) found that, dietary inclusion of probiotics increased protein content and lowered fat content of the whole fish body, without significant differences in ash content of Nile tilapia. Also, Bagheri *et al.,* (2008) reported that application of $3.8\times10^9$ CFU $g^{-1}$ of *Bacillus spp*. probiotic in the diet of rainbow trout fries made a significant increase of fish body protein content compared to the control group. Contaray, El-Dakar *et al.,* (2007) reported that carcass composition was not affected by dietary probiotic inclusion for rabbit fish, *Siganus rivulatus*.

Table 9: Effect of probitics supplemented diets on hematological indices of Nile tilapia

| Treatment | Dry matter | Protein | Lipid | Ash | Dry matter |
|---|---|---|---|---|---|
| T1 | $26.16^{a}$ | $62.73^{a}$ | $20.26^{b}$ | $15.46^{c}$ | $26.16^{a}$ |
| T2 | $25.60^{a}$ | $61.36^{ab}$ | $20.30^{b}$ | $16.30^{b}$ | $25.60^{a}$ |
| T3 | $25.50^{ab}$ | $58.66^{C}$ | $21.16^{a}$ | $15.03^{c}$ | $25.50^{ab}$ |
| T4 | $25.90^{a}$ | $62.43^{a}$ | $18.45^{c}$ | $17.70^{a}$ | $25.90^{a}$ |
| T5 | $25.40^{ab}$ | $61.23^{ab}$ | $18.57^{c}$ | $14.66^{c}$ | $25.40^{ab}$ |
| T6 | $25.60^{ab}$ | $58.63^{C}$ | $21.07^{a}$ | $14.63^{c}$ | $25.60^{ab}$ |
| Standard error | ± 0.28 | ± 0.47 | ± 0.18 | ± 0.26 | ± 0.28 |

**Mortality rate of *O. niloticus***

Results presented in Table (10) showed that, supplementation of the basal diets with each of *E .faecium* (T1) or *Bacillus coagulans* (T2) showed the lowest (4.44%) mortality rate compared to fish group fed the basal diet (6.66%). The infection of *O. niloticus* with *Aeromonas hydrophila* (T4 , T5 and T6) significantly decreased mortality rate to 13.33% (T4), 17.77% (T5) and 62.22% (T6). Results also indicated that probiotics supplemented diets for *O. niloticus* decreased mortality rate specially when the fish groups (T4, T5 and T6) challenged with *A. hydrophila*.

Finally, the addition of E. faecium ZJ4 in aquaria water could increase the growth performances as measured by final weight and DWG and improve the immune responses of the tilapia.

Table 10: Effect of probitics supplemented diets on mortality rate of Nile tilapia

| Treatment | Mortality rate (%) |
|---|---|
| T1 | $4.44^{d}$ |
| T2 | $4.44^{d}$ |
| T3 | $6.66^{d}$ |
| T4 | $13.33^{bc}$ |
| T5 | $17.77^{b}$ |
| T6 | $62.22^{a}$ |
| Standard error | ±2.4 |

All mortalities due to the challenge infection with *A. hydrophila* from the third day post-challenge. The clinical signs observed in fish were hyperemia on the ventral side of the body, swollen abdomen, reddish vent slightly protruding, opaque eyes and loss of equilibrium; all typical signs associated with *A. hydrophila* infection (Schaperclaus 1991). Also with, Major bacterial fish pathogens include *Aeromonas spp.* the logical agents of

furunculosis, vibriosis, cold-water vibriosis and red mouth disease, respectively (Ringø *et al.,* 2010). Culture-dependent and independent approaches have revealed the presence of potentially pathogenic bacteria of *Aeromonas* spp. (Schreier *et al.,* 2010). *E. faecium* is referred to show antagonistic effect on *A. hydrophila* (Panigrahi *et al*., 2007). In the studies that are conducted by *E. faecium*, antagonistic effects against *A. hydrophila* were observed by cross-streaking and the agar spot method. The strain's probiotic effects were confirmed and it was checked for to fish (Gopalakannan and Venkatesan, 2011). *E. Faecium* strong then and enhances the *O. niloticus* immune system response against *A. hydrophila* (Iman *et al;* 2014). Among these options, probiotics, live micro-organisms that confer a health benefit to the host by providing both a nutritional benefit and protection against pathogens (Tania *et al.,* 2013).

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## ARABIC SUMMARY

### دور البروبيوتيك فى تحسين النمو والمناعة ومقاومة بكتريا الايروموناس هيدروفيلا فى أسماك البلطى النيلى

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١- المركز الإقليمي للأغذية والأعلاف مركز البحوث الزراعية، مصر.

٢- كلية الزراعة – جامعة بنها، مصر.

أجريت هذه الدراسة بمعمل تغذية الأسماك بقسم الإنتاج الحيواني بكلية الزراعة جامعة بنها بالتعاون مع المركز الإقليمى للأغذية والأعلاف مركز البحوث الزراعية لدراسة دور البروبيوتك فى تحسين أداء النمو والمناعة فى أسماك البلطى وفى السيطرة على انتشار بكتريا الأيروموناس هيدروفيلا . أستخدم فى هذه الدراسة أسماك البلطى النيلى بمتوسط وزن ٦± ٠,٠٣ جرام وتم اقلمة الأسماك لمدة اسبوعين باستخدام عليقة الكنترول، ٣% من الوزن الحى . بعد هذه الفترة تم تقسيم الأسماك الى ثلاث معاملات كل معاملة ستة مكررات. المجموعة الأولى وفيها تغذت الأسماك على عليقه مضاف اليها بكتريا *Enterococcus faecium* بمعدل ٢٠مل /كجم علف والمجموعة الثانية والتى تغذت على عليقه أضيف اليها بكتريا *Bacillus coagulans* بمعدل ٢٠ مل /كجم علف أما المجموعة الثالثة فهى مجموعة المقارنة التى لم يضاف إليها بكتريا.

وبعد مرور ٧٥ يوم من بداية التجربة ولمدة ١٥ يوم تم وضع الأسماك من كل معاملة فى ٣٠ لتر مياه محتويه على بكتريا الايروموناس هيدروفيلا وكان من أهم النتائج المتحصل عليها مايلى:

١. سجلت المجموعة الاولى (التى غذيت على العليقة الأساسية مضاف إليها بكتريا *Enterococcus faecium)* أعلى مقاييس النمو (وزن وطول الجسم والزيادة فى وزن الجسم ومعدل النمو) وكذلك الغذاء الماكول ومعدل تحويل الغذاء وكفاءة تحويل البروتين).
٢. سجلت المجموعة الاولى أفضل متوسطات لقيم الهيموجلوبين والهيماتوكريت وعدد كرات الدم الحمراء هى مقارنة بالمجموعة الموجبة لبكتريا الايروموناس هيدروفيلا .
٣. بالنسبة لكرات الدم البيضاء سجلت المجموعات التى أصيبت ببكتريا الأيروموناس هيدروفيلا اعلى المجموعات مقارنة بباقى المجموعات وكانت الفروق بين هذه المتوسطات معنوية عند مستوى ٠,٠٠١% .
٤. إشارات النتائج أن المجموعة السادسة (مجموعة المقارنة الموجبة) سجلت أعلى القيم لإنزيمات الكبد ( ALT, AST ٢٩,٦و٩٤) على التوالى مقارنة بباقى المجموعات وكانت الفروق بين هذه المتوسطات معنوية عند مستوى ٠,٠٠١% .
٥. سجلت المجموعة السادسة (مجموعة المقارنة الموجبة) اعلى معدل نفوق (٦٢.٢٢%) مقارنة بباقى المجموعات كانت الفروق بين المجموعات عالية المعنوية عند مستوى ٠,٠٠١% .