Improvement growth and immune status using a potential probiotic bacteria *Micrococcus species* among Culured *Oreochromis niloticus*

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ABSTRACT

Probiotic bacteria *Micrococcus* species isolated from the gonads of apparently healthy *Oreochromis niloticus* was harmless to *O. niloticus* and had antagonistic effect against the pathogenic *Aeromonas hydrophila* invitro. The inhibition zone to *A. hydrohpila* was 47 mm in diameter due to *M.* species. The effect of supplemental dietary probiotic bacteria (pseudomonas sp.10^7 cells/g food) on some heamatological parameter, growth performance and survival rate of *O. niloticus*. were divided to two groups, with three replicates each. Fish were fed frequently on a diet (5% of body weight) three times daily for 60 days. The obtained results showed that the final weight, weight gain, specific growth rate and protein efficiency ratio of *O. niloticus* increased significantly in the treated fish, when compared to the untreated group. Also, feed conversion ratio was lower in the treated group than the control one. Erythrocytic counts and hemoglobin content total serum protein, A/G ratio and phagocytic activity in fish fed on diets containing *M.* species were higher than that of the control group. After feeding sixty days *O. niloticus* was challenged I/P by *A. hydrophila* 0.3×10^7 cells/ml. The survival rate was 75% with *M.* species while, it was 20% in the control group. Thus, the isolated *M.* sp. was harmless to *O. niloticus* and had probiotic effect in vitro and in vivo. [New York Science Journal 2010;3(10):5-11]. (ISSN: 1554-0200).

Key words: *Oreochromis niloticus*- *Micrococcus* species- *Aeromonas hydrophila*- feed conversion ratio- Erythrocyte counts- hemoglobin content- phagocytic activity probiotic

INTRODUCTION

The use of probiotics in aquaculture is proving to be highly effective in improving disease resistance, nutrition and growth of cultured organisms (Macey and Coyne, 2005)**.** *Aeromonas hydrophila,* one of the major bacterial pathogens, is known to cause a variety of diseases in fish such as hemorrhagic septicaemia, infectious dropsy, tropical ulcerative disease and fin rot leading to heavy mortality in aquaculture (Karunasagar et al., 1997)**.** Antibiotics and chemotheraputics are used as either preventive or curative agents with partial success (Stoskopf, 1993). However, such treatment may cause the development of resistant bacteria (Aoki et al., 1985), yields residues in fish and introducing potential hazard to the public health and environment as well. Furthermore, the normal microbial flora in the digestive tract, which is beneficial to fish, may also be killed or inhibited due to oral chemotherapy (Sugita et al., 1991 and Gram et al., 2001**).** In recent years, there is a great interest in combating or controlling disease problems thro-ugh alternative husbandry methods. One method is the use of antagonistic bacterial strains to control populations of potential pathogens through competitive exclusion of enhancement of immunity (Gatesovpe, 1997; Salminen et al., 1999 and Verschuere et al., 2000)**.** Probiotic bacteria have a possible competition for nutrients with pathogens. The antibacterial effect of bacteria is generally due to either singly or incombination of production of antibiotics, bacteriocins, siderophores, lysozymes and proteases, and alteration of pH values by organic acids production (Sugita et al., 1998)**.** The use of probiotics stimulate immunity by stimulating phagocytic activity, complement mediated bacterial killing and immunoglobulin (Ig) production (Nikoselainen et al., 2003, Choudhury et al., 2005; Panigrahi et al., 2005 and Song et al., 2006). Therefore, present study was carried out to evaluate the use of *Micrococcus* species as a growth promoter, antibacterial and immunostimulant for Nile Tilapia *Oreachromis niloticus.*

MATERIALS & METHODS

Bacterial isolation:

Eighty apparent healthy *O. niloticus* (50 ± 5g body weight) were randomly collected from earthen pond in private fish farm the at Kafr El-Sheikh governorate. Bacteriological examinations of the collected fish were done for isolation of probiotics, where samples from the internal organs (liver, kidneys, gonads, stomach and intestine) were cultured on Tryptic Soya Broth (TSB) and incubated at 30°C for 24-48 hours. Purification and identification of the isolates were done using biochemical tests according to Bergey et al. (1984) and Austin and Austin (1993).

The isolated bacteria from the internal organs of the investigated fish (intestine, stomach and gonads) were examined for its inhibitory effects against the pathogenic *Aeromonas hydrophila* (which it was obtained from National Research Center Hydrobiology Dept.). The in vitro probiotic activity

was done using agar diffusion method and the inhibition zone determined as described by Ruiz et al., (1996).

Safety of the suspected probiotic isolate was examined using sixty (25 ± 2 g body weight) apparently healthy fish were acclimated for two weeks. The fish were divided into 2 equal groups (three replicates 10 in each,). Fish of 1st group were I/P inculcated by 0.5 ml of saline containing 10^7 cells / ml of 24 hrs M. species. Fish of 2nd group were I/P inoculated by 0.5 ml of saline as controls according to Irianto and Austin (2002 a).

Preparation of probiotics diet:

The probiotic bacteria were prepared by inoculating the bacterial isolates in TSB and incubated at 30°C for 48 hrs, then centrifuged at 3000 rpm for 30 minutes. After centrifugation, the bacteria were washed twice with saline. The saline containing the probiotic isolate was added to commertial sterilized ration to give an initial number of 10^{10} bacterial cells / g. Sterile saline free from bacteria was added to the commercial diet (control). The two treatment commercial ration were mixed, pelleted and dried in the hot air oven at 40ºC. The number of live probiotic bacterial cells after drying the pellets was 10^6 cells/g diet. The diet was kept in refrigerator at 4ºC at the time of experiment.

Feeding system design:

The feeding experiment was conducted in 6 glass aquaria. The aquaria were supplied with well aerated and dechlorinated tap water. Each aquarium was supplied by compressed air via stone and cleaned daily by siphoning. Water temperature range was adjusted as 26±1ºC.

Oreochromis niloticus average body weight 50 ± 0.5 g were acclimated for two weeks. The fish were distributed randomly at a rate of 20 fish/aquarium. The experimental fish were weighed at the beginning of the experimental period. Fish were fed tow times daily, as 5% of the body weight / day. The experiment continued for two monthes, growth was monitored at 15 day intervals by weighing for each group of fish. Weight gain %, specific growth rate (SGR), feed conversion ratio (FCR) and were determined for each group according to Choudhury et al. (2005).

Haematological studies:

At the end of feeding experiment, three fish from each group were anaesthetised with clove oil (El-Gomhoria Co.) (50 ml/l) and blood was collected from caudal vein using sterile syringe, which was previously rinsed with 2.7% EDTA solution as an anticoagulant. Determination of hemoglobin content and total erythrocytes (Choudhury et al., 2005), haematocrit value (Hct) was calculated according to Britton (1963) Serum was obtained by centrifugation of the blood without anticoagulant by 2 h, at 3000 rpm for 15 min and stored in deep freezer for further biochemical analysis. Total protein content albumine, globuline, A/G ratio and phagocytic activity were determined. (Choudhury et al., 2005).

Challenge test:

After the end of experiment, the fish of each group were divided into two subgroups, the first one was challenged I/P with pathogenic *A. hydrophila* (0.3 ml of 110^7 cells / ml) which was obtained from National Research Center Hydrobiology Department. The second subgroup was injected I/P by 0.3 ml of saline as control. Both subgroups were kept under observation for 14 days to record the survival rate daily.

Statistical analysis:

The results were analyzed statistically by using of the analysis of variance (ANOVA). Dauncan'smultiple range test (Dauncan, 1955) was also used to evaluate the mean differences among different treatments.

RESULTS

Bacteriological examinations:

Bacteriological examinations revealed that, three isolates of Gram-postive cocci of genus *Micrococcus*, were isolated from apparently healthy *O. niloticus.*

They were identified as *M. nihinomyaensis*, and *Micrococcus* species and isolated from spleen, and kidneys respectively.

In vitro probiotic activity:

Gram-positive cocci (*M. nihinomyaensis* and *Micrococcus* species) were examined for thier antagonstic activity against the pathogenic *A. hydrophila* using agar diffusion method. *M. Sp.* gave on inhibition zone 47 mm in diameter, while *M. nihinomyaensis* and *Micrococcus* species had not any effect against *A. hydrophila.*

Safety of the isolated probiotics:

Table (2) revealed that *M.sp.* was harmless to *O. niloticus* as no clinical signs or mortalities were noticed following the challenge via I/P injection. The control group gave mortality 10%.

Table (1): challenge test to the probiotc bacterial isolate among *O. niloticus.*

Isolates	*Micrococcus* sp.	Control
No. of fish	60	60
Route of injection	I/P	I/P
dose	0.5 ml (10^6 cells/ml)	0.5 ml of saline
Mortality rate	0.0	10

Table 2: Probiotic effect of M. sp. on total serum protein, albumin, globulin, A/G ratio and phagocytic activity in *O.niloticus.*

Parameter	Control	Treated
Total serum protein (g/L)	3.62 ± 0.13^a	4.14 ± 0.13^a
Albumin (g/L)	1.94 ± 0.12^a	2.35 ± 0.10^a
Globulin (g/L)	1.68 ± 0.72^a	2.64 ± 0.14^a
A/G ratio	0.20 ± 0.04^a	0.11 ± 0.03^b
Phagocytic activity	21.65 ± 1.06^a	35.22 ± 1.21^b

The same letter in the same row is not significantly different at P<0.05.

Table (3): Survival rate of *O. niloticus* due to challenge with *A. hydrophila* after feeding with diet containing *Micrococcus* species for 2 months.

Items	Control	Treated
No. of injected fish	60	60
Dose of bacteria	0.3 ml of 10^7cells/ml	0.3 ml of 10^7cells/ml
Route of injection	I/P	I/P
Survival rate	23%a	79%b

The same letter in the same row is not significantly different at P<0.05.

Growth:

Results in Table (4) showed that the final weight, weight gain, specific growth rate of *O. niloticus* had significantly increased when fed on a diet containing *M.* sp. (P<0.05., 10.26 g/fish final weight, 7.783 g/fish weight gain, 1.71 % /day SGR and 2.176 PER). While in control group (9.49 g/fish final weight, 6.99 g/fish weight gain, 1.636 %/day SGR and 2.036 PER). Feed conversion ratio (FCR) was lesser in fish group fed on a diet containing *M. sp.* (1.48), than that in control group (1.693).

Table (4): Growth performance of *O. niloticus* fed diet containing *M.*sp.

Items	Control	Micrococcus sp.
Initial weight (g/fish)	50.23 ± 0.01 [a]	50.26± 0.009 [a]
Final weight (g/fish)	60.49± 0.465 [b]	65.33± 0.040 [a]
Weight gain (g/fish)	10.26 ± 0.474 [b]	15.07 ± 0.049[a]
S G R (%/day)	1.636 ± 0.044 ab	1.71 ± 0.007[a]
FCR	1.693 ± 0.067 [a]	1.48± 0.01 [b]

The same letter in the same row is not significantly different at P<0.05.

Hematological analysis:

Erythrocytic counts in fish fed on diets containing *M.* species were significantly higher ($1.159 \pm 0.043 \times 10^6$ / mm^3), than in control group ($0.999 \pm 0.067 \times 10^6/mm^3$). Hemoglobin content was increased slightly in the treated fish (5.32 g/100 ml), than that in untreated group (4.77 g/100 ml). Haematocrit values (Hct) were of non-significant values as 17.8 to 18.3% respectively. The serum total proteins A/G ratio and phagocytic activity showed higher values in treated groups than the control (Table 5&1).

Table (5): Changes in erythrocyte counts, hemoglobin content and haematocrit value, total proteins in the blood of *O.niloticus* fed diet containing *M.* sp.

Items	Control	*Micrococcus* sp.
Erythrocyte count $10^6/mm^3$	0.999 ± 0.067 [a]	1.159 ± 0.043 [a]
Hemoglobin (g/100ml)	4.77 ± 0.222 [a]	5.32 ± 0.151[a]

The same letter in the same row is not significantly different at P<0.05.

Table (3) showed that the survival rate after I/P challenge with *A. hydrophila* was 79 % of *O. nilotecus* fed on a diet containing *M.* Sp. for two months, while it was 23% in the in untreated group. *M.*sp. had antagonistic effect to *A. hydrophila* in vitro and vivo.

DISSCUSION

Probiotics are usually live microorganisms which when administered in adequate amounts confer a health benefits on host. Nowadays, probiotics are also becoming an integral part of the aquaculture practices to obtain high production. The common probiotics that are used for aquaculture practices include Lactobacillus, Lactococcus, Leuconostoc, Enterococcus, Carnobacterium, Shewanella, Bacillus, Aeromonas, Vibrio, Enterobacter, Pseudomonas, Clostridium, and Saccharomyces species. The involvement of probiotics in nutrition, disease resistance and other beneficial activities in fish has proven beyond any doubt. Among the numerous health benefits attributed to probiotics, modulation of immune system is one of the most commonly purported benefits of the probiotics and their potency to stimulate the systemic and local immunity under in vitro and in vivo conditions is noteworthy. Different probiotics either monospecies or multispecies supplementation can eventually elevate phagocytic, lysozyme, complement, respiratory burst activity as well as expression of various cytokines in fish (Nayak 2010). Probiotics are microorganisms or their products that have health benefits to the host, have

been used in aquaculture as a mean of disease control, supplemnting or even in some cases replacing the use of antimicrobial compounds. In the present study, the physical and the biochemical characters of suspected probiotic bacterial isolates were identified as *Micrococcus* sp. according to Bergey et al. (1984)**.** A wide range of Gram-positive (*Bacillus, Carnobacterium, Enterococcus, Lactococcus, Lactobacillus, Micrococcus and Streptococcus*) and Gram-negative bacteria (*Aeromonas, Alteromonas, Photorhodobacterium, Pseudomonas and Vibrio*) had been evaluated as probiotics in aquaculture (Irianto and Austin 2002 b; Choudhury et al., 2005 and Panigrahi et al., 2005).

M. sp. was isolated from gonads of apparently healthy *O. niloticus*, harmless and had inhibitory effects in vitro against *A. hydrophila* as (47 mm) inhibition zone. Irianto and Austin (2002 b) and Sugita et al. (1998**)** isolated *M. luteus* A1-6 from digestive tract of *Oncorhychus mykiss* and seven fish species and recorded that the bacteria had inhibitory effects against *Vibrio Vibrio vulnificus.* Lewus et al. (1991) and Riquelme et al. (1996) noticed that the bacteriocins produced by lactic acid bacteria and *Alteromonas haloplanktis* had an inhibitory activity against *A. hydrophila*. Irianto and Austin (2002 b) used *M. luteus* with feed as a potential combating *A. salmonicida* infection in rainbow trout (*O. mykiss*).

The final weight, weight gain, specific growth rate increased among *O. niloticus* fed on a diet containing *Pseudomonas* sp. and feeding efficiency ratio decreased. This finding is nearly similar to that obtained by Macey and Coyne (2005), Choudhury et al. (2005), Song et al. (2006) and Khattab et al. (2006)**.** Supplementation of the diet with probiotic bacteria that increased digestion and absorption of protein in the distal portion of the gastrointestinal tract may be due to increasing intestinal protease activity (Macey and Coyne, 2005). Appropriate probiotic applications were shown to improve intestinal microbial balance, thus leading to improve food absorption and reduced pathogenic problems in the gastrointestinal tract (Parker, 1974;, Lloyd et al., 1977, Goren et al., 1984 and Fuller, 1989), and/or by the stimulation of host immunity (Irianto and Austin, 2002 b)**.** Probiotics may stimulate appetite and improve nutrition by the production of vitamins, detoxification of compounds in the diet, and by the breakdown of indigestible components (Irianto and Austin, 2002 b). Moreover, Riquelme et al. (1997) studied the naturally occurring bacteria which were able to promote the growth of *Argopecten purpuratus* larvae by inhibiting the activity of other bacteria that flourished in hatchery cultures.

M. species had a good effect on erythrocyte count, heamoglobin content and haematocrit value because of the increasing values than that in the control group. These results are nearly similar to Irianto and Austin (2002 b), Choudhury et al. (2005) and Khattab et al. (2006) who recorded increase in erthrocyte count and heamoglobin content in fish fed on probiotic. The probiotic had great effect on serum total proteins and phagocytic activity; this agrees with Choudhury et al. (2005) who recorded the yeast contained diet gave high total protein. Nikoskelainen et al. (2003), Panigrahi et al. (2005) and Rengpipat et al. (2000) reported that the selected probiotic bacteria may have an impact on the specific and innate immunity of fish.

In the present study *M.* sp. gave survival rate 79% at 10^7 cells / g of feed among *O. niloticus* challenged I/P by *A. hydrophila.* This is nearly similar to Kennedy et al. (1998), Tovar-Ramírez et al. (2004) and Khattab et al. (2006) who recorded that survival rate between fish fed on a diet containing probiotics is higher than that among fish fed on untreated diet. *Haliotis midae* which fed on probiotic had 62% survival compared to 25% for non-teated after challenging with *Vibrio anguillarum* (Macey and Coyne (2005). Dietary supplementation with defined probiotics may be effective biotherapeutic or prophylactic means aquaculture (Nikoskelainen et al., 2003, Panigrahi et al., (2004), and Panigrahi et al., 2005).

In conclusion, *Micrococcus* sp. isolate was clearly beneficial for cultured *O. niloticus* when administrated as a food additive. It is recommended that such probiotic has a role in disease control strategies instead of antibiotics, growth promoter and it also improves the physiological parameters such blood picture among Nile tilapia *O. niloticus.*

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