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EFFECT OF PROBIOTIC ON HAEMATOGICAL PARAMATERS OF DISEASED FISH (*Cirrihinus mrigal*)

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Abstract: Epizootic ulcerative syndrome (EUS) is a dreadful disease of several aquatic animals including mrigal (*Cirrhinus mrigala* Ham.). Prevention of the disease with the help of chemical compounds may prove harmful for the fish as well as the end consumer. Now-a-days the use of probiotics has become firmly established due to their beneficial effects at the nutritional as well as therapeutic levels. Therefore, research efforts have been concentrated on optimizing production with eco-friendly alternatives to the therapeutic use of antimicrobials. Commercially available probiotics were used for controlling this disease in mrigal in the present study. The effect of probiotic on four haemotogical parameters viz. amount of haemoglobin (Hb), hemocrit / packed cell volume (PCV), total erythrocyte count (TEC), and total leucocytes count (TLC) were studied over a period of eight weeks. The level of these parameters decreased significantly in the blood of fish treated with pathogenic bacteria. The fish administrated with probiotics showed significant increase in the hematological parameters thus contributing towards overall health of the fish.

Keywords: Fish, Mrigal, Disease, Bacteria, Probiotic

Özet: **Probiyotiklerin Hasta Balıklarda Hematolojik Özelliklere Etkisi (*Cirrihinus mrigal*)**

Epizzotik ülseratif sendrom (EUS) mrigial'i (Cirrhinus mrigala Ham.) de içeren bazı balıklarda görülen bir hastalıktır. Hastalığın çeşitli kimyasallar bileşikler kullanılarak önlenmesi hem son kullanıcıya hemde balığa zarar verebilir. Son yıllarda, probiyotiklerin kullanımı, hem terepatik hemde besleyici özellikleri açısından kabul edilir hale gelmiştir. Bu nedenle araştırmalar üretimin optimize edilmesinde antimikrobiyallere alternatif olan çevre dostu ürünlere doğru yönelmiştir. Bu çalışmada, hastalığın mrigial balıklarında ticari olarak bulunabilen probiyotikler ile kontrolü konu edilmiştir. Probiyotik kullanımının etkileri, bazı kan parametrelerinin, hemoglobin (Hb), hematokrit/(Packed cell) hacmi, total eritrosit sayımı (TEC) ve total lökosit sayımının sekiz haftalık süreyle çalışılması ile incelenmiştir. Bu sayımlarda, patojen bakteri verilen balıklarda değerlerin düştüğü tespit edilmiştir. Probiyotik verilen balıklarda ise bu değerlerin önemli derecede arttığı ve sonuç olarak balığın sağlığına faydası olduğu görülmüştür.

Anahtar Kelimeler: Balık, Mrigal, Hastalık, Bakteri, Probiyotik

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Introduction

Probiotics, which are micro-organisms or their products, are used for the health welfare of the host. Recently probiotics have found their use as alternative agents to control the fish diseases. A wide range of microalgae, yeasts and bacteria have been isolated and used as probiotic in aquatic medium (Evenberg *et al*., 1985; Cahill, 1990; Mohanty *et al*., 1996; Liu *et al*., 2000; Alcaide, 2003; Ping Chung *et al.,* 2004 and Austin and Brian, 2006). The mrigal (*C. mrigala*) is one of the Indian major carps which are an integral part of aquaculture and an important component of sustainable food security in India. These fishes are infected with wide variety of diseases, including the EUS. In the present context of conservation of environment vis-à-vis ill effects of antibiotics, new generations of preventive/ curative bioagents have come into force. To take the advantage of these bioagents, the present investigations were proposed to ascertain the effect of probiotics on various life parameters of mrigal (*C. mrigala*).

Materials and Methods

The samples of diseased fish were dissected, the affected tissue (skin lesions/muscles) was taken in a test tube, homogenized it into a homogenizer and spread over the nutrient agar medium in Petri plates under aseptic conditions. These plates were incubated in B.O.D at 30 ± 1°C for 24 h. Bacterial growth on the nutrient agar plate was observed after 24 h. Pure colonies of bacteria were isolated and obtained further by sub-culturing of the single colonies on nutrient agar by proper streaking method (OIE, 2006). For the culture and isolation of the pathogenic bacteria, method suggested by OIE (2006) was followed. Commercially available probiotic was tested for their role as disease controlling agents against the infections caused by pathogenic fungi in *C. mrigala*. The composition of probiotic is *Lactobacillus sporogenes*, *Lactobacillus acidophilus, Bacillus subtilis, Bacillus Licheniformis*, *Saccharomyces cervirial*, Sea weed extract; Enzyme complex contains Amylase, Phylase, Protease, Cellulose, Beta-galactosidase, Lipase, C20g vitamin, Vitamin B61g, Sodium Benzoate.

In vitro test of probiotics

In vitro test of available probiotics for their antagonistic potential against fungus was done by using poisoned food technique (Verma *et al*., 2001). The basic principle of this technique was to poison the culture medium with pathogen and then allow the test probiotic to grow on such medium.

In vivo tests of probiotics

Aphanomyces invadans (fungus) were taken as pathogenic organisms for their inoculation in the mrigal (*C. mrigala*). Different treatments were given to the fish. The following treatments were given to the fish.

i) Control: In this treatment, 250µl of physiological buffer saline (PBS) was given into the intraperitoneal cavity of each acclimated fish.

iii) Control+fungus: Here, the motile spores of the fungus were dissolved/submerged into 1 ml of PBS solution. The concentration/µl of the spores was determined utilizing hemocytometer. The solution was then diluted up to 100 spores/250 µl of PBS. It was inoculated into the intraperitonal cavity of fish.

vi) Control+fungus+probiotic: In this case, 0.1gm probiotic1 and 100 motile spores of fungus, taken in 250 µl of PBS, were inoculated into the intraperitonal cavity of fish.

xi) Control+probiotic: Here, 0.1gm probiotic1 dissolved in 250 µl of physiological buffer saline was inoculated into the intraperitonal cavity of fish.

Hematological Studies

Different blood parameters viz. level of haemoglobin (Hb), total erythrocyte count (TEC), total leucocytes count (TLC) hemotocrit/packed cell volume (PCV) were determined with help of a haemocytometer and calculated from the equations given by Anderson and Klontz (1965).

Collection of blood

Blood samples of treated fish were taken at weekly interval after initiation of treatments. Sampling was also done at the same time from control group. Blood was drawn from the caudal peduncle region using a sterile syringe of 2 ml rinsed with 2.7% Ethylene dimethyl tetra amine (EDTA) solution. Blood was collected in small glass vials after drying the vials in hot air oven.

a) Haemoglobin estimation in the blood of mrigal (C.mrigala) under different treatments: The hemoglobin contents of blood were analyzed following the Cyanmethemo-

globin methods using Darbkins Fluid. Twenty micro liter of blood was mixed with 5 ml Darbkin's working solution. The absorbance was measured using a spectrophotometer at wavelength of a 540 nm. Hemoglobin contents were expressed as g / d1.

b) Total erythrocyte count: The blood was drawn from the caudal vein and EDTA was used as an anticoagulant to prevent the blood cells from lysis and clotting. The blood was diluted to 1:200, with RBC counting pipette. The mixture was shaken well to suspend the cells uniformly in the solution. Then the cells were counted using a haemocytometer as follows:

Number of RBC/mm^3 = N x 10000 (where, N= total number of red blood cells counted in 5 squares of the haemocytometer slide and 10,000 is the dilution factor).

c) Total leukocyte count: The blood was drawn from the caudal vein and EDTA was used as an anticoagulant. Blood was diluted 1:20 with WBC diluting fluids using WBC counting pipette. The mixture was shaken well to suspend the cells uniformly in the solution. Then the cells were counted using a haemocytometer as follows:

Number of WBC/mm^3 = N x 50 (where, N = total number of white blood cells counted in 4 squares of the hemocytometer slide and 50 is dilution factor).

Statistical analysis

The obtained results were analyzed statistically using completely randomized design (CRD) to evaluate differences among different treatments means at 0.05 significant levels following Snedecor and Cochran (1989).

Results and Discussion

Level of hemoglobin in the blood of mrigal (C. mrigala) under different treatments

The results on hemoglobin level in the blood of mrigal (*C. mrigala*) under different treatments over a period of eight weeks are presented in table 1. The hemoglobin level of normal fish remained in the range of 6.27 to 6.55 g/ 100ml. However, in fishes inoculated with fungus alone, the level of hemoglobin fell drastically and remained in the range of 4.17 to 2.34 g/ 100ml. The hemoglobin level increased in the range of 4.91 to 6.62 in fish inoculated with fungus + probiotic, respectively. On the other hand, the fish given the treatment of probiotic showed maximal value of haemoglobin level as compared to all other treatments including control. The hemoglobin level was in the range of 6.67 to 7.35 in fish administrated with probiotic. These results revealed that probiotic gives better results in increasing the hemoglobin level of fish.

Level of total erythrocyte count (TEC) in the blood of mrigal (C. mrigala) under different treatments

The results on erythrocyte count level in the blood of mrigal (*C. mrigala*) under different treatments over a period of eight weeks are presented in table 1. The erythrocyte count level of normal fish remained in the range of 2.21 to 2.25. However, in fishes inoculated with pathogenic alone, the level of erythrocyte count fell and remained in the range of 1.23 to 1.03. The erythrocyte count level increased in the range of 1.32 to 2.50 in fish inoculated with fungus along with probiotic. However, the erythrocyte count level was in the range of 1.49 to 2.58 in fish inoculated fungus along with probiotic. On the other hand, the fish given the treatment of probiotic showed maximal value of erythrocyte count level as compared to all other treatments. The erythrocyte count level was in the range of 2.49 to 3.10 in fish administrated with probiotic.

Level of total leukocyte count (TLC) in the blood of mrigal (C. mrigala) under different treatments

The results on leukocyte count level in the blood of mrigal (*C. mrigala*) under different treatments over a period of eight weeks are presented in table 1. The leukocyte count level of normal fish remained in the range of 2.29 to 2.65. However, in fishes inoculated with fungus alone, the level of leukocyte increased and remained in the range of 3.16 to 3.91.

The leukocyte count remained in the range of 2.46 to 2.94 in fish inoculated with fungus + probiotic. However, the leukocyte count level was in the range of 2.53 to 3.18 in fish inoculated with fungus along with probiotic. On the other hand, the fish given the treatment of probiotic showed maximal value of leukocyte count as compared to all other treatments including control. The level of leukocyte count was in the range of 2.40 to 3.07 in fish administrated with probiotic. These results revealed that probiotic gives better results in increasing the leukocyte count of fish.

Hematological parameters of fish reflect the gravity of these changes. Values of hematological parameters of fishes can be affected by environmental and biological factors such as age, weight, sex, food, bacteria, viruses, fungi and water quality parameters (Das and Das, 1993). Palikova *et al.* (2004) observed decrease in the level of blood in the common carp after exposure to Cyanobacteria extract. Results of all these studies resemble those of the present investigation showing that fish inoculated with pathogenic bacteria and fungus showed a decrease in its blood parameters. Hemoglobin level reduced approximately to 50% in its value; erythrocytes count reduced approximately to 40% in its value; leucocytes count reduced to approximately 40% in its value and packed cell volume reduced approximately to 40% in its value (Table 1) in three weeks. This clearly indicated a marked decline in the hematological parameters of diseased fishes.

An increase in erythrocyte count in fish, fed on probiotic bacteria than control group observed in fish species (Irianto and Austin, 2002; Selvaraj *et al*., 2005; Rengpipat *et al.*, 1998 and Prabhu *et al.*, 1999). The results of present study revealed that probiotic had a positive effect on hemoglobin level increased approximately to 50% in its value; erythrocytes count increased approximately to 40% in its value; leukocyte count increased approximately to 30% in its value (Table 1). This clearly indicated that there was increased in the value of hematological parameters of fish. The mean values of various hematological parameters in different treatments groups of fish during the experiment are presented in table 1. At the end of third week, all the fish died.

Conclusions

The present study also confirmed the findings of Mae da and Liao (1992) and Garriques and Arevalo (1995) who reported a significant increment in growth of *Penaeus monodon* and *Penaeus vannamei* fed with probiotic incorporated feeds. This study therefore, clearly reveals that probiotics are very effective in controlling the fish diseases in order to improve their health status. The present study further shows that fishes treated with probiotics showed increase in the level of different hematological parameters viz. hemoglobin, erythrocytes count, leucocytes count and packed cell volume of fish significantly.

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Table 1. Effect of probiotics on the haematological parameters of mrigal (*C. mrigala*) under *in vivo* induced pathogenicity over a period of eight weeks

Treatment	Level of haemoglobin in the blood (g/100ml)[a]								Erythrocyte count (x10^6 cells/ml)								Total leukocyte count (x 10^3 cells/ml)[a]							
	1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk	7th wk	8th wk	1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk	7th wk	8th wk	1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk	7th wk	8th wk
Control (T1)	6.55 ±0.17	6.47 ±0.05	6.33 ±0.24	6.33 ±0.17	6.53 ±0.05	6.43 ±0.06	6.27 ±0.25	6.53 ±0.06	2.22 ±0.08	2.21 ±0.04	2.22 ±0.05	2.25 ±0.04	2.22 ±0.02	2.22 ±0.02	2.22 ±0.01	2.24 ±0.02	2.29 ±0.15	2.44 ±0.13	2.45 ±0.24	2.53 ±0.12	2.56 ±0.07	2.59 ±0.09	2.64 ±0.04	2.65 ±0.03
Control+ Fungus (T2)	4.17 ±0.26	3.54 ±0.22	2.34 ±0.12	-	-	-	-	-	1.23 ±0.05	1.18 ±0.03	1.03 ±0.08	-	-	-	-	-	3.16 ±0.02	3.31 ±0.09	3.91 ±0.03	-	-	-	-	-
Control+ Fungus+ Probiotic (T3)	4.91 ±0.06	5.19 ±0.05	5.23 ±0.04	5.31 ±1.45	6.02 ±0.05	6.26 ±0.07	6.54 ±0.03	6.62 ±0.03	1.32 ±0.04	1.36 ±0.05	1.41 ±0.06	1.44 ±0.06	1.68 ±0.06	1.77 ±0.07	2.27 ±0.09	2.50 ±0.05	3.18 ±0.03	3.09 ±0.01	3.04 ±0.03	2.94 ±0.06	2.77 ±0.08	2.67 ±0.06	2.54 ±0.04	2.53 ±0.03
Control+ Probiotic (T4)	6.67 ±0.12	6.80 ±0.14	6.89 ±0.10	7.10 ±0.22	7.12 ±0.08	7.14 ±0.04	7.36 ±0.14	7.35 ±0.06	2.49 ±0.03	2.54 ±0.02	2.58 ±0.02	2.60 ±0.02	2.63 ±0.02	2.68 ±0.04	2.72 ±0.02	3.10± 0.03	2.40 ±0.42	2.57 ±0.23	2.68 ±0.28	2.69 ±0.23	2.68 ±0.07	2.72 ±0.04	2.97 ±0.03	3.07 ±0.02
CD Value (P≤0.01)	0.417	0.403	0.405	0.916	0.175	0.133	0.168	0.079	0.113	0.101	0.12	0.188	0.061	0.078	0.105	0.097	0.314	0.198	0.339	0.55	0.187	0.117	0.092	0.074

a= Mean ±s.d.,
N=27 (9 fishes x 3 replication),
- Fish died after three weeks